

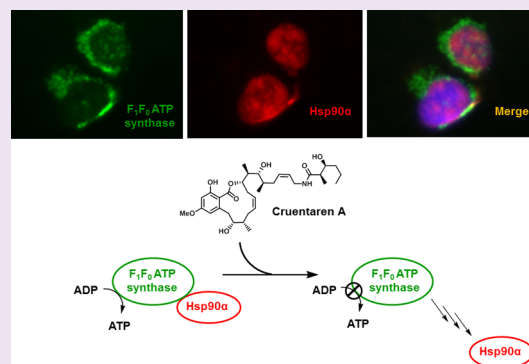
Cruentaren A Binds F₁F₀ ATP Synthase To Modulate the Hsp90 Protein Folding Machinery

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S Supporting Information

ABSTRACT: The molecular chaperone Hsp90 requires the assistance of immunophilins, co-chaperones, and partner proteins for the conformational maturation of client proteins. Hsp90 inhibition represents a promising anticancer strategy due to the dependence of numerous oncogenic signaling pathways upon Hsp90 function. Historically, small molecules have been designed to inhibit ATPase activity at the Hsp90 N-terminus; however, these molecules also induce the pro-survival heat shock response (HSR). Therefore, inhibitors that exhibit alternative mechanisms of action that do not elicit the HSR are actively sought. Small molecules that disrupt Hsp90-co-chaperone interactions can destabilize the Hsp90 complex without induction of the HSR, which leads to inhibition of cell proliferation. In this article, selective inhibition of F₁F₀ ATP synthase by cruentaren A was shown to disrupt the Hsp90-F₁F₀ ATP synthase interaction and result in client protein degradation without induction of the HSR.



Molecular chaperones are an evolutionary conserved class of proteins that prevent the aggregation of and assist in the maturation of cellular proteins. Heat shock proteins (Hsps) are a subset of molecular chaperones that are up-regulated upon exposure to cell stress, including high temperature. Heat shock proteins are also ubiquitously expressed under nonstressed conditions and play vital roles in *de novo* protein synthesis by folding nascent polypeptides, translocating proteins across membranes, and mediating protein turnover.^{1–3} They also serve regulatory functions that include the post-translational regulation of signaling molecules, the activation of transcription factors, and the degradation of proteins *via* the ubiquitin-proteasome pathway.

Hsp90 is the most abundant heat shock protein and represents approximately 1–2% of total cellular protein in unstressed cells. Four isoforms of human Hsp90 exist and include the cytosolic proteins Hsp90 α (inducible) and Hsp90 β (constitutively expressed), the endoplasmic reticulum-associated glucose-related protein 94 (Grp94), and the mitochondrial-associated TNF receptor-associated protein 1 (TRAP1). Hsp90-dependent client proteins play key roles in cellular growth, survival, and development. The list of Hsp90 clients extends beyond 200 reported proteins, many of which contribute to the six hallmarks of cancer (*e.g.*, ErbB2, Raf, Akt and pAkt, steroid hormone receptors, mutant p53, HIF-1 α , survivin, telomerase, *etc.*).⁴

The dependence of so many proteins on Hsp90 for tumor progression has made it an attractive target for anticancer drug development. In contrast to current cancer therapeutics that target at a single signaling pathway, Hsp90 inhibition results in the simultaneous degradation of multiple oncogenic substrates

and leads to a combinatorial attack on cancer. Overexpression of Hsp90 occurs in cancer cells and is responsible for maintaining the homeostasis of the hostile environment caused by neoplastic transformation.^{4–8} Up-regulated Hsp90 levels provide an opportunity to selectively target cancer cells versus noncancerous cells and provides the opportunity to reduce detrimental side effects.

Hsp90 functions as a homodimer, and each Hsp90 monomer consists of three domains: an N-terminal domain that contains an ATP-binding pocket, a middle domain wherein important protein–protein interactions occur, and a C-terminal domain that is responsible for dimerization. Hsp90 facilitates the conformational maturation of client proteins *via* the Hsp90 chaperone cycle, in which the Hsp90 homodimer forms a larger, multiprotein complex that contains other co-chaperones, immunophilins, and partner proteins that together are responsible for folding Hsp90-dependent substrates. The Hsp90 heteroprotein complex folds its client proteins through a number of conformational transitions that are facilitated by ATP hydrolysis at the N-terminus of the protein.⁹ The Hsp90 heteroprotein complex is predominant in cancer cells, whereas the Hsp90 homodimer is abundant in non-transformed cells.^{8,10} In addition, the Hsp90 heteroprotein complex exhibits approximately 200-fold increased affinity for ATP as compared to the Hsp90 homodimer.¹¹ Such attributes have led to the development of small molecule inhibitors of the ATP-binding

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pocket located at the N-terminus and include derivatives of geldanamycin, radicicol, and purine. Although N-terminal inhibitors are effective at inducing client protein degradation, N-terminal inhibition also leads to activation of the Hsp90-associated transcription factor, Heat Shock Factor-1 (HSF-1). HSF-1 activation induces the pro-survival, heat shock response (HSR), in which the cellular concentration of heat shock proteins, such as Hsp90 and Hsp70, is dramatically increased, which can result in dosing and scheduling issues upon the administration of N-terminal inhibitors.^{10,12} Therefore, the development of small molecules that do not exhibit this mechanism of action are actively sought.

Alternative strategies for the inhibition of Hsp90 include the development of small molecules that target the Hsp90 C-terminus as well as the Hsp90 heteroprotein complex. C-Terminal inhibitors derived from the natural product novobiocin inhibit cancer cell proliferation and lead to client protein degradation at concentrations similar to N-terminal inhibitors; however, they do not induce the HSR.^{13–15} In contrast, small molecules that disrupt the Hsp90 heteroprotein complex, specifically interactions with co-chaperones, have been sought to disrupt maturation of select Hsp90 clients at concentrations that do not induce the HSR.^{16–19} Papanthanasia *et al.* have reported that F₁F₀ ATP synthase interacts with Hsp90 to function as a co-chaperone that is important for the maturation of Hsp90 client proteins.²⁰ They showed that interactions between Hsp90 and F₁F₀ ATP synthase could be disrupted upon incubation with the polypeptide natural products, efraeptins (Figure 1A). Incubation with the efraeptins resulted in the degradation of select Hsp90 clients after 48 h and decreased cellular levels of Hsp70, Hsp90, and Hsp27. Efraeptins represent a family of fungal peptides that exhibit potent antiproliferative activity against several cancer cell lines by inhibiting the function of many cellular targets,

including the 20S proteasome, several ATPases, and F₁F₀ ATP synthase.^{21–23} Although efraeptins induced Hsp90 client protein degradation at nanomolar concentrations, the complex peptide structure and nonspecific inhibition hinders the development of these compounds. However, inhibition of F₁F₀ ATP synthase to ultimately disrupt interactions between Hsp90 and F₁F₀ ATP synthase represents a novel and under-investigated approach for disrupting Hsp90-dependent pathways without induction of the pro-survival heat shock response. Therefore, it was proposed that a selective inhibitor of F₁F₀ ATP synthase could inhibit the Hsp90 protein folding machinery *via* disruption of Hsp90-F₁F₀ ATP synthase interactions.

The macrolide cruentaren A was isolated from the myxobacterium *Byssovorax cruenta* and exhibited potent antiproliferative activity against several cancer cell lines^{24,25} (Figure 1B).

Consistent with other cytotoxic natural products isolated from myxobacterium, cruentaren A was shown to be an inhibitor of oxidative phosphorylation and was found to be the only selective inhibitor of F₁F₀ ATP synthase identified. Studies revealed that cruentaren A selectively inhibits the F₁ domain of F₁F₀ ATP synthase and exhibits no inhibitory activity against other V- or P-ATPases. The inhibitory activity of cruentaren A also demonstrates selectivity for eukaryotic F-ATPases, as it was completely inactive against a series of Gram-negative bacteria and did not inhibit the function of purified F₁ from *Escherichia coli*.

Consistent with this hypothesis, we found that incubation with nanomolar concentrations of cruentaren A resulted in Hsp90-dependent client protein degradation after 48 h and furthermore did not induce the HSR after 24 or 48 h of incubation. As shown in these studies, cruentaren A does not bind directly to Hsp90 based on multiple assays. Instead, F₁F₀ ATP synthase was shown to interact directly with Hsp90 in MCF7 cell lysates, which could be disrupted upon 48 h of incubation with cruentaren A. In contrast, the N-terminal inhibitor, geldanamycin, and the C-terminal inhibitor, KU-174, did not affect interactions between F₁F₀ ATP synthase and Hsp90 after 24 and 48 h of incubation, indicating that disruption Hsp90 chaperone function is specifically associated with F₁F₀ ATP synthase inhibition.

RESULTS AND DISCUSSION

Cruentaren A Is a Potent Inhibitor of Cancer Cell Proliferation. Cruentaren A has been previously reported to demonstrate potent antiproliferative activity against multiple cancer cell lines, including the human lung cancer cell line A549. Potent antiproliferative activity against the A549 cell line as well as the estrogen receptor positive, human breast cancer cell line MCF7 was observed; however, cruentaren A was relatively inactive against the normalized HEK293 and MRC5 human cell lines (Table 1 and Supplementary Figure 1).

The observed selectivity is likely a consequence of the increased requirement that cancer cells have upon ATP and Hsp90 chaperone activity as compared to normal cells.²⁶ Transformed cells require higher concentrations of ATP than normal cells to maintain their elevated metabolic rate. In addition, the estrogen receptor and many other oncogenic clients are overexpressed in cancer cells and rely upon the Hsp90 molecular chaperone for activity. Therefore, it was proposed that cruentaren A, a selective and potent inhibitor of F₁F₀ ATP synthase (produces approximately 90% of the cells

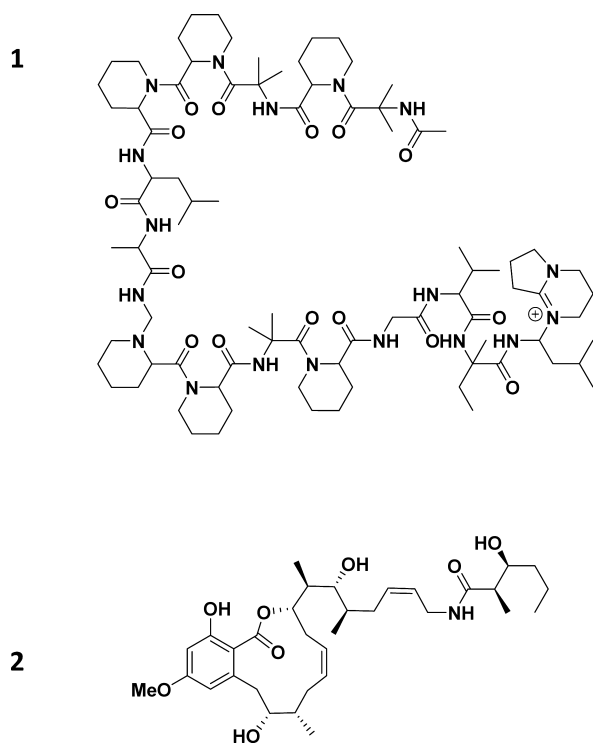


Figure 1. Structures of efraeptins (1) and cruentaren A (2).

Table 1. Calculated EC_{50} Values of Cruentaren A against Cancer Cell Lines MCF7 and A549 and Normal Human Cell Lines HEK293 and MRC5

cell line	EC_{50} value (nM)
MCF7	7.99 ± 4.13
A549	0.188 ± 0.006
HEK293	>500
MRC5	>500

ATP) could decrease cellular levels of Hsp90-dependent client proteins and exhibit potent antiproliferative activity against cancer cell lines with a high differential selectivity.²⁷

Antiproliferation studies using the known Hsp90 inhibitor geldanamycin co-dosed with fixed concentrations of cruentaren A were performed. No synergistic or additive activities were observed for the compounds, which may be due to the overexpression of Hsp90, as geldanamycin induces the heat shock response.

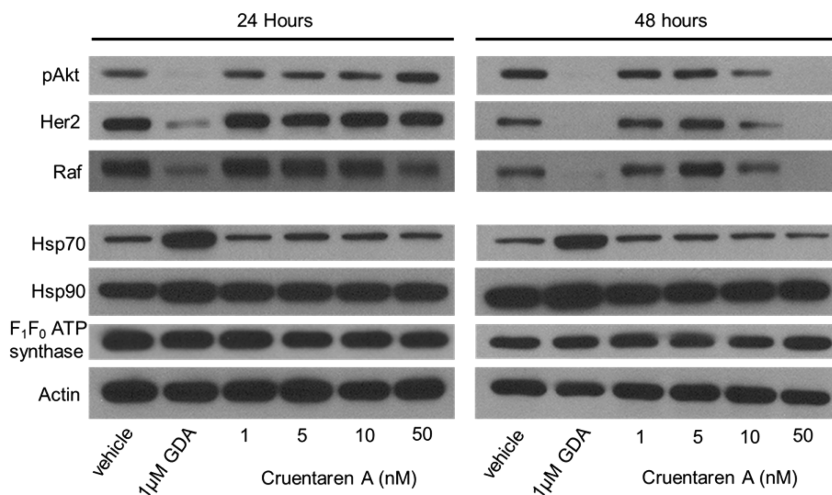
Cellular Levels of Hsp90-Dependent Client Proteins Decreased upon 48-h Incubation with Cruentaren A in a Dose-Dependent Manner. Hsp90 inhibitors induce client protein degradation at concentrations that mirror their antiproliferative IC_{50} value, as client protein degradation manifests a direct effect on cell growth.²⁸ Similarly, cruentaren A exhibits an EC_{50} value of 7.99 ± 4.13 nM against MCF7 cells, and client protein degradation was observed between 5 and 10 nM after 48 h incubation. Cruentaren A also induced a dose-dependent decrease in the levels of Hsp90-dependent client proteins, phosphorylated Akt (pAkt), Her2, and Raf, which were undetectable at 50 nM (Figure 3). When compared to the vehicle control (0.25% DMSO), Hsp90 and F_1F_0 ATP synthase levels remained constant at both the 24 and 48 h time points, whereas a modest decrease in Hsp70 levels was observed at 50 nM cruentaren A after 48 h (Figure 2). Consistent with prior observations using efrapaptins, cruentaren A induced client protein degradation at low nanomolar concentrations without induction of Hsp70 and Hsp90. Levels of F_1F_0 ATP synthase were unchanged after 48 h of incubation with cruentaren A, providing evidence that F_1F_0 ATP synthase is not an Hsp90-dependent substrate.

Incubation with 1 μ M geldanamycin also resulted in client protein degradation at both 24 and 48 h; however, dramatic increases in Hsp70 and Hsp90 levels were observed and indicate induction of the HSR, which is characteristic of geldanamycin and other Hsp90 N-terminal inhibitors. These data indicate that cruentaren A functions as an inhibitor of the Hsp90 protein folding machinery and does not increase levels of Hsp70 and Hsp90, providing evidence that it does not bind Hsp90 in a manner similar to geldanamycin.

Cruentaren A Does Not Protect Hsp90 from Trypsinolysis. Hsp90 inhibitors based on and including novobiocin bind the C-terminus and induce client protein degradation at concentrations that do not induce Hsp70 and Hsp90 levels (*i.e.*, induce the HSR). Because cruentaren A also induced client protein degradation without increasing Hsp70 and Hsp90 levels, cruentaren A was suspected to bind and inhibit the Hsp90 C-terminus similarly to novobiocin. To determine whether cruentaren A binds the Hsp90 C-terminus, trypsinolysis of Hsp90 in TnT rabbit reticulocyte in the presence of 50 nM and 50 μ M cruentaren A, a 1000-fold increase over the concentration needed to induce client protein degradation after 48 h, was investigated.^{29,30}

When Hsp90 is in the semiclosed and closed states, amino acids Lys615 and Arg620 are solvent exposed on an α helix and are susceptible to cleavage by trypsin. However, in the “extended” or “open conformation”, these amino acids are protected and not subject to trypsinolysis.³⁰ Novobiocin binds the Hsp90 C-terminus and alters its conformational state by locking Hsp90 into the “open conformation”, which prevents the cleavage of amino acids Lys615 and Arg620 and produces fragments that differ in molecular weight from the unprotected protein.¹⁴

In the presence of high concentrations of novobiocin, the C-terminal Hsp90 antibody AC88 detects the dose-dependent emergence of a 50 kDa band, while the N-terminal Hsp90 antibody identifies the disappearance of bands at 78 and 30 kDa, as well as doublet bands at 50 kDa and the emergence of a 73 kDa band (Figure 3A).³⁰ Both low and high concentrations of cruentaren A failed to protect Hsp90 from trypsinolysis and instead exhibited proteolytic fingerprints identical to control (Figure 3B). The C-terminal Hsp90 antibody did not detect the presence of a 50 kDa band after incubation with either

**Figure 2.** Western blot for Hsp90 client proteins (pAkt, Her2, and Raf) and Hsp70, Hsp90, and F_1F_0 ATP synthase using MCF7 cell lysates after 24 or 48 h of incubation with the indicated amount of cruentaren A, vehicle (0.25% DMSO), or 1 μ M geldanamycin (GDA).

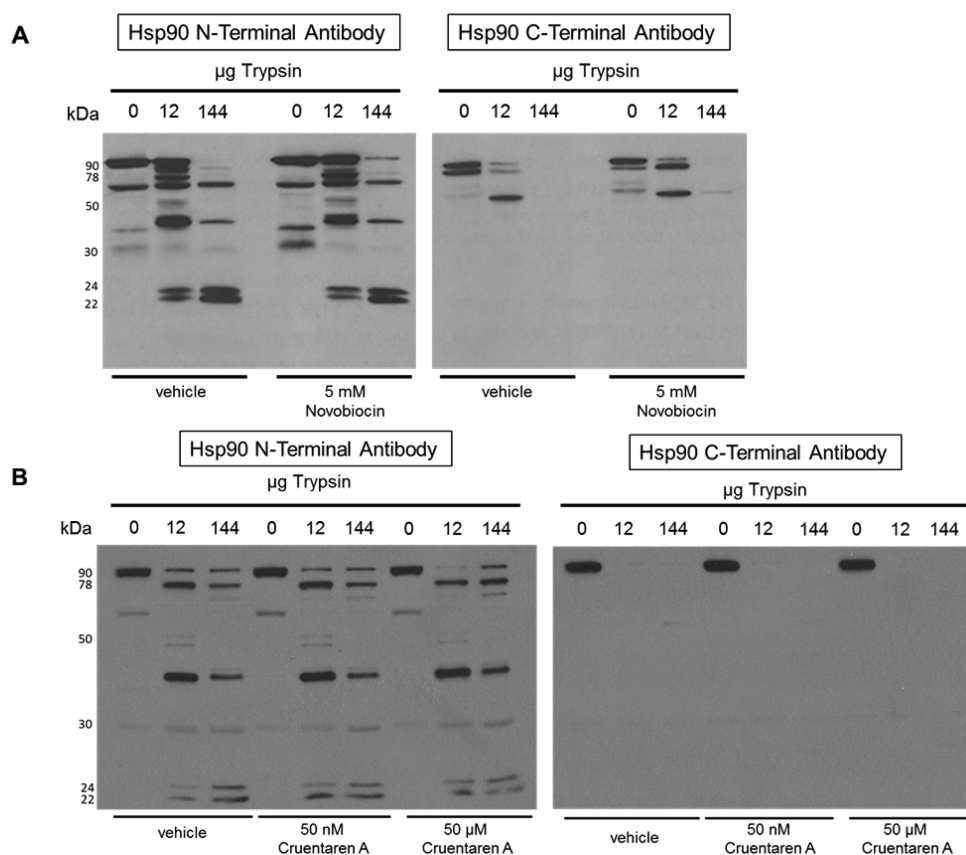


Figure 3. Proteolysis of Hsp90 from TnT reticulocyte lysate incubated under conditions of protein synthesis with (A) vehicle (1% DMSO) and 5 mM novobiocin and (B) vehicle (1% DMSO) or 50 nM or 50 μ M cruentaren A. Antibodies specific to either the N- or C-terminus of Hsp90 were used to identify the Hsp90 fragments produced in the presence of increasing amount of trypsin.

cruentaren A. Likewise, the N-terminal Hsp90 antibody detected bands at 78 and 30 kDa, as well as the 50 kDa doublet bands, and furthermore did not detect the presence of a 73 kDa band following administration of cruentaren A. Consistent with prior studies, the N-terminal antibody detected bands at 40 and 22/24 kDa. These data indicate that cruentaren A does not bind the Hsp90 C-terminus and does not protect the C-terminus from trypsinolysis. In addition, incubation with cruentaren A did not produce a proteolytic fingerprint that differed from vehicle control at low or high concentrations, which provides additional evidence that cruentaren A does not bind Hsp90.

Cruentaren A Does Not Directly Inhibit Hsp90 Function. Firefly luciferase is a Hsp90-dependent substrate that produces bioluminescence upon the conversion of D-luciferin to oxyluciferin and has been used to identify small molecule inhibitors of Hsp90 function.³¹ Heat-denatured luciferase requires functional Hsp90 to refold and, ultimately, produce bioluminescence. Monitoring bioluminescence over a range of concentrations indicates whether a molecule inhibits Hsp90 function in a dose-dependent manner. A cell-based luciferase refolding assay has been used to characterize the Hsp90 C-terminal inhibitor, KU-174. KU-174 is a novobiocin analogue that binds directly to the Hsp90 C-terminus, inducing client protein degradation and cell death at concentrations that do not activate the HSR.¹³ The cell-based luciferase refolding assay was used to determine whether cruentaren A inhibits Hsp90 function in a manner similar to KU-174 and/or geldanamycin.

Cruentaren A did not affect the rematuration of luciferase up to a 1 μ M concentration; however, both geldanamycin and KU-174 prevented the refolding of luciferase in a dose-dependent manner (Figure 4). These data suggest cruentaren A does not

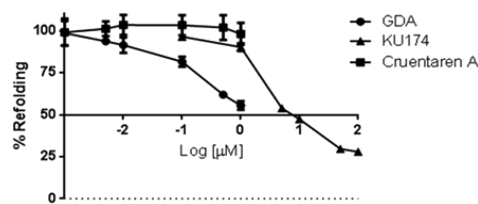


Figure 4. Percent of recovered luciferase activity after incubation of heat-denatured luciferase with geldanamycin (GDA), cruentaren A, or KU174 compared to vehicle (1% DMSO). The concentrations of GDA and cruentaren A used during this assay ranged from 1 to 0.001 μ M, and the concentrations of KU174 ranged from 100 to 0.1 μ M.

inhibit Hsp90 function, even at concentrations well above its EC_{50} value and at concentrations well beyond that needed to induce client protein degradation. When combined, these data demonstrate that cruentaren A does not bind or inhibit Hsp90 yet induces client degradation at low nanomolar concentrations without induction of the HSR. Therefore, cruentaren A inhibits Hsp90 function by inhibiting F_1F_0 ATP synthase and provides evidence that F_1F_0 ATP synthase is important for modulation of the Hsp90 protein folding machinery.

Interaction between Hsp90, Specifically Hsp90 α , and F_1F_0 ATP Synthase Is Disrupted upon 48-h Incubation with Cruentaren A, and Hsp90 α Exhibits Altered

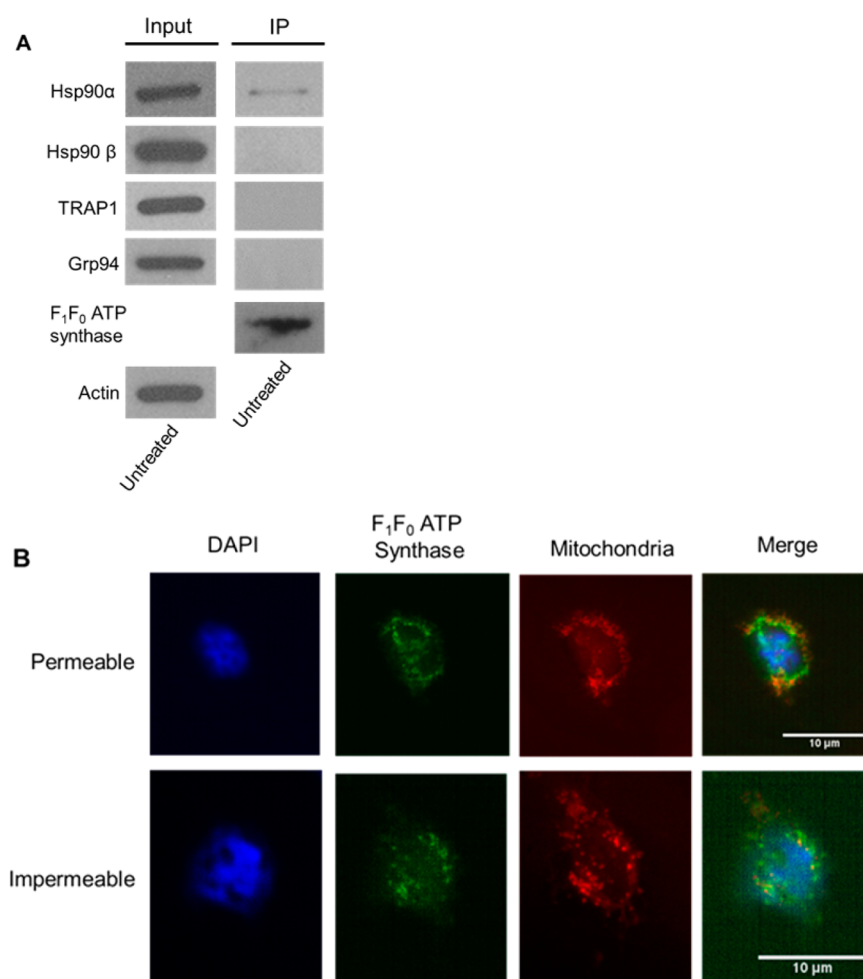


Figure 5. (A) Co-immunoprecipitation of Hsp90 α using an anti-F₁F₀ ATP synthase antibody (Life Technologies) from untreated MCF7 cell lysate. (B) Immunofluorescence images of the nucleus (DAPI), F₁F₀ ATP synthase, the mitochondria, and the merged images of untreated permeable and impermeable MCF7 cells.

Cellular Localization. Small molecules, such as celastrol and gedunin, disrupt the Hsp90 chaperone machinery by selectively targeting co-chaperone components of the heteroprotein complex.^{17,19,32} Disrupting the heteroprotein complex prevents client protein maturation by preventing co-chaperone assistance during the chaperone cycle. In addition, the concentration needed to disrupt the Hsp90 protein folding machinery does not induce the HSR.

It has been shown that F₁F₀ ATP synthase interacts with Hsp90-client protein complexes in several cancer cell lines.²⁰ Inhibition of this interaction with efraptins not only disrupted interactions between F₁F₀ ATP synthase and Hsp90 but also destabilized the Hsp90-client protein complex, which resulted in client protein degradation *via* the ubiquitin-proteasome pathway.^{20,22} Furthermore, it was shown that geldanamycin exhibits no effect on the F₁F₀ ATP synthase-Hsp90 complex.²⁰

We report that F₁F₀ ATP synthase directly interacts with Hsp90 in MCF7 cell lysates, specifically the Hsp90 α isoform (Figure 5 A; also see Supplementary Figure 2). No other Hsp90 isoform was detected following co-immunoprecipitation with F₁F₀ ATP synthase using antibodies specific to Hsp90 isoforms, yet all Hsp90 isoforms were present. There also appears to be a cell surface population of F₁F₀ ATP synthase when immunostaining both permeable and impermeable

MCF7 cells (Figure 5 B). These data suggest the Hsp90 α -F₁F₀ ATP synthase interaction may occur at the cell membrane, as Hsp90 α is located in the cytosol.

Interaction between F₁F₀ ATP synthase and Hsp90 α remained unaffected after 24 h of incubation with 50 nM cruentaren A; however, complete disruption was observed after 48 h (Figure 6 F). Cruentaren A also disrupted interaction between Hsp90 α and F₁F₀ ATP synthase in a dose-dependent manner (Figure 6 E). Interaction between Hsp90 α and other components of the Hsp90 multiprotein complex (*e.g.*, the Hsp90 co-chaperones Hsp70, Cdc37, and p23) were unaffected following 24 or 48 h of incubation with cruentaren A (Supplementary Figure 4). In contrast, the F₁F₀ ATP synthase-Hsp90 α interaction remained intact after 24 and 48 h in the presence of a vehicle control, KU-174, and geldanamycin (Figure 6 A–C). Interactions between F₁F₀ ATP synthase and Hsp90 α increased in the presence of geldanamycin at 24 and 48 h of incubation, which is likely a consequence of increased Hsp90 α levels that result upon induction of the HSR. KU-174 had no effect on the F₁F₀ ATP synthase-Hsp90 interaction, and co-immunoprecipitation results at 24 and 48 h of incubation were comparable to those of the vehicle control. In addition, the cellular distribution of Hsp90 α is dramatically different after 48 h of incubation with cruentaren A (Figure 6 F). When comparing the immuno-

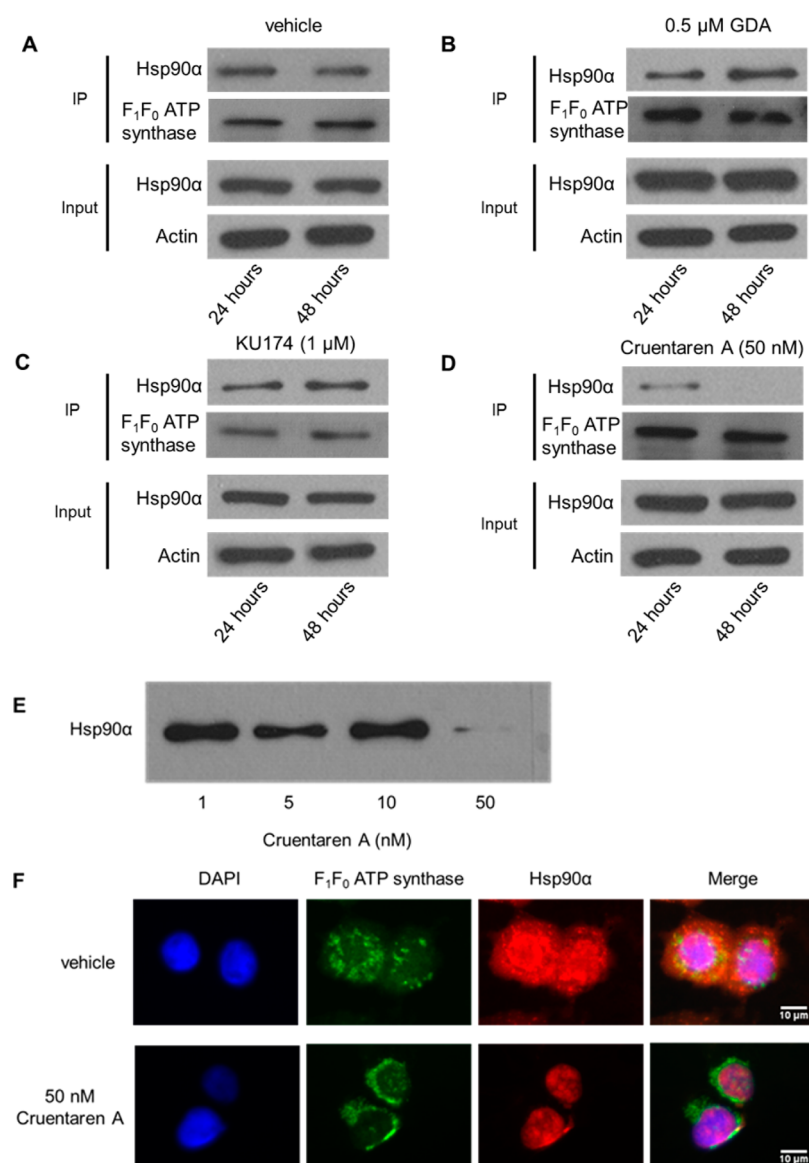


Figure 6. Co-immunoprecipitation of Hsp90 α using an anti-F₁F₀ ATP synthase antibody (Life Technologies) from MCF7 cell lysates treated for 24 and 48 h with (A) vehicle (0.25% DMSO), (B) 0.5 μ M geldanamycin (GDA), (C) 1 μ M KU174, and (D) 50 nM cruentaren A. (E) Dose-dependent disruption between Hsp90 α and F₁F₀ ATP synthase after 48 h of incubation with the indicated concentrations of cruentaren A. (F) Immunofluorescence images of the nucleus (DAPI), F₁F₀ ATP synthase, Hsp90 α , and the merged images after 48 h of incubation with either vehicle (0.1% DMSO) or 50 nM cruentaren A.

fluorescence imaging of Hsp90 α and F₁F₀ ATP synthase after 48 h of incubation with 50 nM cruentaren A to vehicle control, Hsp90 α translocates from an even distribution within the cytosol to localization at the nucleus. F₁F₀ ATP synthase distribution is slightly altered after 48 h and exhibits increased localization around the nucleus. The other cytosolic Hsp90 isoform, Hsp90 β , remained dispersed throughout the cytosol after 48 h of incubation with 50 nM cruentaren A compared to vehicle (Supplementary Figure 5). The cellular distribution of F₁F₀ ATP synthase and the Hsp90-dependent client protein, Raf-1, can be observed in the supplementary data (Supplemental Figure 4). Despite the apparent translocation of both Hsp90 α and F₁F₀ ATP synthase toward the nucleus, there is a distinct difference in the cellular location of these proteins, which corroborates co-immunoprecipitation data that the interaction between Hsp90 α and F₁F₀ ATP synthase is

completely disrupted after 48 h of incubation with 50 nM cruentaren A.

Disruption of F₁F₀ ATP synthase and Hsp90 α interactions resulted in a distinct cellular localization following 48 h of incubation with cruentaren A, which also correlates directly with client protein degradation. Together, these data demonstrate the importance of functional F₁F₀ ATP synthase during client protein maturation and support the promising potential of F₁F₀ ATP synthase as a target for disrupting Hsp90 function.

Due to Hsp90's involvement in multiple oncogenic pathways that contribute to the six hallmarks of cancer, inhibition of the Hsp90 chaperone machinery remains a promising strategy for the development of cancer chemotherapeutics despite the limitations of small molecule Hsp90 N-terminal inhibitors.¹² Alternative approaches to inhibiting Hsp90-dependent client maturation include disruption of interactions between Hsp90

and proteins that assist in client protein maturation during the chaperone cycle. Such small molecules include celastrol, which disrupts the interaction between Hsp90 and Cdc37, a co-chaperone that facilitates the maturation of Hsp90-dependent kinases, and gedunin, which binds co-chaperone p23 and leads to Hsp90-p23 disruption and ultimately induces cancer cell death.^{18,19}

Papathanassiou *et al.* propose that F_1F_0 ATP synthase possesses co-chaperone function, as F_1F_0 ATP synthase directly interacts with Hsp90 and disruption of this interaction *via* efrapreptins prevents client protein maturation.^{20,22} Efrapreptins destabilize the interaction of Hsp90 with client proteins and ultimately cause client protein degradation through F_1F_0 ATP synthase inhibition; however, efrapreptins are nonspecific and are known to inhibit other ATP synthases and the 20S proteasome among others.^{20,22} Kunze *et al.* reported the small molecule cruentaren A not only inhibits F_1F_0 ATP synthase but is selective for this ATP synthase over the Na^+/K^+ and V-type ATP synthases.²⁴ Cruentaren A also demonstrated selectivity for eukaryotic F_1 ATPases from yeast and mammals but did not inhibit the function of F_1 ATPases from *Escherichia coli*.²⁴

We report that cruentaren A indirectly causes Hsp90-dependent client degradation at low nanomolar concentrations through selective inhibition of F_1F_0 ATP synthase. Unlike Hsp90 N-terminal inhibitors, cruentaren A does not induce the pro-survival HSR upon client protein degradation. Hsp90 C-terminal inhibitors also avoid induction of the HSR while causing client degradation; however, cruentaren A did not bind the Hsp90 C-terminus at high concentrations or directly inhibit Hsp90 function. It was demonstrated that F_1F_0 ATP synthase directly interacts with Hsp90 in MCF7 cell lysates and specifically interacts with the Hsp90 α isoform. This interaction remained intact in the presence of the N-terminal inhibitor geldanamycin and the C-terminal inhibitor KU-174 but was completely disrupted upon incubation with cruentaren A. Furthermore, the cellular distribution of Hsp90 α and F_1F_0 ATP synthase is dramatically altered upon incubation with cruentaren A. In the presence of vehicle, the cellular distribution of Hsp90 α and F_1F_0 ATP synthase supports an interaction that occurs within the cytosol; however, upon incubation with cruentaren A, Hsp90 α localizes at the nucleus while F_1F_0 ATP synthase remains relatively unaffected. Destabilization of the F_1F_0 ATP synthase-Hsp90 α interaction and altered localization of these proteins correlates with client protein degradation upon incubation with cruentaren A. Therefore, selective inhibition of F_1F_0 ATP synthase results in client protein degradation by disrupting interactions between Hsp90 α and F_1F_0 ATP synthase.

The hypothesis that ATP depletion affects the interaction between Hsp90 and client proteins has been suggested previously. Peng *et al.* observed that nonmalignant myocytes treated with 2-deoxy-D-glucose or antimycin A, which are inhibitors of glycolysis and oxidative phosphorylation, respectively, disrupted interactions between Hsp90 and the client protein ErbB2, resulting in ErbB2 degradation.³³ It was shown that the mitochondrial ATP synthase inhibitor oligomycin A resulted in decreased levels of Her2 and pAkt after 48 h of incubation comparable to those of cruentaren A; however, no degradation of Raf was observed, and increased Hsp70 levels were observed with high concentrations of oligomycin A (Figure 7).

The mechanism by which cruentaren A inhibits F_1F_0 ATP synthase and whether it prevents proton translocation across

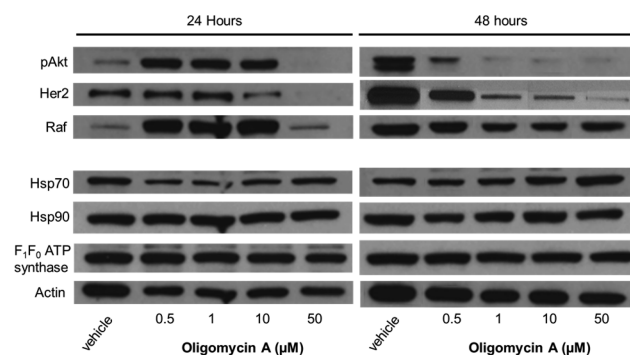


Figure 7. Western blot for Hsp90 client proteins (pAkt, Her2, and Raf) and Hsp70, Hsp90, and F_1F_0 ATP synthase using MCF7 cell lysates after 24 or 48 h of incubation with the indicated amount of vehicle (0.25% DMSO) or oligomycin A. The calculated EC_{50} value of oligomycin A against the MCF7 cell line was 7.35 μ M.

the mitochondrial membrane, similar to oligomycin A, are not known.³⁴ Although treatment with cruentaren A or oligomycin A resulted in decreased levels of Her2 and pAkt after 48 h of incubation, increased Hsp70 levels and unchanged Raf levels indicate that oligomycin A and cruentaren A operate through related but distinct mechanism(s) of action.

Collectively, these data suggest that Hsp90, specifically Hsp90 α , functions as a cellular sensor of ATP and does so by directly interacting with F_1F_0 ATP synthase. Indeed, 80% of ATP from proliferating MCF7 cells occurs through oxidative phosphorylation.³⁵ This contradicts the Warburg hypothesis, which suggests malignancies switch from oxidative ATP production to glycolytic; however, numerous studies have found this to be dependent upon the individual cancer and the cancer cell environment.^{35–37} As previously mentioned, numerous other cancer cells, including the MCF7 cell line, have an increased concentration of Hsp90 α and elevated chaperone activity. Given the ATPase activity of Hsp90, it is plausible that Hsp90 functions as a cellular sensor of ATP and that Hsp90 α , the most abundant Hsp90 isoform in cancer cells, requires a significant amount of ATP and therefore directly interacts with the cell's source of ATP, F_1F_0 ATP synthase. Depriving Hsp90 α of ATP by inhibiting F_1F_0 ATP synthase exhibits a debilitating effect on the chaperone cycle that utilizes oxidative phosphorylation as their ATP source. Simultaneously inhibiting ATP production and Hsp90 chaperone activity may explain the potency of cruentaren A among the many different cancer cell lines and provides a novel approach to modulating the Hsp90 protein folding machinery.

In conclusion, inhibition of F_1F_0 ATP synthase *via* cruentaren A and disruption of the interaction between Hsp90 α and F_1F_0 ATP synthase represents a novel and powerful approach toward inhibiting client protein maturation devoid of the HSR. While work remains to be done, cruentaren A represents a new class of Hsp90 modulators that targets the Hsp90 α - F_1F_0 ATP synthase interaction and represents a new paradigm to modulate the Hsp90 protein folding machinery.

METHODS

Antibodies and Reagents. Antibodies targeting Hsp90 β , Grp94, Raf-1, and actin were purchased from Santa Cruz Biotechnology. Antibodies targeting Hsp90 α -2 and Hsp70 were purchased from Assay Designs. Antibodies targeting Cdc37, p23, and an additional Raf-1 antibody were purchased from abcam. The remaining antibodies are listed and were purchased from the indicated vendors: TRAP1 (BD

Biosciences), pAKT (Cell Signaling), Her2 (*c-erbB-2*) (Invitrogen), and F₁F₀ ATP synthase subunit β (Life Technologies and proteintech). The antibody targeting the N-terminus of Hsp90 was purchased from Thermo Scientific (PA3-013), and the antibody targeting the C-terminus Hsp90 was purchased from Enzo Life Sciences (AC88). KU174 and cruentaren A were synthesized in house, and geldanamycin was purchased from Sigma Aldrich.^{38,39}

Cell Culture. The media for each cell line was supplemented with streptomycin (500 $\mu\text{g mL}^{-1}$), penicillin (100 units mL^{-1}), and 10% FBS. MCF7 cells were maintained in Advanced DMEM/F12 (1:1; Gibco) supplemented with L-glutamine (2 mM). A549 cells were maintained in F12K (Cellgro). MRC-5 cells were maintained in DMEM (Cellgro). Wild type and luciferase-expressing PC3-MM2 cells (a gift from George Vielhauer) were maintained in MEME (Sigma) supplemented with 5 $\mu\text{g mL}^{-1}$ puromycin. Cells were grown in a humidified atmosphere (37 °C, 5% CO₂) and passaged when confluent.

Antiproliferation. Cells were grown to confluence, seeded (2000 cells/well, 100 μL total media) in clear, flat-bottom 96-well plates and allowed to attach overnight. Compound or geldanamycin at varying concentrations in DMSO (1% DMSO final concentration) was added. Cells were returned to the incubator for an additional 72 h. After 72 h, cell growth was determined using a MTS/PMS cell proliferation kit (Promega) per the manufacturer's instructions. Cells incubated in 1% DMSO were used as 100% proliferation (*i.e.*, DMSO = 100% growth), and the relative growth for each compound concentration was compared to that in 1% DMSO. IC₅₀ values were calculated from two separate experiments performed in triplicate using GraphPad Prism 6.0.

Western Blot. MCF7 cells were grown to confluence and seeded at 0.4×10^6 cells/well/2 mL. Cells were incubated for 24 h and treated with varying concentrations of cruentaren A or 1 μM geldanamycin in DMSO (0.25% DMSO final concentration) or vehicle (DMSO) for 24 or 48 h. Cells were harvested in cold PBS and lysed using MPER (Thermo Scientific) supplemented with protease and phosphatase inhibitors (Roche) according to manufacturer's directions. Lysates were clarified at 14,000g for 15 min at 4 °C. Protein concentrations were determined using the Pierce BCA protein assay kit per the manufacturer's instructions. Equal amounts of protein (5 μg) were electrophoresed under reducing conditions (10% acrylamide gels), transferred to PVDF, and immunoblotted with the corresponding antibody. Membranes were incubated with an appropriate horseradish peroxidase-labeled secondary antibody, developed with a chemiluminescent substrate, and visualized.

Proteolytic Fingerprinting Assay. Rabbit reticulocytes (Green Hectares) were incubated under conditions of protein synthesis at 30 °C in the presence of compound or vehicle (1% DMSO) for 10 min. Each reaction mixture contained 66.6% rabbit reticulocytes and 33.3% ATP regenerating system (10 mM creatine phosphate and 20 $\mu\text{g mL}^{-1}$ creatine phosphokinase) and a final concentration of 75 mM KCl. Each reaction mixture contained the indicated amount of compound. After incubating, the samples were immediately placed on ice, and the indicated amount of TPCK-treated trypsin (Worthington) was added to each sample. The samples were digested on ice for an additional 6 min, and the reactions were quenched by the addition of Laemmli sample buffer followed by immediate boiling. Equal amounts of each sample were electrophoresed under reducing conditions (10% acrylamide gels), transferred to PVDF, and immunoblotted with antibodies specific to the N-terminus of Hsp90 or the C-terminus of Hsp90. Membranes were incubated with an appropriate horseradish peroxidase-labeled secondary antibody, developed with a chemiluminescent substrate, and visualized.

Luciferase Refolding Assay. Compound at varying concentrations in DMSO (1% DMSO final concentration) was added to wells of a white, round-bottom 96-well plate containing 50 μL of MEME media. Luciferase-expressing PC3-MM2 cells were grown to confluence, collected, and incubated for 8–12 min at 50 °C in prewarmed MEME media until bioluminescence of luciferase was reduced to 1% of the initial counts. Cells were added (60,000 cells/50 μL) to wells (final concentration of 60,000 cells/100 μL), and the

plate was returned to the incubator for 1 h. After 1 h, 100 μL of luciferase substrate reagent (75 mM tricine at pH7.8, 24 mM MgSO₄, 0.3 mM EDTA, 2 mM DTT, 0.313 D-luciferin, 0.64 mM coenzyme A, 0.66 mM ATP, 150 mM KCl, 10% Triton-X, 20% glycerol, and 3.5% DMSO) was added to wells, and the bioluminescence was immediately read (0.5 s integration time). Cells that were incubated in 1% DMSO were used as 100% bioluminescence (*i.e.*, DMSO = 100% refolding), and the relative refolding for each compound concentration was compared to that in 1% DMSO. The concentrations for each compound were in triplicate, and dose–response curves were generated using GraphPad Prism 6.0.

Co-immunoprecipitation. MCF7 cells were grown to confluence and seeded at 2×10^6 cells/5 mL in 10 cm dishes. Cells were incubated for 24 h and then treated with either 0.5 μM geldanamycin, 1 μM KU174, or 50 nM cruentaren A in DMSO (0.25% DMSO final concentration) or vehicle (DMSO) for the indicated lengths of time. Media and cells were collected with PBS and centrifuged at 200g for 5 min at 4 °C. Supernatant was aspirated, and pellets were washed one time with cold PBS and centrifuged. Supernatant was aspirated, and cell pellets were subsequently suspended in the nondenaturing lysis buffer (10 mM Tris-HCl at pH 7.5 and 0.2% NP-40 (v/v)) and incubated on ice for 2 h. Lysates were clarified at 14,000g for 15 min at 4 °C. Protein concentrations were determined using a Pierce BCA protein assay kit per the manufacturer's instructions. Equal protein (400 or 500 μg) was incubated with 1 μg of anti-F₁F₀ ATP synthase antibody in 500 μL total volume lysis buffer for approximately 16 h with rocking at 4 °C. Following incubation, 30 μL of resuspended Dynabeads Protein A (Invitrogen) was added and incubated with rocking for 1 h at 4 °C. Protein A beads were washed 3 times with lysis buffer (500 μL), suspended in Laemmli sample buffer (15 μL), and were boiled for 15 min to dissociate proteins from beads. Samples were electrophoresed under reducing conditions (10% acrylamide gels), transferred to PVDF, and immunoblotted with the indicated antibodies. Membranes were incubated with a species-appropriate horseradish peroxidase-labeled secondary antibody, developed with a chemiluminescent substrate, and visualized.

Immunofluorescence Analysis. MCF7 cells were grown to confluence, seeded (1000 cells/well, 100 μL total media) in black/clear well, flat-bottom 96-well plates, and allowed to attach overnight. Cruentaren A in DMSO (0.1% DMSO final concentration) was added and incubated at 48 h. Cells were washed with PBS, fixed with freshly made 4% (w/v) paraformaldehyde in PBS for 25 min, washed with PBS, permeabilized with 0.1% (v/v) Tween-20 in PBS for 5 min, washed with PBS, blocked with 3% (w/v) BSA in PBS for 1 h, washed with PBS, and incubated with primary antibody targeting F₁F₀ ATP synthase subunit β and/or Hsp90 α -2 at a 1:200 and a 1:400 concentration, respectively, in 3% BSA in PBS at 4 °C overnight. The cells were then washed with PBS, incubated with secondary antibody conjugated with Alexa Fluor 488 or 568 for 3 h at 4 °C, washed with PBS, and counterstained the DNA with DAPI. Cells that underwent mitochondrial staining were incubated with MitoTracker Red CMXRos prior to fixing according to the manufacturer's instructions. Confocal images were acquired sequentially with SlideBook Version 5.0 software on a 3I Spinning Disk Confocal Inverted Microscope (Olympus) using 40x long working distance air lenses. Images were processed using Image J software (NIH).

■ ASSOCIATED CONTENT

📄 Supporting Information

Supplementary figures and additional experimental information. This material is available free of charge *via* the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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