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Cisplatin differently affects amino terminal and carboxyl terminal domains of HSP90

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Abstract **The 90-kDa heat shock protein (HSP90) is a molecular chaperone that assists in the folding and assembly of proteins in the cytosol. We previously demonstrated that the antineoplastic reagent, cisplatin, inhibits the aggregation prevention activity of mammalian HSP90. We now show that cisplatin binds both the amino terminal and carboxyl terminal domains of the human HSP90 and differently affects these two domains. Cisplatin blocks the aggregation prevention activity of HSP90C, but not HSP90N. In contrast, cisplatin induces a conformational change in HSP90N, but not HSP90C. These results indicate that cisplatin modulates the HSP90 activities through two different mechanisms using the two distinct binding sites of the HSP90 molecule.**

Keywords: HSP90; Molecular chaperone; Cisplatin; Aggregation prevention; Conformational change

1. Introduction

The 90-kDa heat shock protein (HSP90) is a molecular chaperone highly conserved from prokaryotes to eukaryotes. In mammalian cells, HSP90 is the most abundant molecular chaperone in the cytosol and exerts a variety of essential functions. These functions include aggregation prevention, maintenance, transport, assembly and degradation of client proteins and are performed through an ATP-dependent conformational change [1–4].

HSP90 is a multi-domain protein consisting of three domains: the N-terminal flexible domain (HSP90N) that binds ATP, the middle domain (HSP90M), and the C-terminal domain (HSP90C) required for dimerization. The HSP90N and HSP90C domains are both reported to have aggregation prevention activities [5,6]. Recently, X-ray crystallographic and electron microscopic analyses revealed that HSP90 undergoes a large conformational change using these domains during the ATPase cycle [7,8].

HSP90 associates with a variety of co-chaperones including the activator of HSP90 ATPase (Aha1) [9], p23 [10] and HSP90/HSP70-organizing protein (Hop) [11]. These co-chaperones activate the ATPase activity of HSP90. Many signaling protein kinases (e.g., Cdk4, Cdk6 and Raf family kinases) are stabilized by HSP90 in the presence of Cdc37 [12,13]. HSP90 associates with steroid hormone receptors (e.g., glucocorticoid receptor, androgen receptor and estrogen receptor) and regulates the transport of these receptors [14,15]. During this process, a multi-chaperone complex containing HSP90, HSP70, Hop, HSP40, and p23 associates with steroid hormone receptors, and HSP90 binds HSP70 via the TPR domains of Hop in this complex [16,17]. Since this complex is abundant in tumor cells, inhibition of the HSP90 function by antineoplastic agents like geldanamycin and its derivative, 17-allylamino-17-demethoxygeldanamycin (17-AAG), is effective for treating cancers [18].

Cisplatin (*cis*-diamminedichloroplatinum) is a widely used antineoplastic drug for clinical cures. Cisplatin possesses the ability to bind DNA and inhibits the replication of DNA, thereby it inhibits tumor cell growth [19]. Previously, we found that cisplatin directly binds HSP90 and inhibits its aggregation prevention activity in vitro [20]. The cisplatin affinity chromatography of tryptic peptides of HSP90 suggested that there is a cisplatin binding site in the HSP90C domain. Cisplatin is reported to stimulate the dimer formation of HSP90 and inhibit the HSP90-dependent activation of the glucocorticoid receptor in vitro [21]. Since cisplatin inhibits glucocorticoid receptor-dependent and androgen receptor-dependent transcriptional activities in a dose-dependent manner in human cultured cells [22], inhibition of the HSP90 by cisplatin is considered to occur in vivo as well as in vitro. However, the molecular mechanism in which cisplatin inhibits the HSP90 activity is largely unknown.

We analyzed the aggregation prevention activity and conformational change in the purified recombinant HSP90N and HSP90C domains in the presence of cisplatin in vitro. Cisplatin blocked the aggregation prevention activity of HSP90C, but not HSP90N, whereas this drug induced a conformational change in HSP90N, but not HSP90C. We will discuss the role of HSP90N and HSP90C during the cisplatin-dependent inhibition of the HSP90 activities.

2. Materials and methods

2.1. Purification of HSP90, HSP90N and HSP90C

Full-length HSP90 was purified from porcine brain by ammonium sulfate fractionation, Q-Sepharose and hydroxyapatite column chromatography [23]. The amino terminal domain (1–236) or carboxy

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terminal domain (627–732) of the human HSP90α tagged with 6xHis were expressed in *Escherichia* coli BL21. These domains were purified by Ni-affinity column chromatography and dialyzed against 10 mM Tris–HCl (pH 7.4).

2.2. Cisplatin affinity column chromatography

Cisplatin–sepharose column chromatography was basically carried out as previously described [20]. HSP90 was applied to a cisplatin–sepharose column equilibrated with 10 mM Tris–HCl (pH 7.4) and washed with 10 column volumes of 10 mM Tris–HCl (pH 7.4) containing 0.15 M NaCl. The bound proteins were eluted with free cisplatin. The eluted proteins were analyzed by SDS–PAGE (9% gel) followed by Coomassie Brilliant Blue staining. Transplatin-affinity column chromatography was carried out as described above except that transplatin (Sigma, St. Louis, MO) was used instead of cisplatin.

2.3. Far-UV circular dichroism (CD)

The CD measurements were performed by a J-720 spectropolarimeter (Jasco, Tokyo, Japan). The CD spectrum (190–240 nm) of HSP90N (1.1 μM) or HSP90C (1.1 μM) in 50 mM HEPES-NaOH buffer (pH 7.4) in the presence or absence of cisplatin (11.0 μM) were recorded at 25 °C using a cuvette with a 0.5-mm path length. The observed specific ellipticity after normalization against a blank was converted to the mean residue ellipticity [θ] (degrees cm^2 $dmol^{-1}$).

2.4. Measurement of protein aggregation

Thermal aggregation of 0.05 μM mitochondrial citrate synthase (CS) (Roche, Mannheim, Germany) in 10 mM Tris–HCl buffer (pH 7.4) at 50 °C was monitored by optical density at 500 nm using a Pharmacia Ultrospec 3000 UV–vis spectrophotometer equipped with a semi-micro-cuvette (0.5 ml) with a path length of 10 mm and a temperature control unit. In this study, 1 arbitrary unit denotes the absorbance of 0.15. HSP90, HSP90N or HSP90C (0.05 μM) was added to the CS solution in the presence or absence of 2 mM cisplatin.

2.5. Protease resistance assay

HSP90, HSP90N, or HSP90C (1.0 μM) was incubated in the presence of 4.3 nM trypsin in 10 mM Tris–HCl (pH 7.4) at 37 °C for 30 min. ATP (5 mM) or cisplatin (2.33 mM) were added to the reaction to evaluate their effect. After incubation, these samples were analyzed by SDS–PAGE and Coomassie Brilliant Blue staining.

3. Results

3.1. Cisplatin binds both HSP90N and HSP90C

We previously reported that the wild type HSP90 binds cisplatin as analyzed by cisplatin affinity column chromatography [20]. This binding was confirmed in the present study with a control experiment showing that transplatin, a geometric isomer of cisplatin, does not bind HSP90 (Fig. 1A). These results confirmed that cisplatin specifically binds to HSP90. We also previously reported that the C-terminal tryptic fragment of HSP90 could be purified by cisplatin affinity chromatography, suggesting the existence of a cisplatin binding site at the C terminus. To analyze whether cisplatin exclusively binds the C-terminal domain, we prepared recombinant HSP90N and HSP90C proteins and carried out cisplatin column chromatography (Fig. 1B). The elution patterns indicated that cisplatin can bind both HSP90N and HSP90C, and suggested that cisplatin binds HSP90C, slightly more strongly than HSP90N.

3.2. Cisplatin inhibits aggregation prevention activity of HSP90C, but not HSP90N

HSP90 inhibits CS aggregation and cisplatin blocks the HSP90-dependent aggregation prevention [20]. To determine which domain is affected by cisplatin, aggregation prevention by HSP90N or HSP90C against CS was tested in the presence

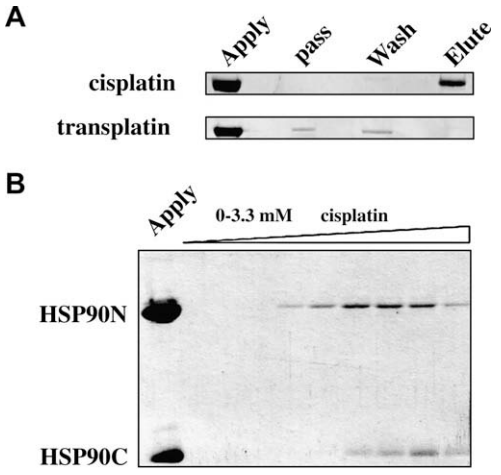

Fig. 1. Cisplatin specifically binds HSP90N and HSP90C. (A) HSP90 purified from bovine-brain was applied to cisplatin or transplatin-affinity columns and washed with 0.15 M NaCl in 10 mM Tris–HCl, pH 7.4. Proteins were eluted with free cisplatin (3.33 mM) or transplatin (1.67 mM). Fractions were analyzed by SDS–PAGE (9% gel) followed by Coomassie brilliant blue staining. (B) Recombinant human HSP90N or HSP90C domains were applied to cisplatin affinity column and washed. Proteins were eluted with a linear gradient of cisplatin (0–3.33 mM). Fractions were analyzed by SDS–PAGE (11% gel).

of cisplatin. The CS aggregation was inhibited in the presence of HSP90C and this aggregation prevention effect was significantly reduced by the cisplatin (Fig. 2B) although the effect of cisplatin was slightly weaker than the case of the full-length HSP90 (Fig. 2A). In contrast, the HSP90N-dependent aggregation prevention was not affected by the presence of cisplatin (Fig. 2C). These results appear to be consistent with the observation that cisplatin binds more strongly to HSP90C than HSP90N. Despite the fact that full-length HSP90 posses the HSP90N domain that is potentially unable to be inhibited by cisplatin for aggregation prevention activity, this drug did inhibit full-length HSP90. This may due to global conformational changes in full-length HSP90 (e.g. interaction between HSP90N and HSP90C) in the presence of cisplatin, because it induces alteration of secondary structure of HSP90N as described bellow.

3.3. Cisplatin alters secondary structure of HSP90N, but not HSP90C

We also previously reported that cisplatin induces changes in the secondary structures of the full-length HSP90 as determined by the CD spectrum. To analyze which domain is responsible for the conformational change, structural changes in the HSP90N and HSP90C were analyzed by CD measurements in the presence of cisplatin. Intriguingly, HSP90N, but not HSP90C, underwent structural changes in the presence of cisplatin (Fig. 3). These results indicate that cisplatin differently affects the HSP90N and HSP90C.

3.4. Cisplatin induces protease resistance of HSP90N but not HSP90C

To examine whether the binding of cisplatin to HSP90N and HSP90C induces conformational changes, the protease resistance was analyzed in the presence and absence of cisplatin (Fig. 4A). We checked the effect of ATP for protease resistance