Characterization of Celastrol to Inhibit Hsp90 and Cdc37 Interaction*

Received for publication, August 3, 2009, and in revised form, September 29, 2009 Published, JBC Papers in Press, October 26, 2009, DOI 10.1074/jbc.M109.051532

Tao Zhang, Yanyan Li, Yanke Yu, Peng Zou, Yiqun Jiang, and Duxin Sun

From the Department of Pharmaceutical Sciences, College of Pharmacy, University of Michigan, Ann Arbor, Michigan 48109

The molecular chaperone heat shock protein 90 (Hsp90) is required for the stabilization and conformational maturation of various oncogetic proteins in cancer. The loading of protein kinases to Hsp90 is actively mediated by the cochaperone Cdc37. The crucial role of the Hsp90-Cdc37 complex has made it an exciting target for cancer treatment. In this study, we characterize Hsp90 and Cdc37 interaction and drug disruption using a reconstituted protein system. The GST pull-down assay and ELISA assay show that Cdc37 binds to ADP-bound/nucleotide-free Hsp90 but not ATP-bound Hsp90. Celastrol disrupts Hsp90-Cdc37 complex formation, whereas the classical Hsp90 inhibitors (e.g. geldanamycin) have no effect. Celastrol inhibits Hsp90 ATPase activity without blocking ATP binding. Proteolytic fingerprinting indicates celastrol binds to Hsp90 C-terminal domain to protect it from trypsin digestion. These data suggest that celastrol may represent a new class of Hsp90 inhibitor by modifying Hsp90 C terminus to allosterically regulate its chaperone activity and disrupt Hsp90-Cdc37 complex.

Heat shock protein 90 (Hsp90) is a highly abundant and essential molecular chaperone in eukaryotic cells, accounting for as much as 1–2% of the cytosolic protein even under non-stressed conditions (1). Hsp90 protects cells not only through correcting the misfolded proteins under stress conditions, but also plays a key role under normal conditions in regulating the stability, maturation, and activation of a wide range of client substrates, including kinases, hormone receptors, and transcription factors (2). There is strong evidence that Hsp90 plays an important role in disease states, particularly in cancer. Hsp90 is expressed 2–10-fold higher in cancer cells compared with their normal counterparts, implying its crucial role in tumor cell growth or survival (3). The largest subset of Hsp90 clients is the protein kinase, many of which are mutated and/or overexpressed signaling proteins in cancers (4–6). Furthermore, cancer cells are significantly more sensitive to Hsp90 overexpressed signaling proteins in cancers (4–6). Further- more, cancer cells are significantly more sensitive to Hsp90 than non-transformed cells (7). Therefore, Hsp90 has emerged as a promising target for cancer treatment.

The crystal structure reveals that Hsp90 consists of three highly conserved domains: an N-terminal ATP-binding domain (25 kDa), a middle domain (35 kDa), and a C-terminal dimerization domain (12 kDa) (8–10). Hsp90 exists as a homodimer (11). The N-terminal domain contains a specific ATP-binding pocket, which has been well characterized (9, 12). The middle domain is highly charged, and its major role is to distinguish various types of client proteins and adjust the molecular chaperone for proper substrate activation (13). The C-terminal domain strengthens the weak association between the two N-terminal domains of the Hsp90 dimer (10). A second ATP-binding site is located in the C terminus, which does not exhibit ATPase activity (14).

Hsp90 chaperone function depends on the conformational changes driven by its ATPase activity (15). Numerous Hsp90 inhibitors, ranging from the original natural products and their derivatives to fully synthetic small molecules, have been discovered or developed to inhibit its chaperone function by binding to the ATP/ADP pocket (16). The antibiotic benzoquinone ansamycins, represented by geldanamycin (GA), are the first identified Hsp90 inhibitors (17). Binding of GA in the N-terminal ATP pocket restrains Hsp90 in its ADP-bound conformation and prevents the subsequent “clamping” of Hsp90 around a client protein, resulting in ubiquitination and proteasomal degradation of the client proteins (18–20). GA has exhibited potent anticancer effect, but the strong hepatotoxicity prevented its clinical development (21). As a result, many GA derivatives have been generated to maintain its anticancer activities but decrease toxicity (22–26), among which 17-AAG (17-allylamino-17-demethoxygeldanamycin), 17-DMAG (17-(dimethylaminoethylamino)-17-demethoxygeldanamycin), and IPI-504 (17-allylamino-17-demethoxy-geldanamycin hydroquinone hydrochloride) are currently in clinical trial for various solid tumors and leukemia (27–30). Inhibitors binding to the newly discovered Hsp90 C-terminal ATP-binding site have also been identified, such as novobiocin, cisplatin, epigallocatechin-3-gallate (EGCG), and taxol (31). Inhibition of Hsp90 by novobiocin induces similar cellular responses as N-terminal inhibitors to destabilize a range of Hsp90 client proteins via the ubiquitin-proteasome pathway (32, 33). Although the biochemical and molecular modeling techniques have made considerable advancements in understanding the Hsp90 C terminus, much still remains speculative or controversial due to the lack of co-crystal structures. Currently, most of the Hsp90 inhibitors are targeting the ATP-binding site in the N-terminal region (34).

The wide-ranging functions of Hsp90 result from its ability to chaperone many client proteins through an ordered forma-
tion of multichaperone complexes with cochaperones (2, 34). With the increasing understanding of the Hsp90 function cycle and the promising results of ATP-binding blockers of Hsp90, interest in Hsp90 inhibition has expanded from the central component Hsp90 to various modulators in the chaperone machinery. Inhibition of cochaperones (Aha1, Cdc37, CHIP, Hop, Hsp70, and PP5) has exhibited therapeutic anticancer potentials as well (34). Silencing of Aha1, the only known Hsp90 ATPase activator, decreases cell protein activation and increases cellular sensitivity to the Hsp90 inhibitor 17-AAG (35). Simultaneous knockdown both Hsc70 and Hsp72 induces proteasome-dependent degradation of Hsp90 client proteins, G1 cell-cycle arrest, and extensive tumor-specific apoptosis (36). Cdc37 silencing promotes the proteasome-mediated degradation of kinase clients via a degradation pathway independent of Hsp90 binding, and enhances apoptosis in combination with 17-AAG (37). Not only targeting cochaperones has exhibited pharmacological benefits, the interference of Hsp90-cochaperone complex has shown therapeutic potential as well. The compounds disrupting Hsp90 and Hop interaction have been identified and shown activity in human breast cancer cells (38, 39). More recently, we have demonstrated that celastrol, a natural triterpene compound isolated from the plant family Celastraceae (40), can disrupt Hsp90 and Cdc37 interaction in pancreatic cancer cells, resulting in Hsp90 client protein degradation and cell apoptosis (41). These findings have highlighted the pharmacological potential of the cochaperone Cdc37.

Cdc37, also known as p50, was originally discovered in yeast as an essential cell cycle protein (42). Later studies proved it to be an Hsp90 cochaperone, acting as an adaptor to load protein kinase to Hsp90 complex (34, 43). Most of the Hsp90 clients Cdc37 associated with are crucial elements implicated in signal transduction, cell proliferation and survival (34). In addition, Cdc37 overexpression is found in cancer cells and tissues, and its induction can promote tumorigenesis (44, 45). Cdc37 can be dissected into three domains (34, 46). The N-terminal domain is the kinase-binding domain containing a conserved phosphorylated residue (Ser-13) (47–49). The middle region of Cdc37 is the most stable domain (50), which binds the N terminus of Hsp90 (4). The C-terminal domain is also involved in Hsp90 binding and probably required for the dimer formation (4, 51).

In previous studies we have shown that celastrol disrupts Hsp90 and Cdc37 interaction in pancreatic cancer cells and exhibited anticancer effect (41). Here, using a purified protein system, we present that Cdc37 binds to ADP-bound/nucleotide-free Hsp90, which can be directly inhibited by celastrol. Through the comparison of celastrol with nucleotides and other Hsp90 inhibitors, we prove that celastrol functions via a distinct mechanism. It interacts with Hsp90 C-terminal domain and inhibits its ATPase activity without blocking the ATP-binding pocket.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Celastrol was purchased from Sigma-Aldrich. Dihydrocelastrol diacetate (DDCel) was from Gaia Chemical Corporation. Geldanamycin (GA) was kindly provided by Dr. George Wang (Department of Chemistry, The Ohio State University). The mixture of celastrol and DTT was analyzed using proton nuclear magnetic resonance spectra (1H NMR) on a 300 MHz Bruker DPX-300 NMR spectrometer. The nucleotides ATP, ADP, and AMP-PNP were from Sigma-Aldrich.

**Protein Purification**—The expression plasmids pET15b-hHsp90β, pET28a (+)-hHsp90β (530–724), pGEX4T.1-Cdc37 for expression of human full length His-Hsp90β, C-Hsp90β, and GST-Cdc37 protein were kindly provided by Dr. Thomas Ratajczak (University of Western Australia); plasmid pET15b-hHsp90α for expression of human His-Hsp90α was provided by Dr. Wei Li (University of Southern California Keck School of Medicine). The plasmids were transformed into Escherichia coli strain Rosetta 2(DE3) (EMD Biosciences Inc., San Diego, CA) according to the protocol provided by manufacturer. Primary cultures of transformed cells were grown overnight, pelleted by centrifugation, resuspended in new culture medium and grown for 1–2 h at 37 °C until A560 = 0.6. Then protein expression was induced by 0.2 mM IPTG (isopropyl-β-D-thio-galactopyranoside) (GE Healthcare, Piscataway, NJ) for 2 h. Cells were collected by centrifugation and washed once by cold PBS. His-tagged proteins were purified by affinity chromatography through mixing with HisPurTM Cobalt Resin (Pierce), and GST-tagged proteins with glutathione 4B-Sepharose (GE Healthcare, Piscataway, NJ). The GST tag of Cdc37 was removed by thrombin cleavage. Purified proteins were dialyzed against PBS, the purity was assessed by SDS-PAGE, and concentrations were determined by BCA assay (Pierce). Proteins were stored at −70 °C after adding glycerol to 10%. The purified yeast Hsp82 protein was kindly provided by Dr. Dan Bolon (University of Massachusetts Medical School).

**GST Pull-down Assay**—Purified Hsp90 protein (~5 μg) was preincubated with different compounds or control (DMSO) at 30 °C for 30 min in 200 μl of incubation buffer (10 mM Tris-HCl, 50 mM KCl, 5 mM MgCl2, 0.01% Nonidet P-40, pH 7.5). Following incubation, GST-Cdc37 protein (~10 μg) was added and further incubated at 4 °C for 1 h. After that, glutathione-Sepharose 4B (20 μl) pre-equilibrated with incubation buffer was added and incubated at 4 °C for 2 h. The incubation was performed with gentle shaking. Sepharose was then pelleted by centrifugation, washed five times with 1 ml of incubation buffer, analyzed by SDS-PAGE and Western blotting.

**ELISA Microtiter Plate Assay**—The ELISA assay was modified from the previous reported method (52). Purified hHsp90α/β protein (80 nm) or control (bovine serum albumin) in ELISA coating buffer (Biolegend, San Diego, CA) was applied to Immulon 4HBX 96-well microtiter plates with 50 μl/well (triplicate per sample) and incubated at 4 °C overnight. Wells were washed three times with TBST (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.075% Tween 20) and blocked with 100 μl of 1% bovine serum albumin in PBS overnight at 4 °C. Afterward wells were washed three times again with TBST. Compound in 100 μl of TBST was added at indicated concentrations and incubated at 30 °C for 30 min. Depending on different experimental design, the compounds were either removed by washing five times, or this step was excluded, before adding 0.25 μM GST-Cdc37 protein in TBST. The mixture was incubated at 4 °C for 1 h. Unbound proteins were removed by washing with TBST for five times. Rabbit anti-Cdc37 antibody (H271, Santa Cruz Bio-
Celastrol Disrupts Hsp90-Cdc37 Complex

RESULTS

Cdc37 Binding to Hsp90—Previous studies about Hsp90 and cochaperones interaction have shown that the ingredients and various conditions of the incubation buffer can dramatically affect the protein interactions (55–57). To test these conditions, we performed GST pull-down assay by mixing Hsp90 and Cdc37 in different buffers (Fig. 1A). We started with pure water, in which no obvious interaction between Hsp90 and Cdc37 was noticed (Fig. 1A, lane 1). A weak interaction was detected using the basic incubation buffer previously reported for characterizing Hsp90 and p23 interaction (Fig. 1A, lane 2), which is surprising because Hsp90 and p23 cannot bind to each other under the same condition (55). The maximal interaction was noticed when Nonidet P-40 was added (Fig. 1A, lane 3). In fact, the Hsp90-Cdc37 complex was present upon simply mixing them in a PBS buffer, although the interaction is slightly weaker (Fig. 1A, lane 4). These results demonstrate that Hsp90 and Cdc37 can form complex in the absence of other proteins.

Hsp90 exists at two different functional states, the ADP-bound and ATP-bound forms (55). The nucleotides have proved to be effective to switch the conformation of Hsp90 (55). We tested these effectors in the Hsp90-Cdc37 complex formation by GST pull-down assay. The addition of ADP has no influence on Hsp90 and Cdc37 interaction (Fig. 1B), indicating Hsp90 is in its ADP-bound state in the complex. It has been shown that molybdate maintains Hsp90 in a pseudo-ATP-bound state, which is completely dependent on the presence of ATP (55). Indeed, the addition of molybdate alone has no effect on Hsp90 and Cdc37 interaction (Fig. 1B). The addition of both molybdate and ATP completely disrupted Hsp90-Cdc37 complex (Fig. 1B), suggesting that the ATP-bound state of Hsp90 is not capable of binding Cdc37. This was further confirmed when AMP-PNP, the nonhydrolysable analog of ATP, was added (Fig. 1B). Because molybdate is only effective with a readily metabolized nucleotide (55), we added AMP-PNP without molybdate. Similar to ATP, AMP-PNP inhibited Hsp90-Cdc37 complex formation (Fig. 1B).

Celastrol Inhibits Hsp90-Cdc37 Complex Formation—We first examined the effect of Hsp90 inhibitors on Hsp90-Cdc37 interaction using GST pull-down assay. As one of the most well-known Hsp90 inhibitors, geldanamycin (GA) blocks Hsp90 function by binding to the N-terminal ATP-binding pocket (2). Our previous results have shown that GA has no effect on Hsp90 and Cdc37 interaction in pancreatic cancer cells (41). Consistent with the in vivo studies, as high as 500 μM of GA was not able to change Hsp90 and Cdc37 binding in the purified protein system, as shown in Fig. 1C. No disruption was noticed with its derivatives 17-AAG and 17-DMAG either (Fig. 1C).

In contrast, celastrol can disrupt Hsp90-Cdc37 complex in the same GST pull-down assay. A progressive decline in the complex formation was observed with increasing concentrations of celastrol (Fig. 1D). As low as 1 μM of celastrol has exhibited moderate effect, 10 μM inhibited more than 70% of complex formation, and up to 100 μM completely abrogated Hsp90 and Cdc37 interaction (Fig. 1D). These results are consistent with what we observed in pancreatic cancer cells upon celastrol treatment (41). Moreover, the inhibitory effect was not restricted to human Hsp90β protein, but applicable to human Hsp90α and yeast Hsp82 proteins as well (Fig. 1D).

To confirm these results, we performed ELISA microtiter plate assay using both Hsp90α and Hsp90β proteins. Purified Hsp90α/β was coated on the Immulon 4HBX 96-well Microtiter plate and incubated with celastrol before Cdc37 was added.
Celastrol Disrupts Hsp90-Cdc37 Complex

Bound Cdc37 was detected by antibody specific to Cdc37. The results showed that celastrol (1–200 μM) was able to disrupt Hsp90 and Cdc37 interaction in a concentration-dependent manner (Fig. 1E), while GA did not interfere with Hsp90-Cdc37 complex.

Inhibitory Activity Resides within the A/B Rings of Celastrol—It has been predicted there are two electrophilic centers residing within the A and B rings of celastrol (58–60). They are active structures responsible for the biological activity of celastrol (58–60). To test the importance of these moieties in compromising Hsp90-Cdc37 complex formation, we used the derivative of celastrol, dihydrocelastrol diacetate (DDCel). Compared with celastrol, DDCel has an altered double bond arrangement within A/B ring, and two acetoxyl groups attached to the A ring carbonyl and hydroxyl groups of celastrol (Fig. 2A). DDCel showed a significantly weaker effect in disrupting Hsp90-Cdc37 complex. A minor disruption of the complex was observed only in response to as high as 100–200 μM concentrations (Fig. 2B), which indicates the importance of the unique quinone methide structure of celastrol.

Previous studies have shown that the activation of the heat shock response by celastrol can be inhibited by mixing with excess thiols (61), therefore we tested whether thiols can affect the effect of celastrol on Hsp90-Cdc37 complex. Celastrol was premixed with DTT before incubation with Hsp90. It seems that these two chemicals react with each other as the red color of celastrol faded away right after DTT was added. The results showed that the mixture was incapable of affecting Hsp90-Cdc37 complex formation (Fig. 2C). The NMR analysis of the mixture indicated altered bond arrangement in the A and B rings of celastrol (data not shown). Therefore, the reactive activity of the A/B rings may contribute to the inhibitory effect of celastrol on Hsp90-Cdc37 complex formation.

Inhibitory Effect of Celastrol on Hsp90-Cdc37 Complex Formation Is via Hsp90—The ELISA microtiter plate assay can be applied either by coating Hsp90 on the well and detecting bound Cdc37, or by coating Cdc37 on the well and detecting bound Hsp90. The data above (Fig. 1E) have shown that when Hsp90 was immobilized on the plate, Cdc37 binds immobilized Hsp90. Similarly, when Cdc37 was immobilized onto the plate, purified Hsp90 also binds to immobilized Cdc37 (Fig. 3A). The addition of celastrol to the mixture disrupts Hsp90-Cdc37 interaction, whereas GA did not, regardless of which protein was immobilized onto the plate (Figs. 1E and 3A).

To investigate which protein in the Hsp90-Cdc37 complex is the main target of celastrol, we modified the ELISA microtiter plate assay by adding one washing step to remove the free drug after incubating celastrol with the coated protein on the wells. First, Hsp90 was coated on the wells and incubated with celastrol at the indicated concentrations or GA (0.2 mM) at 30 °C for half an hour. Purified human Hsp90 protein was preincubated with DMSO or celastrol at the indicated concentrations, and the disruption was analyzed by GST pull-down assay. Bound Cdc37 was detected by antibody specific to Cdc37. The data above (Fig. 1E) have shown that when Hsp90 was immobilized on the plate, Cdc37 binds immobilized Hsp90. Similarly, when Cdc37 was immobilized onto the plate, purified Hsp90 also binds to immobilized Cdc37 (Fig. 3A). The addition of celastrol to the mixture disrupts Hsp90-Cdc37 interaction, whereas GA did not, regardless of which protein was immobilized onto the plate (Figs. 1E and 3A).

Inhibitory Effect of Celastrol on Hsp90-Cdc37 Complex Formation Is via Hsp90—The ELISA microtiter plate assay can be applied either by coating Hsp90 on the well and detecting bound Cdc37, or by coating Cdc37 on the well and detecting bound Hsp90. The data above (Fig. 1E) have shown that when Hsp90 was immobilized on the plate, Cdc37 binds immobilized Hsp90. Similarly, when Cdc37 was immobilized onto the plate, purified Hsp90 also binds to immobilized Cdc37 (Fig. 3A). The addition of celastrol to the mixture disrupts Hsp90-Cdc37 interaction, whereas GA did not, regardless of which protein was immobilized onto the plate (Figs. 1E and 3A).
Celastrol followed by washing. Under such conditions, as shown in Fig. 3B, Cdc37 was incapable of binding immobilized Hsp90, which indicates celastrol has rendered Hsp90 unable to bind Cdc37. In contrast, when the immobilized Cdc37 was incubated with celastrol, Hsp90 was still able to form the Hsp90-Cdc37 complex after drug removal (Fig. 3C). This suggests that either celastrol does not affect Cdc37, or the effect is weaker or transient, which can be abrogated by washing. However, the effect of celastrol on Hsp90 was persistent, implying that celastrol may mainly target Hsp90 to impair Hsp90-Cdc37 complex formation.

Celastrol Inhibits ATPase Activity of Hsp90—As a molecular chaperone, the intrinsic ATPase activity of Hsp90 is critical for its function in the folding and maturation of client proteins (15). Because Hsp90 is likely to be the target of celastrol, we next examined whether celastrol could interfere with the essential ATPase activity of Hsp90. As shown in Fig. 4A, 10 μM celastrol exhibited a marked inhibitory effect on ATPase activity of Hsp90, while 100 μM celastrol completely abolished the activity, comparable to that of GA.

Given that celastrol inhibits ATPase activity, it is reasonable to suspect that celastrol binds to the ATP site because the Hsp90 inhibitors (e.g. GA) occupy the ATP-binding pocket of Hsp90 to inhibit its ATPase activity. However, our previous results have shown that celastrol did not inhibit ATP binding to Hsp90 with the ATP-Sepharose binding assay (50). We further confirmed the phenomenon with the biotinylated GA binding assay. Purified Hsp90 protein was first incubated with DMSO, 17-AAG, or celastrol before the GST pull-down assay. A mouse anti-His antibody was used to detect bound Hsp90β.

FIGURE 2. Chemical specificity of celastrol on Hsp90-Cdc37 complex disruption. A, chemical structure of celastrol and its analog dihydrocelastrol diacetate (DDCel). The rings are identified by capital letters (A–E). B, DDCel showed a much weaker effect on Hsp90-Cdc37 complex disruption. Purified human Hsp90β protein was preincubated with DMSO or DDCel, and the disruption was analyzed by the GST pull-down assay. C, effect of celastrol was blocked by free thiols. Celastrol was premixed with DTT (1 mM) before the GST pull-down assay.

FIGURE 3. Celastrol targets on Hsp90. A, validation of the ELISA microtiter assay when Cdc37 was coated on the plate as the bound protein. Purified Cdc37 protein was bound to the wells and incubated with celastrol (0.2 mM) or GA (0.2 mM) for the ELISA assay. B, formation of Hsp90-Cdc37 complex was inhibited after preincubating Hsp90 with celastrol and removing the compound before adding Cdc37. Purified human Hsp90β protein was bound to the wells and incubated with celastrol at 30 °C for half an hour. The wells were washed five times with TBST before Cdc37 was added and subjected to the same procedure of the ELISA assay. C, formation of the Hsp90-Cdc37 complex was not affected after preincubating Cdc37 with celastrol and removing it before adding Hsp90. Purified Cdc37 protein was bound to the wells and incubated with celastrol. The wells were washed five times with TBST before Hsp90β was added. A mouse anti-His antibody was used to detect bound Hsp90β.
ity of Hsp90, the inhibition can be achieved by alteration or interaction with other domains of the molecule far from the ATP-binding pocket (62). Recent studies have provoked the importance of the Hsp90 C terminus in the regulation of its function (63, 64). Therefore, we tested whether celastrol can interact with C-terminal domain of Hsp90 by performing proteolytic fingerprinting assay with purified full-length and C-terminal (residues 530–724) Hsp90 proteins. The proteins were incubated with DMSO or celastrol, and the fingerprint was obtained by treatment with increasing concentrations of trypsin. The fragments of the C-terminal domain were detected by antibodies specifically recognizing epitopes within this region. As shown in Fig. 4C, in the absence of celastrol, the full-length Hsp90 protein is highly sensitive to trypsin digestion. After the protein was preincubated with celastrol, the stabilization of a 50-kDa fragment was observed, which was detected by the antibody specific to the C terminus. This indicates that celastrol induces the conformational changes of full-length Hsp90, resulting in the altered susceptibility to trypsin-mediated degradation. To further investigate the location of interaction, we examined the effect of celastrol on the proteolysis of the purified Hsp90 C terminus protein containing residues 530–724. As expected, the recombinant protein was sensitive to trypsin digestion (Fig. 4D). Celastrol protected the entire C-terminal region from trypsin cleavage (Fig. 4D). Therefore, we interpret that celastrol binds directly to a site on the C-terminal region of Hsp90.

DISCUSSION

The molecular chaperone Hsp90 has become an exciting target for cancer therapeutics. A number of Hsp90 inhibitors have exhibited anticancer activity in xenograft models, and several of them are in clinical trials (65). Although Hsp90 remains the main target, there is increasing interest in the functionally related cochaperones involved in the multichaperone complex, such as Hop, Cdc37, p23, and Aha1 (34). Our previous studies have shown that celastrol disrupts Hsp90 and Cdc37 interaction in pancreatic cancer cells, and it showed potent anticancer activity in pancreatic cell lines and animal models. The present study establishes a reconstituted protein system to characterize the effect of celastrol on Hsp90-Cdc37 complex. Using purified proteins, we find that Cdc37 binds to Hsp90 in the ADP-bound/nucleotide-free state but not in the ATP-bound state. Celastrol directly disrupts their interaction in the purified protein system in a dose-dependent manner, excluding the possibility that in pancreatic cancer cells the disruption of Hsp90-Cdc37 complex upon celastrol treatment was merely indirect consequence of other factors. Moreover, celastrol completely abrogates the
Celastrol Disrupts Hsp90-Cdc37 Complex

ATPase activity of Hsp90 but does not inhibit GA binding to Hsp90. The functional group of celastrol may reside in the A/B rings and its inhibitory effect may result from its interaction with Hsp90 C-terminal domain.

The chaperoning cycle of Hsp90, which depends on an ordered assembly and disassembly of various cochaperones driven by the ATPase activity, has been studied extensively (2). However, because it is a dynamic process with many cochaperones (e.g. Hsp70, Hsp40, Hop, Hip, Cdc37, p23, IP, and Aha1) involved and new members being identified (66), much detailed information remains inconclusive and controversial. Our results show that the Hsp90-Cdc37 complex forms by simply mixing Hsp90 and Cdc37 in the incubation buffer (Fig. 1A). When the agents (molybdate, ATP, and AMP-PNP) that promote the conversion of Hsp90 into the ATP-bound state were included, Cdc37 can no longer associate with Hsp90 (Fig. 1B). These results reveal that Cdc37 prefers to bind the nucleotide-free or ADP-bound Hsp90. The same conclusion has been drawn on the cochaperone Hop as well (56). In addition, it has been proposed that Cdc37 acts as an adaptor or scaffold that loads client/Hsp70/Hsp40 to Hsp90 with the help of Hop in the early complex during the chaperoning cycle (4, 67). Therefore, Cdc37 and Hop are likely to bind Hsp90 at the same stage. This is consistent with the recent data that Cd37 co-associate with Hop in the early complex (43, 68, 69). Another similarity of Cdc37 and Hop is that neither of them can associate with the ATP-bound Hsp90 in the mature complex (Fig. 1B) (56). The crystal structure has shown that Cdc37 binds to Hsp90 by inserting its C-terminal side chain into the nucleotide-binding pocket of Hsp90, suggesting the binding of ATP would have to eject this side chain out of the ATP-binding pocket (2). Therefore, it is possible that Cdc37 and Hop are removed from the Hsp90 early complex simultaneously due to the binding of ATP to Hsp90. However, more direct evidence is required to confirm these hypotheses.

The disruption of Hsp90-Cdc37 complex by celastrol could be through Hsp90, Cdc37, or both. Our results confirmed that Hsp90 definitely contributes to the disruption (Fig. 3B), and thus we consider celastrol as an Hsp90 inhibitor. There are two well-known classes of Hsp90 inhibitors. The first identified are ATP pocket inhibitors at the N-terminal region, represented by GA and its derivatives (15). GA blocks ATP binding to Hsp90 and locks the chaperone cycle in the early complex containing Hsp70, Hop, and Cdc37 (15) (41). Therefore, it should not disrupt Hsp90 and Cdc37 interaction, which is clearly supported by our results (Fig. 1, C and E). A second nucleotide-binding domain has been reported to exist in the C terminus of Hsp90, and specific inhibitors to this domain are represented by novobiocin and the related coumarin antibiotics (32). Similar with novobiocin, celastrol interacts with a domain in the C-terminal portion of Hsp90 (Fig. 4, C and D). They both interfere with the chaperone function of Hsp90 and deplete a series of Hsp90-dependent signaling proteins in tumor cells (32, 41). However, novobiocin and celastrol showed major differences in their effects on the interaction of cochaperones with Hsp90. Novobiocin (4 mM) significantly reduced the interaction of Hsc70 and p23 with Hsp90 in reticulocyte lysate (54). The same result was reported by Marcu et al. (70) with a lower concentration (1 mM) of novobiocin. Novobiocin (10 mM) moderately decreased the interaction of Hop with Hsp90 (54). However, novobiocin (5 mM) has little effect on the interaction of Cdc37 with Hsp90, while higher concentrations (10 mM) of novobiocin even caused a slight increase in Hsp90-Cdc37 complex formation (54). In contrast, the effect of celastrol is quite different from that of novobiocin. In a reconstituted protein system, 0.001–0.01 mM celastrol induced a dramatic reduction of Cdc37 binding to Hsp90, while 0.1 mM celastrol blocked their interaction completely (Fig. 1D). Celastrol (0.01 mM) also disrupted Hsp90 and Cdc37 interaction in cancer cells while no inhibition of Hsp90-Hsc70-Hop and Hsp90-p23 complexes was noticed under the same condition (41). Therefore, the mechanism by which celastrol inhibits Hsp90 function appears to be distinct from that of Hsp90 C-terminal inhibitors.

Hsp90 function depends on its ability to bind and hydrolyze ATP, and its ATPase activity can be regulated not only by small molecules, but also other modulators such as cochaperones or post-translational modifications (62). Cochaperones preferentially bind to a specific conformation of Hsp90 to modulate its ATPase activity, either by inhibition (such as p23, Cdc37) or activation (such as Aha1) (62). Although the ATP binding and hydrolysis occur at the N-terminal domain, its ATPase activity can be modulated through interaction beyond this site. Sti1, the yeast homolog of Hop, binds to the C terminus of Hsp90 and completely inhibits the ATPase activity of Hsp90 without affecting ATP binding (63, 71). The reason for Sti1 to function like this is because it binds a specific conformation of Hsp90 to prevent the dimerization of N terminus and association of the N- and M-domains (63) (62). Similarly, celastrol interacts with the C terminus of Hsp90 protecting it from cleavage by trypsin digestion (Fig. 4, C and D) and inhibits Hsp90 ATPase activity (Fig. 4A) without inhibiting ATP binding (Fig. 4B). In addition, other modifications of Hsp90 in the C terminus have also shown altered ATPase activity (64). For instance, S-nitrosylation in the Hsp90-C-terminal domain inhibits its ATPase activity (64), although whether it affects ATP binding to Hsp90 was not reported. All these findings reveal the importance of the C terminus in the regulation of Hsp90 ATPase activity. Therefore, we speculate that the inhibition of the ATPase activity by celastrol may relate to its interaction with the Hsp90 C terminus. Thus, celastrol may represent another class of Hsp90 inhibitor that “allosterically” modulates Hsp90 chaperone activity and the superchaperone complex. While this manuscript was under review, Sreeramu et al. (72) showed that celastrol does not bind to Hsp90 N terminus but modifies the cysteine residues in Cdc37. Interestingly, the susceptible target of S-nitrosylation is also a cysteine residue in the Hsp90 C-terminal domain (64). Moreover, our recent data proved that withaferin A, which has been reported to react with protein thiol-nucleophiles, is able to affect Hsp90 and Cdc37 interaction in cells as well (73–75). Therefore, we would not be surprised if celastrol modifies Hsp90 C terminus cysteine residues to inhibit ATPase activity and disrupt Hsp90-Cdc37 complex. However, more work is warranted to map the exact site in Hsp90 where celastrol interacts.

Our study reveals that Cdc37 can only bind ADP-bound or nucleotide-free Hsp90 but not ATP-bound Hsp90, which indi-
Celaspol Disrupts Hsp90-Cdc37 Complex

cates that Cdc37 can only associate with the early but not the mature complex of Hsp90. The data demonstrate that celaspol directly disrupts Hsp90 and Cdc37 interaction in a reconstituted protein system, which confirmed the previously reported in vivo data (41). Celaspol interacts with Hsp90 C terminus, inhibiting its ATPase activity without blocking the ATP-binding site, which is distinctly from the current Hsp90 inhibitors. Additional work is required to locate the exact residues with which celaspol interacts.

Acknowledgments—We thank Dr. Thomas Ratajczak (University of Western Australia) and Dr. Wei Li (University of Southern California Keck School of Medicine) for the generous gifts of expression plasmids, pET15b-hHsp90β, pET28a(+) -hHsp90β (S307–724), and pET15b-hHsp90α. We also acknowledge Dr. Dan Bolon (University of Massachusetts Medical School) for the generous gift of purified yeast Hsp82 protein.

REFERENCES

1. Lai, B. T., Chin, N. W., Stanek, A. E., Keh, W., and Lanks, K. W. (1984) Mol. Cell. Biol. 4, 2802–2810
2. Kamal, A., Boehm, M. F., and Burrows, F. J. (2004) Trends Mol. Med. 10, 283–290
3. Ferrarini, M., Heltai, S., Zocchi, M. R., and Rugarli, C. (1992) Int. J. Cancer 51, 613–619
4. Roe, S. M., Ali, M. M., Meyer, P., Vaughan, C. K., Panaretou, B., Piper, P. W., Prodromou, C., and Pearl, L. H. (2004) Cell 116, 87–98
5. Whitesell, L., and Lindquist, S. L. (2005) Nat. Rev. Cancer 5, 761–772
6. Chiosis, G., and Neckers, L. (2006) ACS Chem. Biol. 1, 279–284
7. Solit, D. B., and Chiosis, G. (2008) Drug Discov. Today 13, 38–43
8. Stebbins, C. E., Russo, A. A., Schneider, C., Rosen, N., Hartl, F. U., and Pavletich, N. P. (1997) Cell 89, 239–250
9. Schmitz, H., and Neckers, L. (1994) Curr. Med. Chem. 1, 280–290
10. Donnelly, J. P., O'Brien, A. T., Morin, M. J., Foster, B. A., Pollack, V. A., Savage, D. M., Sloan, D. E., Pustilnik, L. R., and Moyer, M. P. (1995) J. Biol. Chem. 270, 8385–8395
11. Sullivan, W., Stensgard, B., Caucutt, G., Bartha, B., McMahon, N., Alnenmii, E. S., Litwack, G., and Toft, D. (1997) J. Biol. Chem. 272, 8007–8012
12. Ward, B. K., Allen, R. M., Mok, D., Temple, S. E., Taylor, P., Dornan, J., Mark, P. I., Shaw, D. J., Kumar, P., Walkinshaw, M. D., and Ratcliff, T. (2002) J. Biol. Chem. 277, 40799–40809
13. Ali, J. A., Jackson, A. P., Howells, A. J., and Maxwell, A. (1993) Biochemistry 32, 2717–2724
14. Yun, B. G., Huang, W., Leach, N., Hartson, S. D., and Matts, R. L. (2004) Biochemistry 43, 8217–8229
15. Sullivan, W., Stensgard, B., Cau cott, G., Bartha, B., McMahon, N., Alnenmii, E. S., Litwack, G., and Toft, D. (1997) J. Biol. Chem. 272, 8007–8012
16. Johnson, B. D., Schumacher, R. J., Ross, E. D., and Toft, D. O. (1998) J. Biol. Chem. 273, 3679–3686
17. Sullivan, W. P., Owen, B. A., and Toft, D. O. (2002) J. Biol. Chem. 277, 45942–45948
18. Huang, F. C., Chan, W. K., Moriarity, K. J., Zhang, D. C., Chang, M. N., He, W., Yu, K. T., and Zilberstein, A. (1998) Bioorg. Med. Chem. Lett. 8, 35388 • JOURNAL OF BIOLOGICAL CHEMISTRY

35388 VOLUME 284 • NUMBER 51 • DECEMBER 18, 2009

35388 • JOURNAL OF BIOLOGICAL CHEMISTRY
2. Yang, H., Chen, D., Cui, Q. C., Yuan, X., and Dou, Q. P. (2006) Cancer Res. 66, 4758–4765
3. Trott, A., West, J. D., Klaić, L., Westerheide, S. D., Silverman, R. B., Morimoto, R. I., and Morano, K. A. (2008) Mol. Biol. Cell 19, 1104–1112
4. Lee, J. H., Koo, T. H., Yoon, H., Jung, H. S., Jin, H. Z., Lee, K., Hong, Y. S., and Lee, J. J. (2006) Biochem. Pharmacol. 72, 1311–1321
5. Wandinger, S. K., Richter, K., and Buchner, J. (2008) J. Biol. Chem. 283, 18473–18477
6. Richter, K., Muschler, P., Hainzl, O., Reinstein, J., and Buchner, J. (2003) J. Biol. Chem. 278, 10328–10333
7. Martinez-Ruiz, A., Villanueva, L., Gonzalez de Orduña, C., López-Ferrer, D., Higuera, M. A., Tarín, C., Rodríguez-Crespo, I., Vázquez, J., and Lamas, S. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 8525–8530
8. Onuoha, S. C., Coulstock, E. T., Grossmann, J. G., and Jackson, S. E. (2008) J. Mol. Biol. 379, 732–744
9. Zhao, R., and Houry, W. A. (2005) Biochem. Cell Biol. 83, 703–710
10. Caplan, A. J., Mandal, A. K., and Theodoraki, M. A. (2007) Trends Cell Biol. 17, 87–92
11. Hartson, S. D., Irwin, A. D., Shao, J., Scroggins, B. T., Volk, L., Huang, W., and Mats, R. L. (2000) Biochemistry 39, 7631–7644
12. Lee, P., Shabbir, A., Cardozo, C., and Caplan, A. J. (2004) Mol. Biol. Cell 15, 1785–1792
13. Marcu, M. G., Chadli, A., Bouhouche, I., Catelli, M., and Neckers, L. M. (2000) J. Biol. Chem. 275, 37181–37186
14. Prodromou, C., Siligardi, G., O’Brien, R., Woolfson, D. N., Regan, L., Panaretou, B., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1999) EMBO J. 18, 754–762
15. Seeramulu, S., Gande, S. L., Göbel, M., and Schwalbe, H. (2009) Angew. Chem. Int. Ed. Engl. 48, 5853–5855
16. Fuska, J., Fusková, A., Rosazza, J. P., and Nicholas, A. W. (1984) Neoplasma 31, 31–36
17. Khaled, M., Vanden Berghe, W., Heyerick, A., Horion, J., Piette, J., Libert, C., De Keukeleire, D., Essawi, T., and Haegeman, G. (2007) J. Biol. Chem. 282, 4253–4264
18. Yu, Y., Hamza, A., Zhang, T., Gu, M., Zou, P., Newman, B., Li, Y., Gunatilaka, A. A. L., Whitesell, L., Zhan, C. G., and Sun, D. (2009) Biochem. Pharmacol., in press