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The effect of celastrol, a triterpene with antitumorigenic activity, on conformational and functional aspects of the human 90 kDa heat shock protein Hsp90α, a chaperone implicated in the stabilization of the tumor phenotype

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ABSTRACT

Background: Hsp90 is a molecular chaperone essential for cell viability in eukaryotes that is associated with the maturation of proteins involved in important cell functions and implicated in the stabilization of the tumor phenotype of various cancers, making this chaperone a notably interesting therapeutic target. Celastrol is a plant-derived pentacyclic triterpenoid compound with potent antioxidant, anti-inflammatory and anticancer activities; however, celastrol's action mode is still elusive.

Results: In this work, we investigated the effect of celastrol on the conformational and functional aspects of Hsp90α. Interestingly, celastrol appeared to target Hsp90α directly as the compound induced the oligomerization of the chaperone via the C-terminal domain as demonstrated by experiments using a deletion mutant. The nature of the oligomers was investigated by biophysical tools demonstrating that a two-fold excess of celastrol induced the formation of a decameric Hsp90α bound throughout the C-terminal domain. When bound, celastrol destabilized the C-terminal domain. Surprisingly, standard chaperone functional investigations demonstrated that neither the in vitro chaperone activity of protecting against aggregation nor the ability to bind a TPR co-chaperone, which binds to the C-terminus of Hsp90α, were affected by celastrol.

Conclusion: Celastrol interferes with specific biological functions of Hsp90α. Our results suggest a model in which celastrol binds directly to the C-terminal domain of Hsp90α causing oligomerization. However, the ability to protect against protein aggregation (supported by our results) and to bind to TPR co-chaperones are not affected by celastrol. Therefore celastrol may act primarily by inducing specific oligomerization that affects some, but not all, of the functions of Hsp90α.

General significance: To the best of our knowledge, this study is the first work to use multiple probes to investigate the effect that celastrol has on the stability and oligomerization of Hsp90α and on the binding of this chaperone to Tom70. This work provides a novel mechanism by which celastrol binds Hsp90α.

1. Introduction

Cancer is the generic name for diseases in which abnormal cells divide without control and are invasive (malignant). According to the World Health Organization, cancer is a leading cause of death worldwide, and although important progress has been made toward its treatment, considerable research remains to be undertaken to understand the molecular basis of cancers and to identify suitable methods of treatment. There have been several studies on cancer-identified genes, later named oncogenes, which are often mutated or highly expressed in tumor cells, and many of the oncogenic proteins depend on the 90 kDa heat shock protein Hsp90 to be stable and functional (for reviews see [1–4]). As a matter of fact, Hsps are highly expressed in several cancerous tumors, most likely because they favor cell growth in stressed environments; thus, Hsps have become important targets for cancer therapy.

Hsp90 exist as homodimers (~160 kDa) in which each subunit is discretized into three domains [5–7]. The N-terminal domain contains an ATP-binding site [8] and binds natural compounds with anti-tumoral activities, such as geldanamycin (from *Streptomyces hygroscopicus*) and radicicol (also known as monorden) [9,10]. The central domain is highly charged and has a high affinity for co-chaperones and client proteins [11–13]. The C-terminal domain is essential for dimerization [14] and

Abbreviations: CD, circular dichroism; DLS, dynamic light scattering; DSC, differential scanning calorimetry; DSF, differential scanning fluorimetry; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SEC–MALS, size-exclusion chromatography coupled to multi-angle light scattering; C-Hsp90α, C-terminal domain of Hsp90α; Hsp90α, 90 kDa heat shock protein α-isoform (paralog)

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has also been demonstrated to bind compounds with anti-tumoral activities, such as cisplatin [15], novobiocin [16] and epigallocatechin-3-gallate (EGCG) [17]. It could appear counterintuitive to label an important chaperone such as Hsp90 as a cancer target, when considering its vital role in normal cells. However, cancer drugs such as 17-AAG, which inhibit Hsp90, are well tolerated by patients [18], likely because Hsp90 found in cancer cells has a much higher affinity for 17-AAG than does Hsp90 from normal cells [19,20]. Kamal et al. [19] showed that Hsp90 derived from cancer cells binds to 17-AAG up to 100 times more tightly than does Hsp90 isolated from normal cells. Hsp90 in normal cells would be in a free form while in malignant cells the chaperone would be associated with a multi-protein complex and would have a much higher ATPase activity [19,21,22]. Because cancer cells have high protein (normal and mutant) expression and live in a stressful microenvironment, they would be very dependent on Hsp90 that is in a highly activated state [3]. Other inhibitors apparently are also more effective against Hsp90 isolated from cancer cells than from normal cells [reviewed in 23]. Consequently, studies on the interaction between recombinant Hsp90 and ligands have gained increased attention worldwide, due to the potential of this chaperone as a therapeutic target for cancer [3,4,24].

Humans have two cytosolic isoforms of Hsp90, named α and β, that are highly identical [7,25]. The literature is rich in evidence that Hsp90α, which was investigated in this study, is linked to cancer and may have a diverse action than the β isoform. To mention a few examples: only Hsp90α, but not Hsp90β, has the capacity to activate oncogenic kinases [26]; Hsp90α has elevated expression in brain, skin and thyroid cancers, while Hsp90β has higher expression in bladder cancer [27]; Hsp90α is secreted by a variety of tumor cell lines, enhancing invasiveness and thus being considered a valid target for anticancer therapies (revised in [25]).

Celastrol, a quinone methide triterpene isolated from plants belonging to the family *Celastraceae* (used in Oriental medicine), has demonstrated potential anticancer activity in several cancer models, including prostate cancer, pancreatic cancer, leukemia and melanoma (See the following review and references therein: [28]). Celastrol has some action upon Hsps, the compound induces HSF-1 (heat shock factor-1) in a similar way than a heat shock treatment (42 °C) [29] and it was identified as a ligand of Hsp90 [30]. Although several investigations on the interaction of Hsp90 and celastrol have been performed, the true nature of the molecular mechanism of this chaperone-ligand interaction is still elusive. For instance, studies based on the effect of celastrol on the Hsp90–Cdc37 interaction [31] and ATPase inhibition [32,33] have suggested that celastrol binds to the N-terminal domain as this is the site of Cdc37 and ATP binding. However, trypsinolysis studies [32] suggested that celastrol binds to the C-terminal domain of the Hsp90. In addition, the targets of the celastrol in the Hsp90 complexes are still a matter of intense debate. Some studies have reported the inhibition of the Hsp90 ATPase activity by celastrol [31,33], whereas others have demonstrated no effect [31,34]. By using HSQC NMR experiments, Sreeramulu and co-workers [35] demonstrated that celastrol disrupted the Hsp90–Cdc37 complex not by binding directly to a deletion mutant of Hsp90 representing the N-terminal domain but by targeting Cdc37. Co-immunoprecipitation experiments provide conflicting results, as they have demonstrated that the Hsp90/HOP complex both could [36] and could not [31] be disrupted by celastrol.

Motivated by the need for further investigation regarding Hsp90α-celastrol interaction, we performed an extensive characterization of the interaction between them using biochemical and biophysical complementary techniques, such as size-exclusion chromatography coupled to multi-angle laser light scattering (SEC–MALS), native-PAGE, dynamic light scattering (DLS), differential scanning calorimetry (DSC), differential scanning fluorescence (DSF) and chaperone and protein–protein assays. We found that celastrol affected the oligomeric state of Hsp90α by binding to the C-terminal domain. Moreover, we report here that celastrol destabilized the C-terminus of Hsp90 but had neither an effect on the interaction of Hsp90α with its co-chaperone Tom70, which binds to the C-terminus of Hsp90α nor on the functional activity of Hsp90α against protein aggregation. To the best of our knowledge, this study is the first work to use multiple probes to investigate the effect that celastrol has on the stability and oligomerization of Hsp90α and on the binding of this chaperone to Tom70.

2. Materials and methods

2.1. Protein expression and purification

The expression and purification of the C-terminal domain of human Hsp90α (C-Hsp90α, residues 566–732) and the soluble fraction of human Tom70 (residues 111–608) were performed as previously described [37]. *Escherichia coli* strain BL21(DE3) was transformed with a pProExHta vector, in which human full-length Hsp90α was cloned and grown at 37 °C until A_{600} reached 0.8–1.0. After this step the temperature of growth was set to 18 °C, and protein expression was induced with 1.0 mM isopropyl-beta-D-thiogalactopyranoside. After overnight induction, the cells were harvested and disrupted by sonication in a buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 1 mM EDTA, 30 μg/ml lysozyme, 1 mM PMSF and 5 units DNase. The lysate was cleared by centrifugation, and the proteins were purified using nickel metal affinity chromatography (eluted by 500 mM imidazole and dialyzed afterwards) followed by size-exclusion chromatography (Superdex 200) in a buffer containing 20 mM Tris–HCl, pH 7.4, and 150 mM NaCl. Sample purity was analyzed by SDS-PAGE, and protein concentration was determined by measuring UV absorbance at 280 nm. Celastrol was kept soluble by dilution into dimethyl sulfoxide (DMSO), and the final concentration of DMSO was never higher than 2% in the experiments described below.

2.2. Circular dichroism

A JASCO model J-810 CD spectropolarimeter coupled to a thermoelectric sample temperature controller (Peltier system) was used to record circular dichroism (CD) spectra using parameters previously described [38]. Briefly, experiments were conducted with 15 μM C-Hsp90α or 10 μM of full-length Hsp90α and 9 μM Tom70 in a buffer containing 20 mM Tris–HCl, pH 7.4, and 150 mM NaCl at 20 °C. Data were collected from 260 to 200 nm using cuvettes with a 1 mm path length, and the results reported are the average of at least three experiments.

2.3. Differential Scanning Fluorescence

DSF (Differential Scanning Fluorescence) was performed using an iCycler iQ Real-Time PCR detection system with excitation and emission wavelengths of 580 and 623 nm, respectively, and monitoring with a CCD camera. Samples were 2.5 μL solutions of Sypro Orange (8×), 12.5 μL of buffer alone (control) or protein containing ligand (celastrol) and 5 μL of buffer reaction. The protein concentrations were 2 μM for either full-length Hsp90α or C-Hsp90α. Samples were added to a PCR plate with 96 wells that was sealed and heated from 20 to 90 °C. With the transition temperature of protein unfolding, Tm, a Boltzman equation was used to fit the fluorescence curve:

$$I = (A + (B\text{-}A) / (1 + \exp((Tm\text{-}T)/C)$$

where I is the fluorescence intensity at temperature T; A and B is the fluorescence intensity before and after of the transition, respectively; C is the slope of the curve [39]. DMSO (2%) was present in all samples and did not interfere with the results as confirmed by control experiments.