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Article

ITZ-1, a Client-Selective Hsp90 Inhibitor, Efficiently Induces Heat Shock Factor 1 Activation

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SUMMARY

ITZ-1 is a chondroprotective agent that inhibits interleukin-1β–induced matrix metalloproteinase–13 (MMP-13) production and suppresses nitric oxide–induced chondrocyte death. Here we describe its mechanisms of action. Heat shock protein 90 (Hsp90) was identified as a specific ITZ-1–binding protein. Almost all known Hsp90 inhibitors have been reported to bind to the Hsp90 N-terminal ATP-binding site and to simultaneously induce degradation and activation of its multiple client proteins. However, within the Hsp90 client proteins, ITZ-1 strongly induces heat shock factor–1 (HSF1) activation and causes mild Raf-1 degradation, but scarcely induces degradation of a broad range of Hsp90 client proteins by binding to the Hsp90 C terminus. These results may explain ITZ-1's inhibition of MMP-13 production, its cytoprotective effect, and its lower cytotoxicity. These results suggest that ITZ-1 is a client-selective Hsp90 inhibitor.

INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease characterized by slow but irreversible progressive destruction of articular cartilage. The sustained production of interleukin (IL)–1 in the affected joint may play a key role in OA pathogenesis (Aigner et al., 2006; Ge et al., 2006). IL-1 induces up-regulation of matrix metalloproteinases (MMPs) that degrade principal matrix macromolecules, such as proteoglycans and collagens, in cartilage. Proteoglycans have a high turnover rate, whereas type II collagen has a low turnover rate; thus, type II collagen degradation is a committed step in OA progression. Within the approximately 20-member MMP-family, MMP-1, MMP-8, and MMP-13 are interstitial collagenases that degrade type II collagen; in particular, MMP-13 may play a pivotal role in cartilage degradation (Ge et al., 2006; Takaishi et al., 2008).

IL-1 has also been reported to induce death in chondrocytes, which regulate cartilage metabolism, probably via nitric oxide (NO) production (Yasuhara et al., 2005). Because the chondrocytes constitute the only cell type of the articular cartilage, survival of the chondrocytes is essential for the maintenance of normal articular cartilage (Presle et al., 1999; Kühn et al., 2004). Because there are no known therapeutic agents proven to prevent erosion of articular cartilage or slow disease progression, agents that interfere with cartilage destruction would be of considerable therapeutic value (Kühn et al., 2004; Aigner et al., 2006; Ge et al., 2006).

To develop therapeutic drugs for OA, we have searched for chondroprotective agents and found *N*-{(1*Z*)-5,6-Dimethyl-3-oxo-8-[(4,4,5,5,5,-pentafluoropentyl)thio]-2,3-dihydro-1*H*-imidazo[5,1-*c*][1,4]thiazin-1-ylidene}-4methylbenzenesulfonamide, ITZ-1 (Kimura et al., 2009), which suppresses IL-1β–induced cartilage degradation in vitro and in vivo (Pettipher et al., 1986; Cawston et al., 1995; Bottomley et al., 1997). ITZ-1 selectively inhibits IL-1β–induced MMP-13 production with a half-maximal inhibitory concentration (IC_{50}) of 0.51 μM via inhibition of extracellular signal–regulated protein kinase (ERK) activation (Vincenti and Brinckerhoff, 2002; Ahmed et al., 2004, 2005), and suppresses NO-induced death in human articular chondrocytes (HACs) (Terauchi et al., 2003). Thus, ITZ-1 could be a promising lead compound for a disease-modifying anti-OA drug program. However, the precise mechanisms of action of ITZ-1 have been poorly understood.

Here, we describe the molecular mechanisms of ITZ-1. We identified heat shock protein 90 (Hsp90) (Whitesell and Lindquist, 2005; Sharp and Workman, 2006; Neckers, 2007) as a target protein of ITZ-1 by use of drug-affinity chromatography (Harding et al., 1989; Taunton et al., 1996; Shimizu et al., 2000; Sato et al., 2007) and have further elucidated its downstream molecular mechanisms. Hsp90 is involved in the folding, activation, and assembly of several proteins, known as Hsp90 client proteins, and the inhibition of Hsp90 function has been considered to simultaneously induce degradation and activation of these multiple client proteins (Whitesell and Lindquist, 2005; Sharp and Workman, 2006; Neckers, 2007). However, several recent reports have suggested the existence of small molecule inhibitors of Hsp90 with client selective properties (Yu et al., 2005; Salehi et al., 2006; Gallo, 2006; Ansar et al., 2007; Lu et al., 2009). Interestingly, ITZ-1 strongly induced heat shock factor–1 (HSF1) activation and caused mild Raf-1 degradation without affecting other Hsp90 client proteins by binding to the Hsp90 C terminus (Whitesell and Lindquist, 2005). Considering the definitive evidence that Hsp90 plays pivotal roles in multiorgan physiology and pathology, the discovery we present here may provide an approach to the development of therapeutic drugs targeting Hsp90.

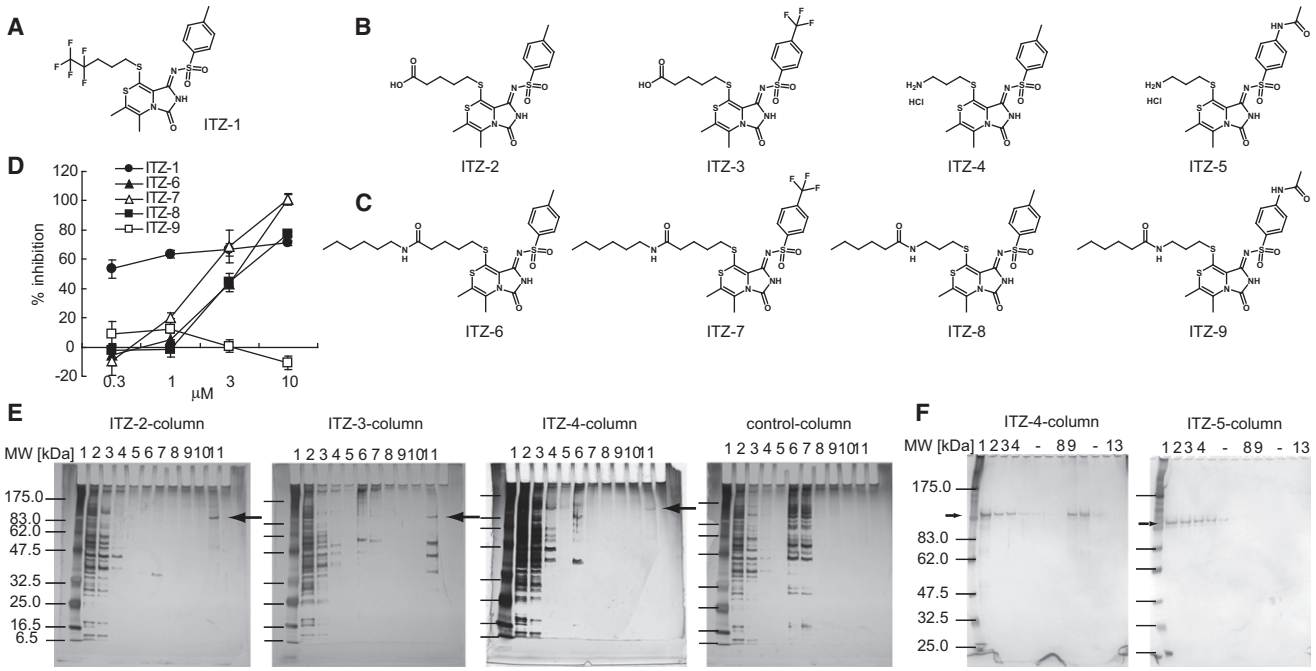

Figure 1. Identification of Hsp90 as an ITZ-1-Binding Protein

(A) Chemical structure of ITZ-1.

(B) Chemical structures of ITZ-1 derivatives used for drug-affinity chromatography.

(C) Chemical structures of ITZ-1 derivatives used to estimate the potencies of derivatives shown in (B) on the matrix.

(D) Inhibitory activities of the ITZ-1 derivatives against IL-1β–induced MMP-13 production in HACs. Results shown are means ± SD (n = 3).

(E) Identification of Hsp90 from protein extracts as an ITZ-1–binding protein by drug-affinity chromatography. ITZ-2-, ITZ-3-, and ITZ-4-columns were used as positive columns. As a control, a matrix on which no compound was immobilized was used (control-column). Lane 1, molecular weight (MW) marker; lane 2, protein extract; lane 3, flow-through fraction; lanes 4 and 5, binding buffer wash fractions; lanes 6–10, 1 M NaCl-eluted fractions; and lane 11, glycine-HCl buffer-eluted fraction. Arrows indicate the Hsp90 protein.

(F) Direct binding of His-hHsp90α to the ITZ-4-column. The ITZ-5-column was used as a negative control. Lane 1, MW marker; lane 2, His-hHsp90α; lane 3, flow-through fraction; lanes 4–8, binding buffer wash fractions; and lanes 9–13, 6 M urea buffer-eluted fractions. Arrows indicate the His-hHsp90α protein. See also Table S1.

RESULTS

Identification of Hsp90 as a Possible ITZ-1–Binding Protein

The initial goal of the study was to identify the possible target proteins of ITZ-1 (Figure 1A; see Table S1 available online) by use of drug-affinity chromatography. A terminal carboxyl or amino group was needed to couple small molecules to AF-amino or AF-carboxy TOYOPEARL 650 matrix; however, ITZ-1 does not possess these groups. Thus, two carboxyl derivatives, ITZ-2 and ITZ-3, and two amino derivatives, ITZ-4 and ITZ-5, of ITZ-1 were prepared for affinity chromatography (Figure 1B; Table S1). ITZ-2 and ITZ-4 are structurally similar to ITZ-1. ITZ-3 and ITZ-5 were selected because previous studies have shown that introduction of a trifluoromethyl group to the 4-position of benzene ring in the (phenylsulfonyl)imino group retained activity, whereas introduction of an acetylamino group to the same position resulted in a decrease in activity. Because each compound was immobilized to the matrix by coupling with a spacer arm at the terminal carboxyl or amino group, we estimated the potency of each compound on the matrix by using compounds coupled with a long alkyl chain instead of a spacer arm. Potencies of ITZ-2, ITZ-3, ITZ-4, and ITZ-5 on the matrix were estimated with ITZ-6, ITZ-7, ITZ-8, and ITZ-9, respectively (Figure 1C; Table S1). ITZ-6, ITZ-7, and ITZ-8 inhibited IL-1β–induced MMP-13 production and exhibited an IC_{50} of 3.2 μM, 2.0 μM, and 4.0 μM, respectively, whereas ITZ-9, even at 10 μM, did not show any detectable activity (Figure 1D). We hypothesized that common binding proteins of matrix coupled with ITZ-2, ITZ-3, and ITZ-4, but not ITZ-5, would also bind to ITZ-1. Thus, columns of ITZ-2–, ITZ-3–, and ITZ-4–coupled matrixes (ITZ-2-column, ITZ-3-column, and ITZ-4-column) were used as positive columns, and an ITZ-5–coupled matrix column (ITZ-5-column) was used as a negative column. We also utilized a column of AF-amino TOYOPEARL 650 matrix on which no compound was immobilized as another negative control column (control-column).

There was a possibility that the binding affinity of ITZ-5 to the target proteins was only 10 times less than that of ITZ-2, ITZ-3, and ITZ-4 (Figure 1D), which could result in the binding of target proteins to both positive and negative columns in some condition. Therefore, we used control-column instead of ITZ-5-column for initial search. Chondrocyte protein extracts were applied to the ITZ-2-, ITZ-3-, ITZ-4-, and control-column. After washing the columns with binding buffer, bound proteins were eluted with 1 M NaCl-containing buffer followed by glycine-HCl buffer.

By analyzing each fraction, a common binding protein with a molecular mass of ~90 kDa (indicated by arrows) was identified in the glycine-HCl buffer-eluted fractions of the three positive columns (Figure 1E). This 90-kDa protein was not obtained from the control-column, indicating that it was retained in these positive columns by binding to the compound, but not to the solid support. Amino acid sequence analysis revealed that the 90-kDa protein was Hsp90. The two closely related cytosolic isoforms of Hsp90 (Hsp90α and Hsp90β) were identified (Whitesell and Lindquist, 2005).

To exclude the possibility that the interactions between the ITZ-1 derivatives and Hsp90 are indirect or nonspecific, we analyzed the binding of ITZ-1 derivatives to purified recombinant Hsp90α (His-hHsp90α) using the drug-coupled columns. The ITZ-4-column was used as a positive column, and the ITZ-5-column was used as a negative column. His-hHsp90α was diluted with binding buffer and then applied to the drug-coupled columns. After washing the columns with binding buffer, bound protein was eluted with the elution buffer containing 6 M urea. Analysis of each fraction by SDS-PAGE followed by silver staining revealed that His-hHsp90α was retained in the positive ITZ-4-column, but not in the negative ITZ-5-column, and was eluted with elution buffer (Figure 1F). These results suggest that ITZ-1 derivatives interact directly with Hsp90α, and the rank order of the derivatives in the inhibition of MMP-13 production might agree well with that of the binding affinity for Hsp90.

Direct Interaction Between ITZ-1 and Hsp90α

Next, we investigated the direct interaction between ITZ-1 derivatives and Hsp90α by two distinct binding assays. To obtain further insights into the structure-activity relationship for ITZ-1 derivatives, we selected ITZ-1 and three other derivatives without the terminal carboxyl/amino group or the long alkyl chain (ITZ-10, ITZ-11, and ITZ-12; Figure 2A; Table S1). ITZ-1 was most potent, followed by ITZ-10, ITZ-11, and ITZ-12, in the inhibition of IL-1β–induced MMP-13 production in HACs (Figure 2B).

First, we analyzed the ITZ-1-Hsp90α interaction with the ITZ-3–immobilized matrix. To test the functionality of the ITZ-3–immobilized matrix, ITZ-3–immobilized matrix or control matrix was incubated with His-hHsp90α with agitation, and then bound protein was analyzed by SDS-PAGE followed by Coomassie brilliant blue R250 (CBB) staining. His-hHsp90α was recovered from the ITZ-3–immobilized matrix, but not from the control matrix (Figure 2C). This result shows that ITZ-3–immobilized matrix can be used to measure the affinity of ligands that bind to the ITZ-3–binding site of Hsp90α. Under these conditions, the binding reactions were performed in the presence of free compounds. Analysis of the bound protein to the ITZ-3–immobilized matrix revealed that ITZ-1 was most potent, followed by ITZ-10, ITZ-11, and ITZ-12, in the competitive inhibition of Hsp90α-ITZ-3 binding (Figure 2D).

Next, we developed a scintillation proximity assay (SPA assay) based on the detection of the radioligand [^{3}H]-ITZ-1 binding to a 6His-tagged Hsp90α protein (His2-hHsp90α), which was captured by Ysi (2–5 μm) copper His-tag SPA beads (Graziani et al., 1999). In this assay, [^{3}H]-ITZ-1 binding to Hsp90α led to the excitation of the scintillant contained in the SPA beads, and the concomitant light emission was measured. Using 40 nM of [^{3}H]-ITZ-1, specific interactions between His2-hHsp90α and ITZ-1 were detected; however, when other 6His-tagged proteins were used instead of His2-hHsp90α, [^{3}H]-ITZ-1 binding was undetectable (Figure S1A). These data indicate that ITZ-1 itself interacts specifically with Hsp90α.

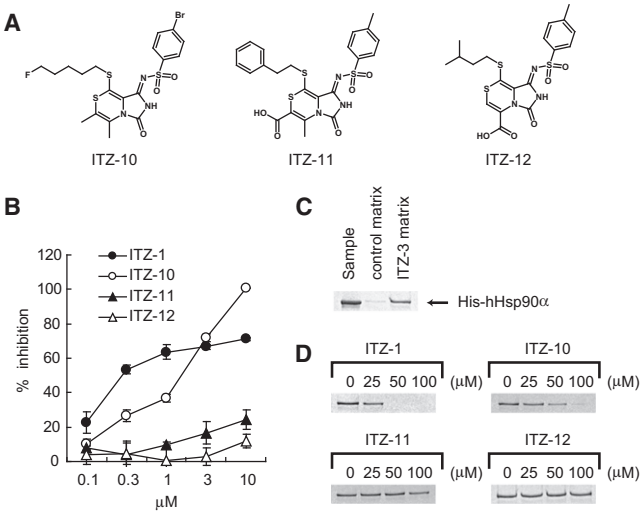

Figure 2. Direct Interaction between ITZ-1 and Hsp90

(A) Chemical structures of ITZ-1 derivatives.

(B) MMP-13 production inhibitory activities of ITZ-1 derivatives. Results shown are means ± SD (n = 3).

(C) Specific binding of His-hHsp90α to ITZ-3–immobilized matrixes. ITZ-3–immobilized matrix or control matrix was incubated with His-hHsp90α with agitation, and then bound protein was analyzed by SDS-PAGE followed by CBB staining.

(D) Displacement studies with ITZ-1 derivatives by the binding assay using ITZ-3-immobilized matrixes. The binding reactions were performed as in (C) in the presence of free compounds. See also Table S1and Figure S1.

In the SPA assay measuring the competitive inhibition of [^{3}H]-ITZ-1 binding to His2-hHsp90α, ITZ-1 was the most potent, followed by ITZ-10, ITZ-11, and ITZ-12, and exhibited IC_{50} of 1.9 μM, 3.9 μM, 86 μM, and 100 μM, respectively (Figure S1B). Note that the rank order of the derivatives in the inhibition of MMP-13 production agrees well with the binding affinities for Hsp90α (Figures 2B and D and Figure S1B). ITZ-1 might inhibit MMP-13 production via binding to Hsp90. Interestingly, geldanamycin (GA), which binds to the Hsp90 N-terminal ATP-binding site (Whitesell and Lindquist, 2005; Sharp and Workman, 2006; Neckers, 2007), hardly inhibited the ITZ-1-Hsp90α interaction at concentrations less than 100 μM (Figure S1B). ITZ-1 might not bind to the N-terminal ATP-binding site of Hsp90.

ITZ-1 Inhibits IL-1β–Induced ERK Activation via Raf-1 Degradation

We have reported that ITZ-1 selectively inhibited IL-1β–induced extracellular signal–regulated protein kinase (ERK) activation without affecting p38 kinase or c-Jun N-terminal kinase (JNK) activation (Kimura et al., 2009). As an Hsp90 client protein, Raf-1, which triggers ERK activation, is one IL-1β–signaling intermediate (Vincenti and Brinckerhoff, 2002), and Raf-1 protein levels are known to be decreased by Hsp90 inhibition (Stancato et al., 1997; Sharp and Workman, 2006; Neckers, 2007). We examined whether ITZ-1 could decrease Raf-1 protein levels

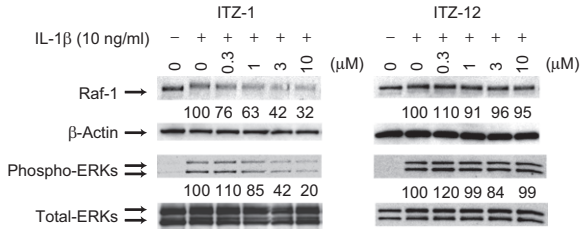

Figure 3. Effects of ITZ-1 and ITZ-12 on the Raf-1 Protein Levels and IL-1β–Induced ERK Activation in HACs

HACs were treated with ITZ-1 or ITZ-12 for 24 hr followed by stimulation with IL-1β for 30 min. Raf-1 protein levels and total or phosphorylated protein levels of ERK in equal amounts of whole-cell lysates were detected by western blot analysis and quantitated by densitometry. β-actin was used as a control.

using ITZ-12 with a lower Hsp90 binding affinity as a control (Figure 2D; Figure S1B). HACs were pretreated with compounds for 24 hr and then stimulated with 10 ng/ml of IL-1β for 30 min to induce ERK activation. ITZ-1, but not ITZ-12, decreased Raf-1 protein levels and inhibited the IL-1β–induced ERK activation at similar concentrations (Figure 3). ITZ-1, by binding to Hsp90, might inhibit the MMP-13 production through an inhibition of ERK activation via decreased Raf-1 protein levels.

ITZ-1 Binds to the Hsp90 C Terminus Without Inhibiting Hsp90 ATPase Activity

Hsp90 consists of three domains that have important functional interactions: the N-terminal domain contains the ATP-binding site and has ATPase activity, the middle domain interacts with client proteins and cochaperons and activates the ATPase activity, and the C-terminal domain is responsible for its inherent dimerization. In eukaryotes, a flexible, highly charged region (CR) connects the N-terminal domain to the middle domain (Whitesell and Lindquist, 2005). GA, 17-allylaminogeldanamycin (17-AAG), radicicol (RAD), and CCT018159 (CCT) bind to the N-terminal ATP-binding site, whereas novobiocin (NOV) has been reported to bind with poor affinity to a second ATP-binding site on the Hsp90 C-terminal domain (Marcu et al., 2000a, 2000b; Whitesell and Lindquist, 2005; Sharp and Workman, 2006; Neckers, 2007).

To obtain insights into the ITZ-1–binding site, we assessed whether ITZ-1 could inhibit ATPase activity. A colorimetric assay for inorganic phosphate, based on the formation of a phosphomolybdate complex and subsequent reaction with malachite green, was used to measure the ATPase activity of human Hsp90α (Rowlands et al., 2004). The N-terminal ligands exhibited potent inhibitory activities (IC_{50} of GA, 17-AAG, RAD, and CCT were 3.1 μM, 4.6 μM, 4.1 μM, and 3.7 μM, respectively), whereas neither ITZ-1 nor NOV showed detectable activities at concentrations <100 μM (Figure 4A). These data suggest that ITZ-1 does not bind to the N-terminal ATP-binding site.

In the competitive inhibition of Hsp90α-ITZ-3 binding, GA, 17-AAG, and RAD at 100 μM and NOV at 1000 μM did not exhibit any activity, whereas CCT at 100 μM showed partial inhibition (Figure 4B). These results suggest that ITZ-1 does not bind to Hsp90 at the same site as GA, 17-AAG, RAD, and NOV do in solution, or the binding affinity of ITZ-1 for Hsp90 is much stronger compared to those compounds.

The ITZ-1–binding site on Hsp90 was further examined by analyzing several deletion fragments for binding to the ITZ-3–immobilized matrix (Whitesell and Lindquist, 2005). Interestingly, removal of 77% of the N-terminal domain and CR between residues 48 and 290 (designated Δ1) or removal of CR and 70% of the middle domain between residues 197 and 528 (designated Δ2) from His-hHsp90α retained ITZ-3 binding, whereas removal of the C-terminal domain between 645 and 732 (designated Δ3) markedly impaired ITZ-3 binding (Figure 4C). The binding of Δ1 and Δ2 to the ITZ-3–immobilized matrix was also competitively inhibited by ITZ-1, suggesting that the ITZ-1–binding domain on Hsp90 is within its C-terminal domain.

ITZ-1 Is Comparable to GA in the Induction of Hsp Expression

Because the Hsp90-binding domain of ITZ-1 seemed to be different from those of other Hsp90 inhibitors, including GA, 17-AAG, and RAD, we determined to investigate the Hsp90 inhibitory properties of ITZ-1. Hsp90 inhibitors have been reported to induce Hsp expression by dissociating the HSF1/Hsp90 complex (Zou et al., 1998; Sittler et al., 2001; Mimnaugh et al., 2004; Shen et al., 2005). To test whether ITZ-1 could

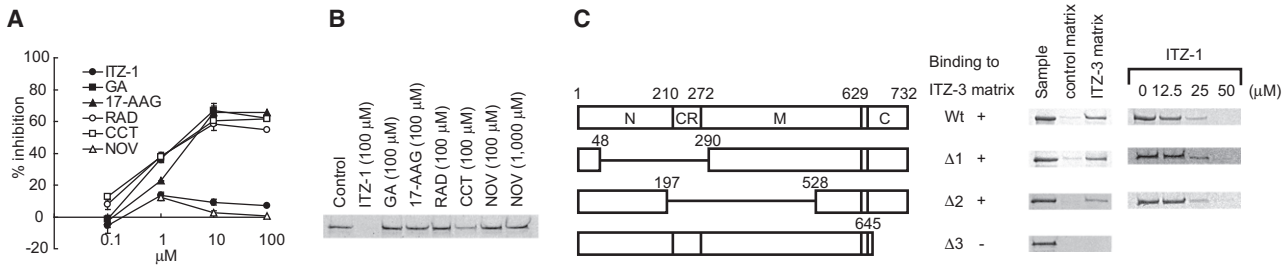

Figure 4. ITZ-1 Binds to the C-terminal Domain of Hsp90 Without Inhibiting Hsp90 ATPase Activity

(A) Effects of ITZ-1 and various Hsp90 inhibitors on Hsp90α ATPase activity. A colorimetric assay for inorganic phosphate, based on the formation of a phosphomolybdate complex and subsequent reaction with malachite green, was used to measure the ATPase activity of human Hsp90α. Results shown are means ± SD ($n = 3$).

(B) Displacement studies with ITZ-1 and various Hsp90 inhibitors by the binding assay using ITZ-3–immobilized matrixes.

(C) Schematic representation of the human Hsp90α (His-hHsp90α; Wt) and its deletion mutants (Δ1 – Δ3). N, N-terminal domain; CR, flexible, highly charged region; M, middle domain; C, C-terminal domain. Specific binding of Wt, Δ1, Δ2, and Δ3 to ITZ-3–immobilized matrixes was evaluated as in Figure 2C. Effect of ITZ-1 on the interaction between ITZ-3–immobilized matrix and these Hsp90 proteins were assessed as in Figure 2D.

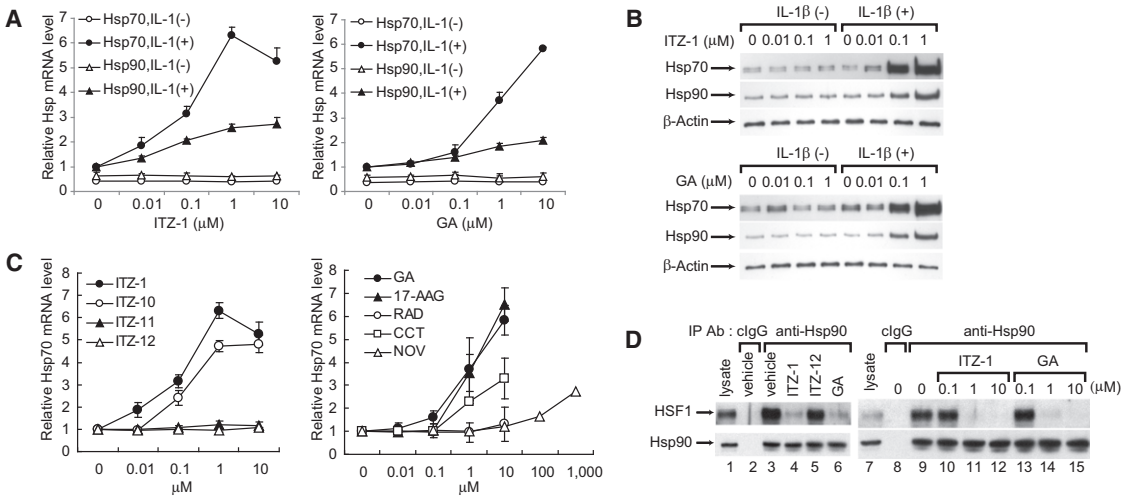

Figure 5. HSF1 Activation and Hsp70 and Hsp90 Induction by ITZ-1

(A) Up-regulation of Hsp70 and Hsp90 mRNA by ITZ-1 or GA. HACs were treated with ITZ-1 or GA in the presence or absence of IL-1β (10 ng/ml) for 24 hr. Messenger RNA levels of Hsp70 and Hsp90 were measured by real-time quantitative PCR. Results shown are means ± SD (n = 3).

(B) Up-regulation of Hsp70 and Hsp90 protein by 48 hr treatment with ITZ-1 or GA. Protein levels of Hsp70 and Hsp90 were detected by western blot analysis. β-actin was used as a control.

(C) Induction of Hsp70 mRNA expression by ITZ-1 derivatives or various Hsp90 inhibitors in the presence of IL-1β (10 ng/ml) costimulation. Results shown are means ± SD (n = 3).

(D) ITZ-1 and GA, but not ITZ-12, disrupted the HSF1-Hsp90 complex in the cell lysates obtained from IL-1β–stimulated HAC. Coimmunoprecipitation using an anti-Hsp90 antibody followed by western blot analysis of HSF1 (top) and Hsp90 (bottom) was performed. Coimmunoprecipitation was performed in the presence or absence of 1 μM ITZ-1, ITZ-12, or GA (lanes 1–6) and various concentrations of ITZ-1 or GA (lanes 7–15). See also Figure S2.

induce Hsp expression, HACs were stimulated with ITZ-1 or GA in the presence or absence of IL-1β costimulation. Both ITZ-1 and GA increased mRNA and protein levels of Hsp70 and Hsp90 in HACs in the presence of IL-1β costimulation (Figures 5A and 5B). It is well known that there are various approaches to induce Hsp expression using pharmacological interventions. Therefore, we investigated in detail whether ITZ-1 induced Hsp expression by binding to Hsp90.

We first examined the structure-activity relationship in the induction of Hsp70 expression for the ITZ-1 derivatives. With IL-1β costimulation, ITZ-1 and ITZ-10 dramatically up-regulated Hsp70 mRNA, whereas ITZ-11 and ITZ-12 showed little or no Hsp70 up-regulation at concentrations <10 μM (Figure 5C). This rank order was in good agreement with their order in the binding affinity for Hsp90α (Figure 2D; Figure S1B). Thus, ITZ-1 may induce Hsp70 expression via binding to Hsp90. ITZ-1 was more potent than known Hsp90 inhibitors in the induction of Hsp70 expression; the concentrations of ITZ-1, GA, 17-AAG, RAD, CCT, and NOV required to double the Hsp70 mRNA level (the minimum effective concentration, $MEC_{2.0}$) were 0.042 μM, 0.19 μM, 0.55 μM, >10 μM, 0.97 μM, and 240 μM, respectively (Figure 5C). On the other hand, ITZ-1 was the least toxic to HACs (Figure S2); the effective concentration 50 (EC_{50}) of ITZ-1, GA, 17-AAG, RAD, CCT, and NOV were >10 μM, 0.32 μM, 1.2 μM, 13 μM, >10 μM, and 200 μM, respectively; thus, this potent Hsp induction was not a byproduct of cytotoxicity.

The coimmunoprecipitation (IP) study conducted in the presence of free compounds in the lysates revealed that ITZ-1 at 1 μM, but not ITZ-12, drastically decreased the amount of HSF1 coprecipitated with Hsp90 (Figure 5D). The rank order was in good agreement with their order of binding affinity for Hsp90α. Moreover, the potency of ITZ-1 in the dissociation of HSF1/Hsp90 complexes was equal to that of GA (Figure 5D). This result agreed well with the results shown in Figures 5A and 5B. These results suggest that ITZ-1 induced Hsp expression via binding to Hsp90 and that the potency of ITZ-1 was at least equivalent to that of GA.

ITZ-1 Is Over Three Orders of Magnitude Less Potent in the Degradation of Several Hsp90 Client Proteins than GA

Hsp90 inhibitors have been reported to induce degradation of its multiple client proteins (Whitesell and Lindquist, 2005; Sharp and Workman, 2006; Neckers, 2007). To further address the Hsp90 inhibitory property of ITZ-1, HACs were treated with ITZ-1 or known Hsp90 inhibitors for 24 hr, and then degradation of Hsp90 client proteins, such as Raf-1, glucocorticoid receptor (GR), Akt, epidermal growth factor receptor (EGF-R), and receptor interacting protein (RIP), was analyzed (Stancato et al., 1997; Lewis et al., 2000; Bagatell et al., 2005; Zhang et al., 2007a, 2007b). Interestingly, ITZ-1 was less potent by over three orders of magnitude than was GA in the degradation of a majority of these client proteins (Figure 6A). ITZ-1 did not induce ≥50% degradation of client proteins except Raf-1 at 10 μM, whereas GA simultaneously reduced these client proteins at concentrations >0.01 μM. Moreover, ITZ-1 at 0.1 μM induced more than a 40% decrease of Raf-1, whereas there were no other client proteins that were decreased by more than 40% with ITZ-1 at 10 μM. Both ITZ-1 and GA required IL-1β costimulation to induce Hsp expression (Figures 5A and 5B); thus, we investigated the

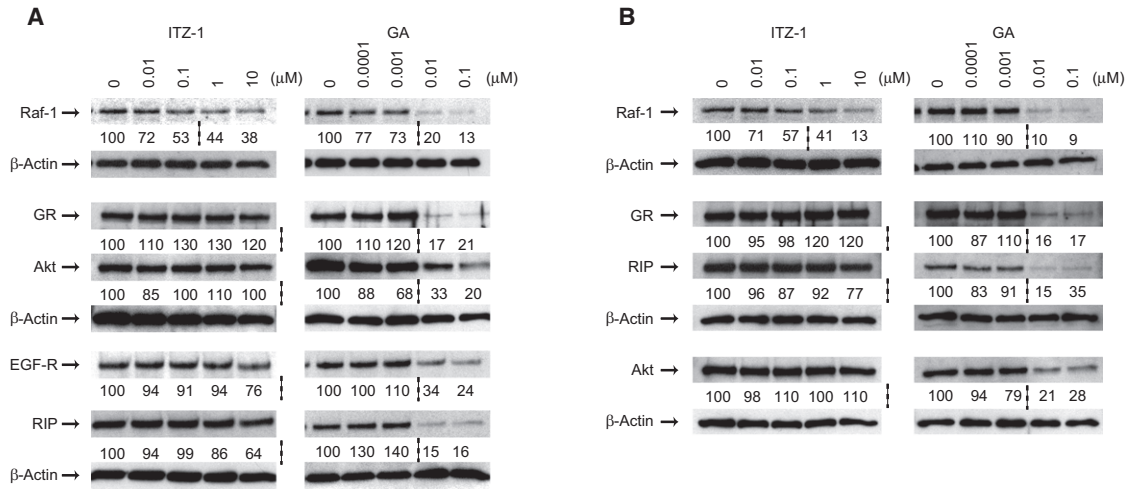

Figure 6. ITZ-1 Selectively Induces Raf-1 Protein Degradation

HACs were treated with ITZ-1 or GA for 24 hr in the absence (A) or presence (B) of IL-1β (10 ng/ml) costimulation. Protein levels of various Hsp90 client proteins in equal amounts of whole-cell lysates were assessed by western blot analysis and quantitated by densitometry. β-actin was used as a control. Dotted lines indicate the concentration of compound required to induce a ≥50% decrease of each client protein level. See also Figure S3.

client protein degradation by ITZ-1 or GA in the presence of IL-1β costimulation. However, IL-1β costimulation did not affect their client protein degradation properties (Figure 6B). Other Hsp90 inhibitors also simultaneously induced degradation of these client proteins (Figure S3A). The ≥50% degradation of these five client proteins by ITZ-1, GA, 17-AAG, RAD, CCT, and NOV was seen at concentrations of >10 μM, 0.01 μM, 0.1 μM, 0.1 μM, 10 μM, and 1000 μM, respectively.

To determine whether ITZ-1 down-regulates Raf-1 at the protein level, HACs were treated with ITZ-1 together with cycloheximide (CHX), a protein synthesis inhibitor (Nomura et al., 2005). Evaluation of cellular Raf-1 protein level by western blot analysis revealed that preexisting Raf-1 protein was markedly decreased by ITZ-1 and CHX. In contrast, when cells were treated with CHX alone, Raf-1 protein decreased to only 44% of the initial amount after 24 hr, indicating a protein half-life of about 24 hr. These data demonstrate that down-regulation of Raf-1 protein occurred at the protein level (Figure S3B).

Previous research has suggested a link between GA and degradation of Hsp90 client proteins via the proteasome pathway (Mimnaugh et al., 2004; Nomura et al., 2005). We examined whether proteasomal degradation mediates the loss of Raf-1 in ITZ-1–treated HACs using the proteasome inhibitor MG132. Pretreatment with MG132 resulted in a remarkable suppression of ITZ-1–induced Raf-1 degradation in HACs (Figure S3C). This result suggests that Raf-1 was degraded by the proteasome.

DISCUSSION

ITZ-1 is a chondroprotective agent that suppresses IL-1β–induced cartilage degradation in vitro and in vivo. ITZ-1 also inhibits IL-1β–induced MMP-13 production and suppresses NO-induced chondrocyte death (Kimura et al., 2009). Here, we show the successful identification of Hsp90 as a target protein of ITZ-1. Drug-affinity chromatography has played an important role in the identification of drug-binding proteins (Harding et al., 1989; Taunton et al., 1996). However, in many cases, nonspecific binding of proteins to solid support or to the compound itself has complicated identification of target proteins, and examination of the precise connection between the function of the selected binding proteins and activity of the drugs was not done sufficiently (Shimizu et al., 2000; Sato et al., 2007). To facilitate drug target identification, we estimated the potency of each compound on the matrix after coupling with a long alkyl chain, instead of a spacer arm, at their terminal carboxyl or amino group. We also adopted a system that can select activity-associated structure-specific binding proteins of compounds using three columns of active compounds immobilized matrixes. These devices might confer our higher purification efficiency of Hsp90 as the target protein of ITZ-1.

We have previously shown that ITZ-1 as well as an ERK-MAPK pathway inhibitor (U0126), but not a p38 kinase inhibitor (SB203580) or a JNK inhibitor (SP600125), selectively inhibited IL-1β–induced MMP-13 production (Kimura et al., 2009). Here we showed that the rank order of ITZ-1 derivatives in the inhibition of MMP-13 production agreed well with their binding affinity for Hsp90α. Furthermore, ITZ-1, but not ITZ-12, decreased Raf-1 protein levels and inhibited the IL-1β–induced ERK activation. Thus ITZ-1, by binding to Hsp90, might inhibit MMP-13 production by suppressing IL-1β–induced ERK activation via decreased Raf-1 protein levels.

The rank order of ITZ-1 derivatives affecting the dissociation of HSF1/Hsp90 complex and up-regulation of Hsp70 expression also agreed well with their binding affinity for Hsp90α. These results suggest that ITZ-1 induced Hsp expression by binding to Hsp90. The Hsp up-regulation might confer the chondroprotective effects of ITZ-1 in vitro and in vivo (Kimura et al., 2009) because Hsp70 induction has been shown to prevent NO-induced apoptosis in chondrocytes and to efficiently help in the recovery from osteoarthritis (Terauchi et al., 2003; Kühn et al., 2004; Ge et al., 2006; Grossin et al., 2006).

Table 1. Hsp90 Inhibitory Properties of ITZ-1 and Several Known Hsp90 Inhibitors

	ITZ-1	GA	17-AAG	RAD	CCT	NOV
Hsp70 mRNA induction, $MEC_{2.0}$ (μM)	0.042	0.19	0.55	>10	0.97	240
Raf-1 degradation, EC_{50} (μM)	1	0.01	0.1	0.1	10	1,000
Client protein degradation, EC_{50} (μM)	>10	0.01	0.1	0.1	10	1,000
Cytotoxicity, EC_{50} (μM)	>10	0.32	1.2	13	>10	200
ATPase inhibition, IC_{50} (μM)	>100	3.1	4.6	4.1	3.7	>100

$MEC_{2.0}$ for Hsp70 mRNA induction was determined as the concentration of each compound to double the Hsp70 mRNA level. EC_{50} for Raf-1 degradation was determined as the concentration of each compound required to induce a $\geq$50% decrease in Raf-1 protein levels. EC_{50} for client protein degradation was determined as the concentration of each compound required to induce a $\geq$50% decrease of the five client protein levels.

So far, Hsp90 inhibitors, especially those that bind at the N-terminal domain, have been considered to simultaneously induce degradation or activation of multiple Hsp90 client proteins (Whitesell and Lindquist, 2005; Sharp and Workman, 2006; Neckers, 2007). In fact, all known Hsp90 inhibitors used in this study simultaneously reduced cellular protein levels of several Hsp90 client proteins in HACs. However, the Hsp90 inhibitory property of ITZ-1 was client protein selective, compared with these other Hsp90 inhibitors. ITZ-1 strongly induced Hsps expression and mildly caused Raf-1 degradation but scarcely induced degradation of client proteins other than Raf-1. ITZ-1, GA, 17-AAG, RAD, CCT, and NOV required >240, 0.053, 0.18, >0.01, 10, and 4.2 times lower concentrations, respectively, for Hsp70 expression, compared with client protein degradation (Table 1). Because HACs were used for these studies, different uptake and metabolism rates of ITZ-1 by cells in each assay had a negligible effect on the experimental results.

This unique property of ITZ-1 might be due to its unique Hsp90-binding site; almost all known Hsp90 inhibitors bind to the N-terminal ATP-binding domain and inhibit the ATPase activity (Rowlands et al., 2004; Whitesell and Lindquist, 2005; Sharp and Workman, 2006; Neckers, 2007), whereas ITZ-1 binds to Hsp90 at a site in the C-terminal domain without affecting ATPase activity. Although NOV has been reported to bind to the Hsp90 C-terminal domain, its binding affinity was too weak to understand the contribution of this site to the regulation of Hsp90 function (Marcu et al., 2000a, 2000b; Whitesell and Lindquist, 2005). ITZ-1 may be useful for better understanding the function of the Hsp90 C-terminal domain. Recently, a NOV analog A4, which could induce client protein degradation at ~70-fold lower concentrations than NOV, has been reported to up-regulate Hsp90 levels at concentrations 1,000–10,000 fold lower than that required for client protein degradation (Yu et al., 2005; Ansar et al., 2007; Lu et al., 2009). AEG3482, an antiapoptotic compound that has been shown to bind Hsp90 at a site different from GA, has also been reported to induce HSF-1–dependent Hsp70 production without causing client protein degradation (Salehi et al., 2006; Gallo, 2006). Therefore, it is conceivable that ITZ-1, a C-terminal inhibitor, possesses a mechanism of action distinct from that of GA and other N-terminal inhibitors. Our results, as well as results with A4 and AEG3482, suggest that the C-terminal domain is suitable for the development of HSF-1–selective Hsp90 inhibitors.

Up-regulation of Hsps, especially that of Hsp70, has been shown to be of great therapeutic potential for various diseases, such as ischemic heart disease, diabetes, stroke, trauma, and various neurodegenerative diseases (Auluck and Bonini, 2002; Sittler et al., 2001; Shen et al., 2005; Söti et al., 2005). Several reports have shown that GA, via Hsp70 induction, is effective as a cytoprotective agent (Sittler et al., 2001; Auluck and Bonini, 2002; Shen et al., 2005). However, it is well known that GA is toxic to cells probably as a result of nonspecific degradation of a broad range of client proteins. ITZ-1 was at least 300 times less toxic than GA for growing HAC, probably because of its client selectivity (Table 1). ITZ-1 type Hsp90 inhibitors may be beneficial as Hsp inducers with lower cytotoxicity.

Both ITZ-1 and GA required IL-1β costimulation to induce Hsp expression in chondrocytes. To become transcriptionally competent, HSF1 requires homotrimerization, translocation from the cytoplasm to the nucleus, hyperphosphorylation, and acquisition of DNA-binding activity (Zou et al., 1998). Both ITZ-1 and GA dissociated HSF1/Hsp90 complexes in the cell lysate; therefore, IL-1β signal may affect the activation step of free HSF1. Actually, IL-1β stimulation has been reported to involve trimer formation of HSF1 in human embryonic lung fibroblast cells (Sasaki et al., 2002).

In summary, our results suggest that ITZ-1 is a client selective Hsp90 inhibitor that selectively induces robust HSF1 activation and mild Raf-1 degradation. ITZ-1 type Hsp90 inhibitors are promising as Hsp70 inducers, as well as disease-modifying anti-OA drugs. It seems difficult to discover Hsp90 inhibitors with such mechanisms of action by classical genetics because Hsp90 null mutants are lethal in eukaryotes (Young et al., 2001). Furthermore, ITZ-1 type Hsp90 inhibitors could not be obtained by conventional assays such as the ATPase assay. Thus, the two binding assays we used here are useful to identify additional ITZ-1 type Hsp90 inhibitors. Our study demonstrates that forward chemical genetics can result in the more efficient identification of new drugs and new molecular targets (Lokey, 2003; Saghatelian and Cravatt, 2005; Spring, 2005; Kawasumi and Nghiem, 2007).

SIGNIFICANCE

ITZ-1, a lead compound for an antiosteoarthritis (OA) drug program, was previously found to have protective effects against interleukin-1β (IL-1β)–induced cartilage degradation in vitro and in vivo by both selective inhibition of IL-1β–induced matrix metalloproteinase–13 (MMP-13) production via inhibition of extracellular signal–regulated protein kinase (ERK) activation and suppression of nitric oxide (NO)–induced chondrocyte death. Here, we report the elucidation of its precise mechanisms of action. Heat shock protein 90 (Hsp90) was identified as a specific ITZ-1–binding protein. ITZ-1 induced degradation of an Hsp90 client protein, Raf-1, which triggers IL-1β–induced ERK activation and up-regulation of a cytoprotective heat shock protein 70 (Hsp70) by dissociating heat shock factor 1 (HSF1)/Hsp90 complexes, which may account for its cytoprotective effect as well

as a selective inhibition of MMP-13 production. Almost all known Hsp90 inhibitors, including geldanamycin (GA), have been reported to bind to the N-terminal ATP-binding site of Hsp90 and simultaneously induce degradation and activation of its multiple client proteins. However, ITZ-1 specifically bound to the C-terminal domain of Hsp90 without affecting Hsp90s ATPase activity. Interestingly, ITZ-1 strongly induced HSF1 activation and caused mild Raf-1 degradation but scarcely induced degradation of a broad range of Hsp90 client proteins besides Raf-1. These results suggest that ITZ-1 is a client-selective Hsp90 inhibitor. ITZ-1 type Hsp90 inhibitors are promising as Hsp70 inducers for various diseases, as well as disease-modifying anti-OA drugs. It seems difficult to discover Hsp90 inhibitors with such a mechanism of action by classical genetics because Hsp90 null mutants are lethal in eukaryotes. Furthermore, an ITZ-1 type Hsp90 inhibitor could not be obtained by conventional assays such as the ATPase assay. Our study demonstrates that forward chemical genetics can result in the efficient identification of new drugs and new molecular targets.

EXPERIMENTAL PROCEDURES

Materials

GA, malachite green, polyvinyl alcohol, ammonium molybdate, CHX, and anti-β-actin antibody (A-5441) were from Sigma. Anti-Raf-1 antibody (sc-133), anti-GR antibody (sc-8992), anti-EGF-R antibody (sc-03), and protein A/G-agarose (sc-2003) were from Santa Cruz Biotechnology. Phosphorylated and nonphosphorylated state specific antibodies for ERK p44/p42 (9100), anti-Akt antibody (9270), and anti-rabbit IgG, HRP-linked antibody (7074) were from Cell Signaling Technology. Anti-Hsp70 antibody (H53220), anti-Hsp90 antibody (H38220), and anti-RIP antibody (551042) were from BD Bioscience. Anti-HSF1 antibody (SPA-901) was from Assay Design. Peroxidase-labeled affinity purified antibody to mouse IgG (γ) (074-1802) was from KPL; 17-AAG, RAD, and NOV were from Wako. CCT was from Tocris Bioscience. ATP was from Roche. IL-1β was from R & D Systems. HACs and Chondrocyte Growth Medium (CGM) were from Lonza Walkersville. A 1:1 (v/v) mixture of Dulbecco's modified Eagle's MEM (DMEM) and Ham's F-12 medium (DMEM/F-12) was from Invitrogen. Detailed methods for the syntheses of ITZ-1 derivatives are described in Supplemental Information.

Assay for MMP Production

IL-1β–induced production of MMPs by HACs was measured as described elsewhere (Kimura et al., 2009).

Drug-Affinity Chromatography

Two milliliters of 100 mM imidazo[5,1-c][1,4]thiazine derivatives dissolved in 75–95% DMF and 20 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were coupled to 1 ml of AF-Amino TOYOPEARL 650M (Tosoh) or AF-Carboxy TOYOPEARL 650M (Tosoh), according to the manufacturer's instructions.

HACs were homogenized in four volumes of binding buffer (10 mM Tris-HCl [pH 7.5], 20% glycerol, 1 mM EDTA, 100 mM NaCl, 0.5 mM p-APMSF, and 1 mM DTT) with a teflon homogenizer. The homogenate was spun at 100,000 × g for 60 min at 4°C, and then the supernatant was recovered and applied to the columns of drug-coupled matrixes. Columns were washed twice with five volumes of binding buffer, and then binding proteins were eluted with five volumes of elution buffer (10 mM Tris-HCl [pH 7.5], 20% glycerol, 1 mM EDTA, 1 M NaCl, 0.5 mM p-APMSF, and 1 mM DTT) by collecting 5 fractions equal to column volume, and with three volumes of glycine-HCl buffer (0.1 M glycine-HCl [pH 2.7]).

His-hHsp90α was diluted to 50 μg/ml with binding buffer (10 mM Tris-HCl [pH 7.5], 20% glycerol, 1 mM EDTA, 500 mM NaCl, and 1 mM DTT) and applied to the columns. After a wash with five volumes of binding buffer, binding proteins were eluted with five volumes of elution buffer (10 mM Tris-HCl [pH 7.5], 20% glycerol, 1 mM EDTA, 500 mM NaCl, and 6 M Urea). The eluted proteins were analyzed by SDS-PAGE followed by silver staining.

Protein Identification by LC-MS/MS

The eluted proteins were subjected to SDS-PAGE, and then the gels were stained with CBB. Protein spots were excised from the gel and subjected to in-gel digestion followed by LC-MS/MS sequence analysis at APRO Life Science Institute.

Expression and Purification of Recombinant Proteins

Expression and purification of recombinant proteins were accomplished by the pET expression system (Merck) with His Bind Resin according to the manufacturer's instructions. Detailed methods are described in the Supplemental Experimental Procedures.

In Vitro Binding Assay with ITZ-3–Immobilized Matrix

One microgram of His-hHsp90α or deletion mutants was incubated with sample in 200 μl of binding buffer (10 mM Tris-HCl [pH 7.5], 20% glycerol, 1 mM EDTA, 500 mM NaCl, and 1 mM DTT) at room temperature for 30 min. After addition of 5 μl of ITZ-3–immobilized matrix or control matrix, the mixtures were further incubated for 40 min with agitation. The matrixes were washed five times with 200 μl of binding buffer and then heated for 5 min at 95°C in SDS-PAGE sample buffer. The samples were subjected to SDS-PAGE followed by CBB staining.

Evaluation of MAPK Activation and Hsp90 Client Protein Degradation

ERK activation was evaluated as described elsewhere (Kimura et al., 2009). To evaluate client protein degradation, HACs pr-cultured in CGM at a density of 4×10^4 cells/ml in 6-well plates for 1 day were further cultured in DMEM/F-12 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 μg/ml gentamicin at 37°C in 5% CO_2/95% humidified air for 1 day, and then treated with samples for 24 hr. HACs were lysed with lysis buffer (Kimura et al., 2009) and then subjected to western blot analysis.

Western Blot Analysis

Samples were subjected to SDS-PAGE, transferred onto nitrocellulose filters (Hybond-ECL; GE Healthcare), and immunoblotted with the primary antibodies followed by horseradish peroxidase–conjugated secondary antibodies. The immune complexes were visualized with an enhanced chemiluminescence system (ECL Detection Kit; GE Healthcare) and Hyperfilm ECL (GE Healthcare), according to the manufacturer's instructions. Protein levels were calculated from densitometrical analysis by GS-800 Calibrated Densitometer (Bio-Rad).

ATPase Activity Assay

The ATPase activity assay was performed as described elsewhere, using human Hsp90α (ATGen) (Rowlands et al., 2004).

Evaluation of the Induction of Hsp Expression

HACs maintained in CGM were suspended in DMEM/F-12 supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin at a density of 5.5×10^4 cells/ml and were cultured in 96-well or 12-well plates at 37°C in 5% CO_2/95% humidified air for 1 day. After medium exchange, HACs were treated with samples and 10 ng/ml of IL-1β for 24 or 48 hr. Real-time quantitative PCR was performed as described elsewhere (Kimura et al., 2009). Primers used for Hsp70 analysis were Hsp70-F (5′-CGAGAGGGTGTCAGCCAAGA-3′) and Hsp70-R (5′-AGCCACGAGATGACCTCTTGAC-3′). Primers used for Hsp90 analysis were Hsp90-F (5′-TTGGTCCTGTGCGGTCACT-3′) and Hsp90-R (5′-CCATC GGTTGGTCTTGGG-3′). For western blot analysis, HACs were washed with PBS and then lysed with lysis buffer (Kimura et al., 2009).

Immunoprecipitation

HACs were cultured in the presence 10 ng/ml of IL-1β for 24 hr, were washed with PBS, were lysed with RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholic acid, 1 mM sodium orthovanadate V, and 1 μM microcystin-LR) with proteinase inhibitors (Complete; Boeringer

Mannheim), and were centrifuged at 16,000× *g* for 10 min, and then supernatant (lysates) was recovered. Next, 600 μl of lysates (2 mg/ml) were preincubated with samples for 30 min and then were incubated with 30 μg of anti-Hsp90 antibody (SPA-840; Assay Design) or control IgG (I4131; Sigma) for 30 min. Subsequently, the lysates were incubated with a 50-μl slurry of protein A/G-agarose with rotation for 3 hr at 4°C. The immunoprecipitates were collected by centrifugation, washed seven times with RIPA buffer, and then subjected to SDS-PAGE followed by western blot analysis using anti-HSF1 or anti-Hsp90 antibodies.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2009.12.012.

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