Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 345 (2006) 419–429
BBRC
www.elsevier.com/locate/ybbrc

F_1F_0-ATP synthase functions as a co-chaperone of Hsp90–substrate protein complexes

Adonia E. Papathanassiu *, Nicholas J. MacDonald [1], Akos Bencsura [2], Hong A. Vu [3]

Ergon Pharmaceuticals LLC, PO Box 1001, Silver Spring, MD 20910, USA

Received 30 March 2006
Available online 2 May 2006

Abstract

Inhibition of heat shock protein 90 (Hsp90) has emerged as a novel intervention for the treatment of solid tumors and leukemias. Here, we report that F_1F_0-ATP synthase, the enzyme responsible for the mitochondrial production of ATP, is a co-chaperone of Hsp90. F_1F_0-ATP synthase co-immunoprecipitates with Hsp90 and Hsp90–client proteins in cell lysates of MCF-7, T47D, MDA-MB-453, and HT-29 cancer cells. Inhibition of F_1F_0-ATP synthase by efrapeptins results in the disruption of the Hsp90 complexing with its substrate proteins and, in most cases, in the degradation of the latter. Hsp90–client proteins affected by the inhibition of F_1F_0-ATP synthase included ERα, mutated p53 (m.p53), Hsp70, Hsp27, and caspase-3 but not Raf-1. This is the first report identifying caspase-3 as a substrate protein of Hsp90. Unlike typical Hsp90 inhibitors, efrapeptin treatment triggers Hsp70 downregulation in parallel with depletion of Hsp90. This suggests that suppression of Hsp90 chaperone function through inhibition of F_1F_0-ATP synthase does not result in activation of transcription factor HSF-1, a generally unfavorable consequence of anti-cancer treatments based on Hsp90 inhibition.

Keywords: Hsp90; Hsp70; Hsp27; Mutated p53; Caspase-3; Efrapeptins; ATPase

Heat shock proteins (Hsps) are a family of housekeeping molecules that function as chaperones to recognize proteins with abnormal conformations, prevent them from non-specific aggregation, and support their conversion to a native, functional structure [1]. Hsps are particularly vital to cells under conditions conducive to the production of abnormal proteins. For example, they are upregulated in cells as a response to environmental stress [2–4]. Because deleterious environmental conditions are commonly found in tumors, Hsps appear to play a significant role in cancer growth and progression, possibly by allowing cancer cells to successfully survive and adapt to a harmful milieu created by hypoxia, nutrient deprivation, accumulation of harmful metabolic by-products, and often exposure to chemotherapy and radiation [4–7].

Hsp90 is a prominent member of the Hsp family and one that is abundantly expressed in cells under physiological as well as environmentally stressful conditions. As a part of a super-chaperone complex, which also includes Hsp70, Hsp40, Hop, and p23, Hsp90 specifically binds to substrate proteins that are near their native state [8]. The majority of Hsp90 substrates are involved in signal transduction and include steroid receptors such as estrogen receptor (ER), soluble kinases such as Akt and Raf-1, oncogenes such as m.p53, transmembrane kinases such as ErbB2, and transcription factors such as HIF-1 [9–11].

Hsp90 is an ATPase and, as such, it is known to exist in different conformations. ADP-bound conformation

* Corresponding author. Fax: +1 202 723 0058.
E-mail address: adoniap@ergon-pharmaceuticals.com (A.E. Papathanassiu).
[1] Present address: Malaria Vaccine Development Branch NIAID, Twinbrook I, Rm 1123, 5840 Fishers Ln, Rockville, MD 20852, USA.
[2] Present address: Department of Theoretical Chemistry, Chemical Research Center HAS Pusztaszeri u. 59-67, Budapest, Hungary.
[3] Present address: IOMAI Corporation, 20 Firstfield Road, Gaithersburg, MD 20878, USA.

0006-291X/$ - see front matter
doi:10.1016/j.bbrc.2006.04.104

promotes the assembly of the Hsp90–Hsp70–Hsp40–Hop super-chaperone complex and the binding of Hsp90 to substrate proteins, while ATP-bound conformation promotes the association of the substrate-bound Hsp90–Hsp70–Hsp40–Hop complex with p23 and the release of co-chaperones Hop and Hsp70–Hsp40 [12]. Formation of the substrate-bound Hsp90–Hsp70–Hsp40–Hop complex is thought to promote client ubiquitination, while p23 binding contributes to appropriate structural folding, stable expression, and activity of the client proteins. Inhibitors of Hsp90 such as geldanamycin (GA) deplete client proteins by stabilizing Hsp90 super-chaperone complexes that promote ubiquitination and proteasomal degradation of the substrate proteins [13,14].

F_1F_0-ATP synthase is a multi-subunit protein localized in the mitochondria of eukaryotic cells, where it utilizes the electrochemical gradient, established across the inner mitochondrial membrane by oxidative phosphorylation, for the synthesis of ATP from ADP and inorganic phosphate [15]. It consists of two major domains: the catalytic moiety F_1, comprising subunits α, β, γ, δ, and ε in a stoichiometry of 3:3:1:1:1, and the proton channel component F_0, comprising subunits a, b, and c in a stoichiometry of 1:2:12. Like other mitochondrial enzymes, the majority of F_1F_0-ATP synthase subunits are subject to nuclear transcription, translocation from the cytosol to the mitochondria, and assembly into a macromolecular complex in the inner mitochondrial membrane. The assembled enzyme physically interacts with Hsp60, which is known to stabilize numerous mitochondrial proteins [16]. In the present study, we report that, in addition to Hsp60, F_1F_0-ATP synthase co-immunoprecipitates with Hsp90 and its client proteins and plays a role in modulating Hsp90 chaperone function. Inhibition of F_1F_0-ATP synthase by efrapeptins, oligomycin A or 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) leads to dissociation of the F_1F_0-ATP synthase-Hsp90–substrate protein complexes and degradation of the latter in an ATP-independent fashion. The majority of the experiments were performed with efrapeptins, a family of small, naturally occurring oligopeptides known to bind to β-subunit of F_1F_0-ATP synthase [17,18]. The amino-acid sequence of efrapeptins is shown in Fig. 1.

Materials and methods

Materials. Efrapeptins used here were isolated as a mixture of efrapeptins D and E from cultures of the fungus *Tolypocladium niveum*. Efrapeptins D and E are nearly identical peptides differing only in a single amino acid: in efrapeptin E, at position 4, α-amino-isobutyric acid of efrapeptin D is substituted by isovaline. Oligomycin A, NBD-Cl, and rotenone were from Sigma–Aldrich (St. Louis, MO). Geldanamycin was from LKT Laboratories Inc. (St. Paul, MN). All cancer cells lines (MCF-7 breast, MDA-MB-453 breast, T47D breast, and HT-29 colon) were obtained from ATCC (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), trypsin–EDTA, and penicillin–streptomycin antibiotic mixture were obtained from Cambrex (Walkerville, MD). Antibodies (abs) against Ubiquitin (Ub), Hsp90, ERα, Hsp70, Hsp27, Raf-1, and caspase-3 were obtained from Lab Vision Corporation (Fremont, CA). The ab against p53 was purchased from Sigma–Aldrich. Secondary antibodies were from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD). Nu–PAGE electrophoresis related materials were purchased from Invitrogen (Carlsbad, CA). Protein A–agarose beads were from Pierce Biotechnology (Rockford, IL). Enhanced chemiluminescence (ECL) reagents were from Amersham Biosciences (Piscataway, NJ).

A Ac-AIB-GLY-LEU-isoVAL-X

B Ac-LEU-isoVAL-X

C Ac-PIP-AIB-PIP-AIB-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-GLY-LEU-AIB-X

D Ac-PIP-AIB-PIP-AIB-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-GLY-LEU-isoVAL-X

E Ac-PIP-AIB-PIP-isoVAL-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-GLY-LEU-isoVAL-X

F Ac-PIP-AIB-PIP-AIB-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-ALA-LEU-isoVAL-X

G Ac-PIP-AIB-PIP-isoVAL-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-ALA-LEU-isoVAL-X

where AIB= α-amino-isobutyric acid, isoVAL=isovaline, PIP= pipecolic acid and X=

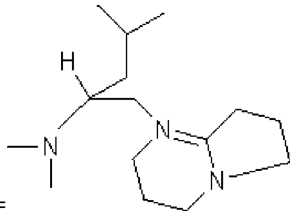

Fig. 1. Amino-acid sequence of efrapeptins. Efrapeptins A and B are hydrolytic fragments of efrapeptins D, E, F, and G.

Cell extracts. MCF-7, MDA-MB-453, T47D, and HT-29 tumor cells were routinely cultured in DMEM supplemented with 10% FCS and antibiotics. For immunoblotting experiments, cells were plated onto 60 cm^2 culture dishes and cultured until they were 30–60% confluent. For co-immunoprecipitation experiments, cells were exposed to various concentrations of efrapeptins, oligomycin A, NBD-Cl or rotenone for 10 min. For client protein degradation experiments, cells were exposed to various concentrations of efrapeptins for 24 h, although, in the case of ERα and caspase-3, other time periods were also used. Following treatment, media were removed and the cells were washed twice with PBS. They were scraped, centrifuged at 1000*g* for 10 min at 4 °C, and lysed in 10 mM Tris–HCl, pH 7.5, containing 0.2% v/v NP-40, and 5 mM phenylmethylsulfonyl fluoride. The cell lysates were centrifuged at 15,000*g* for 10 min at 4 °C. After collection, clarified supernatants were stored at −70 °C or used immediately.

Co-immunoprecipitation. Equal amounts of cell lysates containing approximately 0.25–1.0 mg of total protein were incubated overnight at 4 °C with 0.5 μg of a monoclonal ab (mab) raised against the β-subunit of F_1F_0-ATP synthase or 0.5 μg of a rabbit anti-Hsp90 polyclonal ab in the presence of 10 mM sodium molybdate. Molybdate is known to stabilize the interaction of Hsp90 with m.p53, steroid hormone receptors, and certain kinases possibly by inducing structural changes in the COOH-terminal of the protein [12]. After an additional incubation with 25 μL protein-A agarose beads at room temperature (RT) for 1 h, the samples were microcentrifuged for 30 s and the agarose beads were washed 3× with PBS. Complexes were eluted in an SDS-loading buffer containing 10% mercaptoethanol after brief heating at 95 °C.

Immunoblotting. Equal volumes of immunoprecipitated proteins or equal amounts (50–100 μg) of cell extracts were subjected to SDS–PAGE electrophoresis followed by protein transfer onto nitrocellulose membranes. After blocking in TBS (20 mM Tris–HCl, pH 7.4, 0.9% NaCl) containing 10% non-fat dried milk and exposure to appropriately diluted primary and secondary abs, transferred proteins were detected by ECL. The band density seen on the obtained images was evaluated using ImageJ software (http://rsb.info.nih.gov/ij/). Co-immunoprecipitation and protein degradation experiments were repeated at least twice. Western blots shown here are representative examples. Abs used for immunoprecipitation (IP) and immunoblotting (Dev) are denoted.

ATP assays. Tumor cells were plated onto 96-well plates at a cell density of 2000–5000 cells/well. The cells were then allowed to adhere overnight. The following day, the cells were treated with various concentrations of ATP-depleting agents for 10 min. At the end of the incubation period, cell media were removed and cells were washed with 0.1 M Tris–HCl, pH 7.8. The cells were lysed in 0.1 N NaOH for 5 min. ATP levels of cell lysates previously neutralized with 0.1 N HCl were determined using an ATP Bioluminescent Assay kit from Sigma–Aldrich according to the manufacturer's instructions and a Wallac 1420 VICTOR3 plate reader from Perkin-Elmer (Boston, MA). Each experiment was performed in triplicate and repeated at least thrice. Reported tables show average values and standard deviations obtained from similar experiments.

Results

Co-immunoprecipitation of Hsp90 with ERα in the presence of efrapeptins

Brief (10 min) exposure of MFC-7 breast cancer cells to low levels of efrapeptins resulted in dissociation of Hsp90-ERα complexes as revealed by co-immunoprecipitation experiments (Fig. 2). At increased concentrations of efrapeptins, recovery of the complex was seen. While 0.001 μM efrapeptins inhibited association of Hsp90 with ERα by 70%, the association was inhibited only by 30% at 0.01 μM. No inhibition was observed at 0.1 μM efrapeptins.

To examine whether efrapeptin-driven inhibition of the Hsp90-ERα association was the indirect consequence of a general reduction in the levels of intracellular ATP by efrapeptins, we compared ATP concentrations found in whole cell extracts of untreated (control) and efrapeptin-treated MCF-7 cells. Table 1 shows no significant decrease in ATP at efrapeptin concentrations less than 1 μM. At 0.001 μM efrapeptins, where dissociation of Hsp90-ERα complex was previously observed, a slight increase in the ATP concentration was seen. However, this increase was not statistically significant. This suggests that modulation of Hsp90 chaperone function by efrapeptins utilized an ATP-independent mechanism.

Inhibition of Hsp90-ERα complexing in the presence of ATP-depleting agents

In order to verify that efrapeptins modulated Hsp90 chaperone function by an ATP-independent mechanism,

Table 1
Intracellular ATP levels found in cell lysates of MCF-7 cells treated with F_1F_0-ATP synthase inhibitors or rotenone

Concentration (μM)	Intracellular ATP Concentration (% Control)			
	Efrapeptins	Oligomycin	NBD-Cl	Rotenone
0.001	115 ± 14*	95 ± 9	104 ± 9	101 ± 2
0.01	96 ± 4	95 ± 16	98 ± 2	87 ± 8
0.1	93 ± 7	77 ± 11	100 ± 10	93 ± 17
1	81 ± 9	69 ± 2	116 ± 8	82 ± 8
10	68 ± 8	56 ± 13	35 ± 11	39 ± 8

* Mean ± st dev.

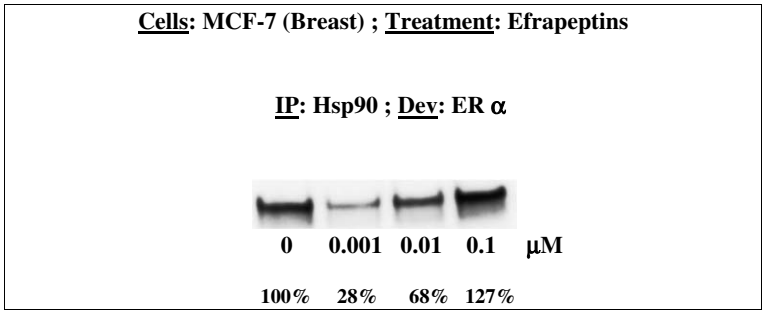

Fig. 2. Co-immunoprecipitation of Hsp90 with client protein ERα after brief exposure (10 min) of MCF-7 cells to various concentrations of efrapeptins.

we examined the effect of other ATP-depleting agents on the Hsp90-ERα complexing. In Fig. 3, oligomycin A and NBD-Cl inhibited co-immunoprecipitation of Hsp90 with ERα in a dose-dependent fashion, whereas rotenone had no effect even at increased concentrations of the drug. Like efrapeptins, oligomycin A and NBD-Cl suppress the synthesis of mitochondrial ATP by inhibiting the enzymatic activity of F_1F_0-ATP synthase: NBD-Cl binds to the β-subunit of the F_1 catalytic moiety of the enzyme, while oligomycin A binds to its proton channel F_0 [19–21]. On the other hand, rotenone reduces ATP production by inhibiting NADH dehydrogenase.

Examination of the intracellular levels of ATP revealed that, at low concentrations of oligomycin A and NBD-Cl, dissociation of Hsp90-ERα complexes was not accompanied by a concomitant decrease in ATP concentration. For example, 0.01 μM oligomycin A or 0. 1 μM NBD-Cl, disrupts Hsp90 complexing with ERα (Fig. 3) without a statistically significant change in the levels of ATP (Table 2). Furthermore, rotenone decreased intracellular ATP concentrations in treated MCF-7 without a parallel dissociation of Hsp90-ERα (Fig. 3). These observations suggest that F_1F_0-ATP synthase inhibitors are able to disrupt the interaction of Hsp90 with its client protein independently of ATP. We then examined the possibility that F_1F_0-ATP synthase is a co-chaperone involved in stabilizing Hsp90–client protein complexes.

Co-immunoprecipitation of F_1F_0-ATP synthase with Hsp90

F_1F_0-ATPase and Hsp90 co-immunoprecipitated from whole cell extracts of MCF-7 cells (Fig. 4A). The physical

Table 2
Intracellular ATP levels found in the cell lysates of various tumor cells treated with efrapeptins

Efrapeptin concentration (μM)	Intracellular ATP concentration (% Control) after efrapeptin treatment		
	T47D	MDA-MB-453	HT-29
0.001	92 ± 9*	93 ± 13	88 ± 16
0.01	110 ± 10	94 ± 17	94 ± 8
0.1	106 ± 4	100 ± 1	89 ± 7
1	113 ± 8	99 ± 4	87 ± 12
10	108 ± 5	112 ± 15	83 ± 12

* Mean ± st dev.

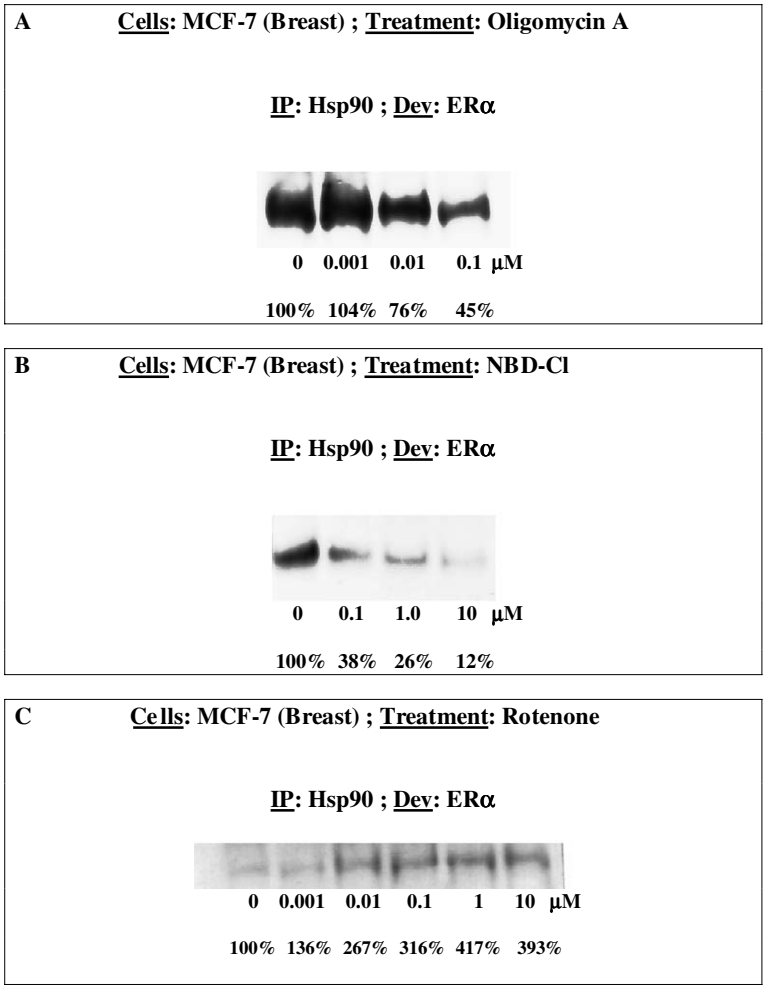

Fig. 3. Co-immunoprecipitation of Hsp90 with client protein ERα after brief exposure of MCF-7 cells to oligomycin A (A), NBD-Cl (B), and rotenone (C).

interaction of F_1F_0-ATPase with Hsp90 was partially disrupted after brief (10 min) treatment of the cells with efrapeptins (Fig. 4A). At 0.01 μM efrapeptins, 70% disruption of the F_1F_0-ATPase–Hsp90 complex was observed. In agreement with previous observations, complex recovery was seen at increased concentrations of efrapeptins: 0.1 μM efrapeptins inhibited F_1F_0-ATPase–Hsp90 association only by 30%. A similar exposure of MCF-7 cells to a wide range of GA concentrations had no effect on the co-immunoprecipitation of F_1F_0-ATP synthase with Hsp90 (Fig. 4B).

Hsp90 client proteins affected by the dissociation of the Hsp90–F_1F_0-ATP synthase complex

To examine which client proteins are affected by the disruption of the F_1F_0-ATPase–Hsp90 interaction, co-immunoprecipitation experiments were performed using whole cell extracts of various tumor cells treated with efrapeptins for 10 min. Hsp90 co-immunoprecipitated with p53 in T47D breast cancer cells, which are known to express m.p53 (Fig. 5A). F_1F_0-ATPase co-immunoprecipitated with ERα in T47D breast cancer (Fig. 5B), Hsp70 in MDA-MB-453 breast cancer (Fig. 5C), Hsp27, and Raf-1 in MCF-7 breast cancer (Figs. 5D and E, respectively), and caspase-3 in HT-29 colon cancer cell lysates (Fig. 5F). This is the first report suggesting that caspase-3 is an Hsp90 substrate.

Co-immunoprecipitation of Hsp90 with m.p53 and F_1F_0-ATP synthase with ERα, Hsp70, Hsp27, and caspase-3 but not Raf-1 were interrupted after exposure of the cells to efrapeptins. The degree to which efrapeptins affected the interaction of F_1F_0-ATPase with Hsp90 client proteins depended on the specific client protein. For example, 0.001 μM of efrapeptins reduced co-immunoprecipitation of F_1F_0-ATPase with ERα in T47D breast cancer cells by 50%. No further reduction was observed when the concentration of efrapeptins increased to 0.01 or 0.1 μM. On the other hand, a dose-dependent decrease in the F_1F_0-ATPase–Hsp90 client protein interaction was observed when the client protein was Hsp70 or Hsp27. In both cases, 0.1 μM efrapeptins seem to greatly disrupt the F_1F_0-ATPase–Hsp90–client protein complex. It is worth noting here that efrapeptin treatment of T47D, MDA-MB-453, or HT-29 tumor cells did not result in any reduction in the intracellular levels of ATP as revealed by ATP bioluminescent assays (Table 2).

Inhibition of the co-immunoprecipitation of F_1F_0-ATP synthase with an Hsp90–client protein was indicative of the dissociation not only of the F_1F_0-ATP synthase–Hsp90 complex but also of the Hsp90–client protein association. Fig. 6A shows disruption of the Hsp90–Hsp27 complex after efrapeptin treatment of MCF-7 cells, while Fig. 6B shows disruption of the Hsp90–caspase-3 complex in HT-29 cells after a similar treatment with efrapeptins. Interestingly, dissociation of Hsp90 with Hsp27 or caspase-3 is seen at concentrations of efrapeptins, which do not disrupt complexing of F_1F_0-ATP synthase with Hsp27 or caspase-3. This may be indicative of the sequence of release of the various components comprising the super-chaperone complex that involves F_1F_0-ATP synthase, Hsp90, and Hsp70 following inhibition of F_1F_0-ATP synthase. In addition, Figs. 6A and B suggest a partial recovery of the Hsp90–client protein complex at increased concentrations (0.1 and 1.0 μM) of efrapeptins. This recovery appears transient since co-immunoprecipitation of Hsp27 or caspase-3 with Hsp90 decreases again at 10 μM efrapeptins.

Efrapeptin-induced degradation of Hsp90–client proteins

With the notable exception of ERα, exposure of cancer cells to efrapeptins led to the degradation of Hsp90 and

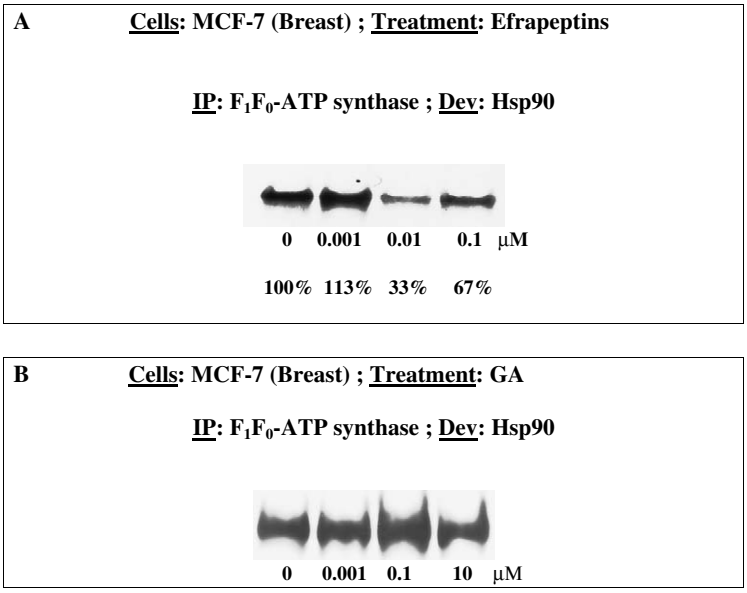

Fig. 4. Co-immunoprecipitation of F_1F_0-ATP synthase with Hsp90 after brief exposure of MCF-7 cells to efrapeptin (A) and GA (B).

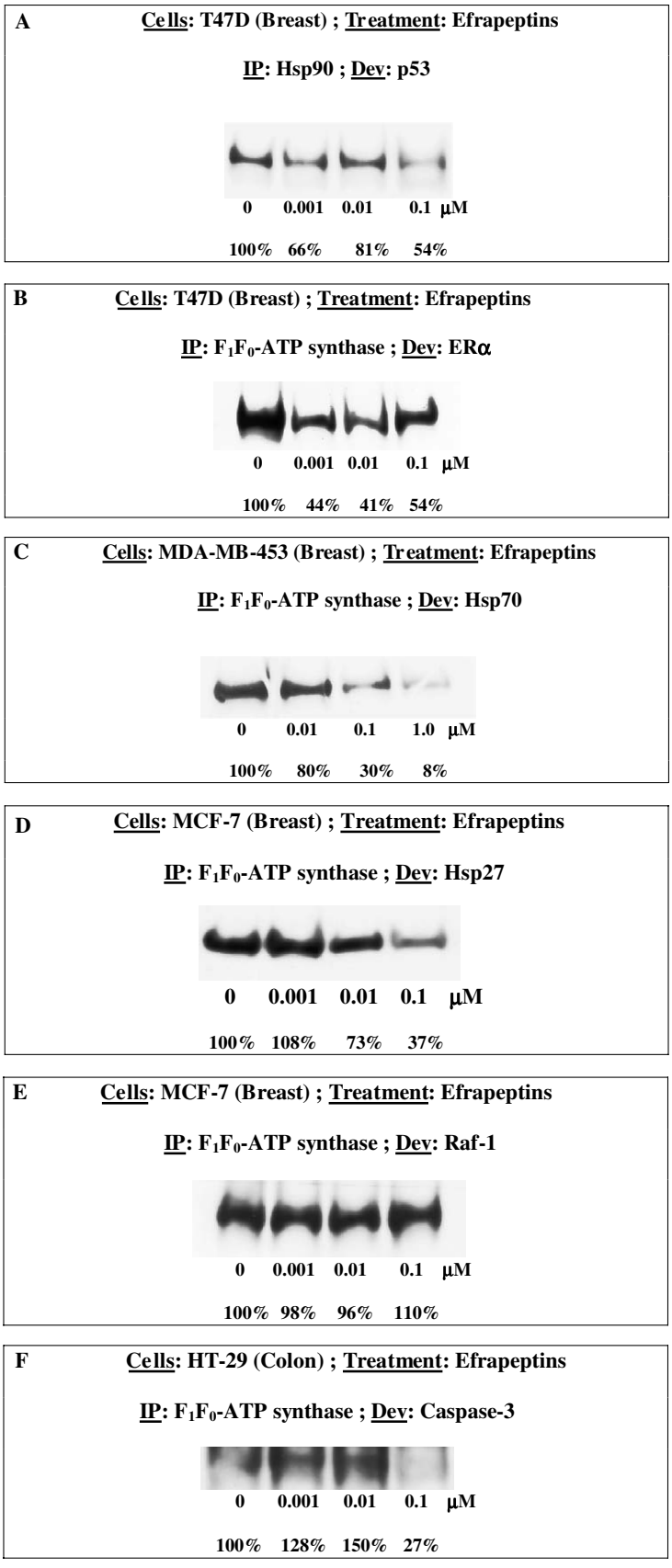

Fig. 5. Co-immunoprecipitation of F_1F_0-ATP synthase or Hsp90 with Hsp90–client proteins m.p53 (A), ERα (B), Hsp70 (C), Hsp27 (D), Raf-1(E), and caspase-3 (F) after exposure of various tumor cell lines to efrapeptins.

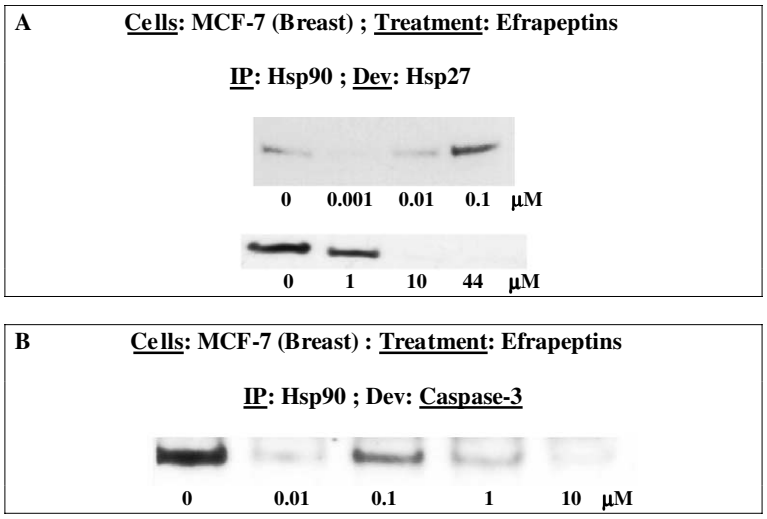

Fig. 6. Co-immunoprecipitation of Hsp90 with client proteins Hsp27 (A) and caspase-3 (B).

Hsp90–client proteins. Fig. 7A shows degradation of Hsp70 after overnight exposure of MDA-MB-453 cells to various concentrations of efrapeptins. Similar degradations were observed for m.p53 in T47D cells (Fig. 7B), Hsp27 in MCF-7 cells (Fig. 7C), and caspase-3 in HT-29 cells (Fig. 7D). Fig. 7D suggests that prolonged exposure of tumor cells to efrapeptins increases client protein degradation. While no degradation of caspase-3 was seen when HT-29 cells were exposed to 0.01 μM efrapeptins for 24 h, a complete depletion of the protein was observed when the cells were exposed to the same concentration of efrapeptins for 48 h.

In agreement with an earlier observation that efrapeptins had no effect on the association of Hsp90 with Raf-1, exposure of MCF-7 to efrapeptins for 24 h resulted in accumulation rather than degradation of Raf-1 (Fig. 7E). On the other hand, depletion of Hsp90 protein levels at very low concentrations of efrapeptins was seen (Fig. 7F). A similar phenomenon has been reported for other Hsp90 inhibitors.

Ubiquitination of ERα after brief exposure of MCF-7 breast cancer cells to efrapeptins

Disruption of the Hsp90-ERα complex by efrapeptins did not result in ERα degradation. On the contrary, when MCF-7 cells were exposed to efrapeptins for 3, 24, or 48 h, an increase in ERα protein levels was seen at 3 and 24 h but not 48 h (Fig. 8A). A similar phenomenon was observed when T47D cells were treated with efrapeptins for 3 or 24 h (data not shown). Accumulation of ERα in cells treated with efrapeptins led us to examine ubiquitination of the protein after exposure of MCF-7 cells to efrapeptins. In Fig. 8B, ubiqutinated proteins were immunoprecipitated from cell lysates of MCF-7 cells treated with various concentrations of efrapeptins for 10 min using an anti-Ub polyclonal ab. Subsequently, immunoprecipitated ubiquitinated proteins were subjected to SDS–PAGE electrophoresis and Western immunoblotting using an anti-ERα mab. Fig. 8B suggests that ERα becomes ubiquitinated following a brief exposure of the cells to 0.01 and 0.1 μM but not 0.001 μM efrapeptins. This may explain the Hsp90-ERα complex recovery reported at increased concentrations of efrapeptins.

Discussion

Co-immunoprecipitation experiments performed here suggest that F_1F_0-ATP synthase physically associates with Hsp90–protein complexes in various tumor cell lines including MCF-7 breast, MDA-MB-453 breast, T47D breast, and HT-29 colon cancer cells, where it plays a role in stabilizing Hsp90–client proteins. Inhibition of F_1F_0-ATP synthase by efrapeptins leads to the dissociation of F_1F_0-ATP synthase from Hsp90 as well as to the disruption of Hsp90 complexing with such substrate proteins as ERα, Hsp-27, m.p53, Hsp70, and caspase-3 but not Raf-1. With the exception of ERα, dissociation of the Hsp90–substrate protein complexes by efrapeptins results in partial or complete degradation of the client proteins. These phenomena are not associated with a general reduction in the intracellular levels of ATP.

Depletion of ATP is expected to have a negative impact on the Hsp90 chaperone function [12]. Hsp90 is a known ATPase, which utilizes ATP hydrolysis to assume conformations that promote substrate stabilization. The significance of ATP for Hsp90 function is highlighted by the fact that many of the Hsp90 inhibitors, such as GA, act as nucleotide mimetics to bind to the ATP binding site on the N-terminal of the protein effectively "locking" Hsp90 in its ADP-bound conformation [22]. This conformation is thought to promote substrate release and degradation. Studies have shown that Hsp90 has an approximately 10-fold higher affinity for ATP than ADP suggesting that a small decrease in the cytoplasmic ATP/ADP ratio will result in a large reduction in the number of Hsp90 molecules bound to ATP [23].

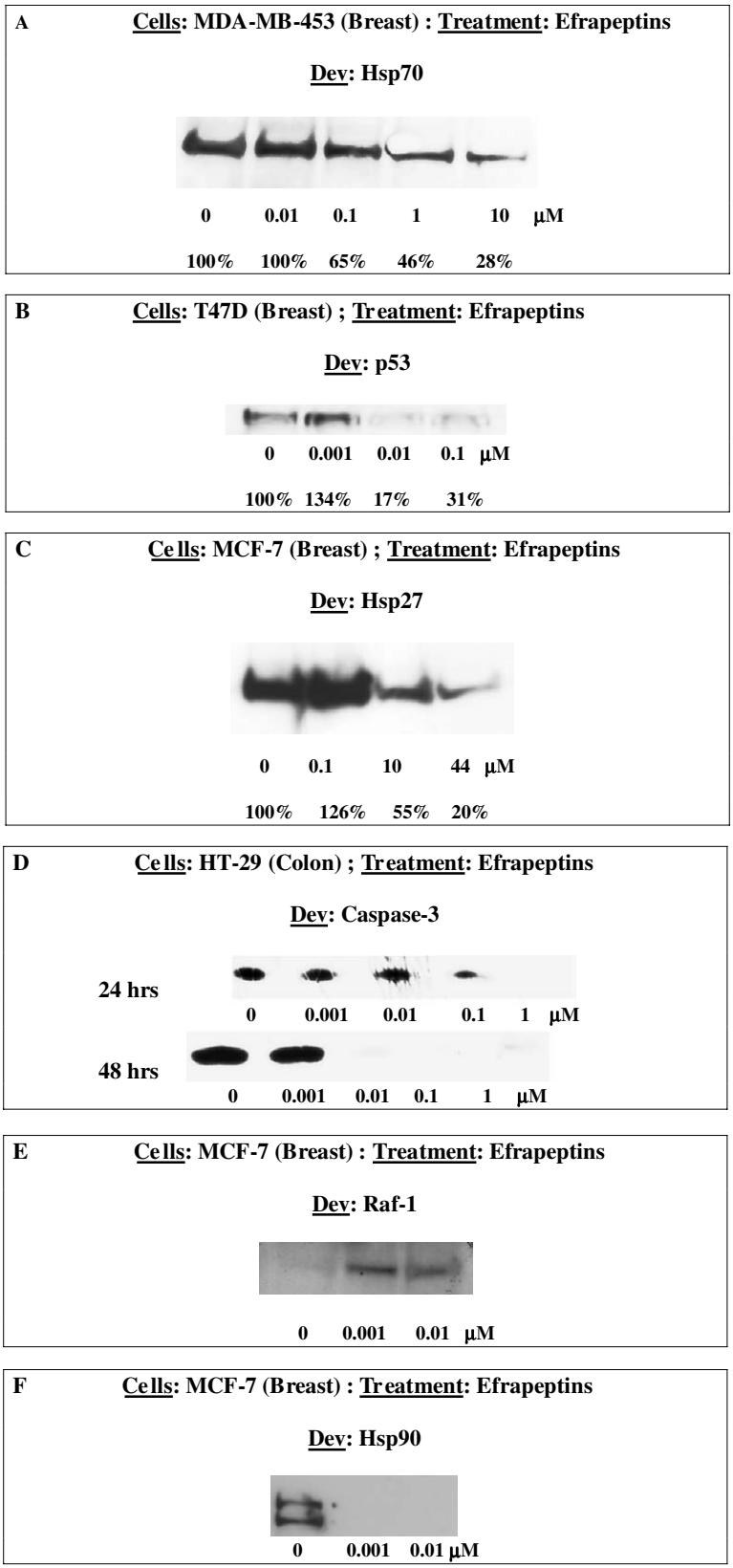

Fig. 7. Expression of Hsp 70 (A), m.p53 (B), Hsp27 (C), caspase-3 (D), Raf-1 (E), and Hsp90 (F) after treatment of tumor cells with various concentrations of efrapeptins. Cells were typically treated with efrapeptins for 24 h except in the case of caspase-3, where HT-29 cells were also treated for 48 h.

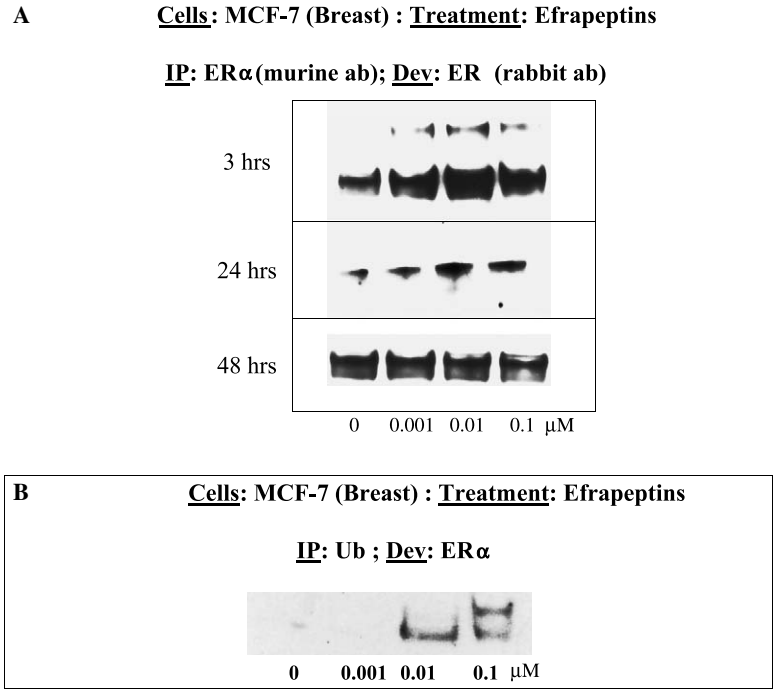

Fig. 8. (A) Expression of ERα after treatment of MCF-7 cells with various concentrations of efrapeptins for 3, 24, or 48 h. (B) Ubiquitination of ERα after brief exposure (10 min) of MCF-7 cells to various concentrations of efrapeptins.

Independent studies have suggested that ATP depletion adversely affects Hsp90–client proteins. In 1996, Loktionova et al. showed that ATP depletion in endothelial cells treated briefly with CCCP, an oxidative phosphorylation uncoupler, or rotenone in the absence of glucose leads to the rapid aggregation of Hsp27 [24]. The authors did not examine ubiquitination of Hsp27 as a possible explanation for this aggregation nor did they investigate proteasomal degradation of Hsp27 aggregates. Seo et al. linked degradation of ER in MCF-7 cells grown in glucose-free media and treated with oligomycin with ATP depletion in these cells [25]. However, the authors did not associate ATP depletion by oligomycin with a dissociation of the Hsp90-ER complex. Here, it is worth noting that, in the presence of glucose, Seo et al. reported only partial degradation of ER after treatment of MCF-7 cells with 0.01 μM oligomycin. On the other hand, Peng et al. showed that treatment of myocytes with 2-deoxy-D-glucose or antimycin A leads to a decrease in the intracellular ATP concentration, disruption of the Hsp90–ErbB2 complexes, and ErbB2 degradation [23]. The authors hypothesized that Hsp90 acts as an ATP sensor that links the cellular response to a growth stimulus with the cellular energy charge by modulating the stability of signaling cascades. Our study proposes that the ATP sensing abilities of Hsp90 may not only stem from its function as an ATPase but also from its interaction with F_1F_0-ATP synthase.

Although it is reasonable to assume that inhibition of oxidative phosphorylation by oxidative phosphorylation inhibitors like antimycin A will lead to ATP depletion in normal cells such as myocytes, the assumption may not be accurate for tumor cells. Tumor cells are known to maintain a high degree of glycolysis even in the presence of oxygen, a phenomenon known as the Warburg hypothesis. In 1924, Warburg suggested that malignant growth is the result of a decrease in the mitochondrial energy metabolism and an increase in the glycolytic production of ATP. Although the universality of this hypothesis has been challenged repeatedly, a number of studies have shown a significant shift towards a glycolytic phenotype with increased tumorigenicity in breast, lung, colon, and other cancers [26,27]. Our ATP bioluminescent assays suggest a cell type-dependent sensitivity to ATP depletion in the presence of inhibitors of oxidative phosphorylation. For example, MCF-7 cells decrease their intracellular levels of ATP in response to F_1F_0-ATP synthase inhibition by efrapeptins and oligomycin or to NADH dehydrogenase suppression by rotenone. This is in agreement with a recent study suggesting that, in proliferating MCF-7 cells, 80% of ATP production was oxidative [28]. On the other hand, ATP concentration in T47D and MDA-MB-453 cells remained unaffected by treatment of the cells with efrapeptins. Thus, the ability of efrapeptins to dissociate Hsp90–client protein complexes does not depend on ATP depletion. Although it is possible that efrapeptins affect Hsp90 chaperone activity by directly interacting with the protein, a similar behavior exhibited by other F_1F_0-ATP synthase inhibitors suggests an indirect event via inhibition of co-chaperone F_1F_0-ATP synthase.

Suppression of Hsp90 function through inhibition of F_1F_0-ATP synthase may represent a new paradigm in the design of therapeutic interventions that target the heat shock proteome. In contrast with drugs that directly inhibit Hsp90, F_1F_0-ATP synthase inhibitors suppress Hsp90 func-

tion without activating HSF-1, since such activation leads to upregulation of Hsp70 [29]. Hsp70 is known to inhibit apoptosis and to render cells resistant to chemotherapy. Efrapeptins reduced expression of Hsp70 by 50% in MDA-MB-453 breast cancer cells treated with 0.1 μM efrapeptins. In contrast, Bagatell et al. reported a dramatic increase in Hsp70 levels after treatment of HL-60 leukemia cells with 0.09 μM GA [29].

Lack of degradation of ERα after treatment of MCF-7 cells with efrapeptins is puzzling. It is well documented that, following dissociation from their complex with Hsp90, substrate proteins become ubiquitinated and are rapidly degraded by 26S proteasome. Our study shows that, although exposure of MCF-7 cells to efrapeptins results in ERα ubiquitination, this ubiquitination is not followed by degradation. This observation led us to a series of experiments suggesting that efrapeptins inhibit 26S proteasome by suppressing the chymotrypsin-like and caspase-like enzymatic activities of its catalytic core. These experiments (to be reported elsewhere) may explain the Hsp90–client protein recovery seen with certain client proteins at increased concentrations of efrapeptins. Mimnaugh et al., who studied the concomitant inhibition of Hsp90 and 26S proteasome through exposure of MCF-7 cells to a combination of 17-*N*-allylamino-17-demethoxy geldanamycin, a GA analogue, and bortezomib, a proteasomal inhibitor, suggested that such an inhibition leads to a massive accumulation of ubiquitinated proteins that are mostly detergent insoluble and likely non-functional [30]. However, it is not clear why efrapeptin treatment results in the accumulation of ERα but not of other Hsp90–client proteins. It is worth noting that small Hsp90–client proteins such as Hsp27 and caspase-3 seem to deplete in the presence of efrapeptins, while larger proteins such as m.p53, Hsp70, and ERα merely decrease or accumulate. It remains to be seen if the accumulated proteins are functional.

In summary, F_1F_0-ATP synthase inhibition appears to provide a new mechanism for inhibition of Hsp90 chaperone functions, a mechanism that is devoid of HSF-1 activation. Considering the significant role that Hsp90 plays in promoting cancer growth and development, inhibition of Hsp90 by F_1F_0-ATP synthase inhibitors such as efrapeptins is not only an intriguing scientific observation but also an event relevant to developing novel, effective, and specific anti-cancer treatments.

References

[1] J. Frydman, Folding of newly translated proteins in vivo: the role of molecular chaperones, Annu. Rev. Biochem. 70 (2001) 603–647.
[2] B.S. Inas, I.R. Brown, Synthesis of a heat shock protein in the microvascular system of the rabbit brain following elevation of body temperature, Biochem. Biophys. Res. Commun. 106 (3) (1982) 881–887.
[3] S.P. Tomasovic, P.A. Steck, D. Heitzman, Heat-stress proteins and thermal resistance in rat mammary tumor cells, Radiat. Res. 95 (2) (1983) 399–413.
[4] B. Hildebrandt, P. Wust, O. Ahlers, A. Dieing, G. Sreenivasa, T. Kerner, R. Felix, H. Riess, The cellular and molecular basis of hyperthermia, Crit. Rev. Oncol. Hematol. 43 (1) (2002) 33–56.
[5] B. Patel, A. Khaliq, J. Jarvis-Evans, M. Boulton, S. Arrol, M. Mackness, D. McLeod, Hypoxia induces HSP 70 gene expression in human hepatoma (HEP G2) cells, Biochem. Mol. Biol. Int. 36 (4) (1995) 907–912.
[6] K. Ohnishi, H. Matsumoto, A. Takahashi, X. Wang, T. Ohnishi, Heat shock transcription factor, HSF, is activated by ultraviolet irradiation, Photochem. Photobiol. 64 (6) (1996) 949–952.
[7] Y.C. Chen, S.Y. Lin-Shiau, J.K. Lin, Involvement of heat-shock protein 70 and P53 proteins in attenuation of UVC-induced apoptosis by thermal stress in hepatocellular carcinoma cells, Photochem. Photobiol. 70 (1) (1999) 78–86.
[8] J.C. Young, I. Moarefi, F.U. Hartl, Hsp90: a specialized but essential protein-folding tool, J. Cell Biol. 154 (2001) 267–273.
[9] L. Neckers, S.P. Ivy, Heat shock protein 90, Curr. Opin. Oncol. 15 (6) (2003) 419–424.
[10] W.B. Pratt, D.O. Toft, Steroid receptor interactions with heat shock protein and immunophilin chaperones, Endocr. Rev. 18 (3) (1997) 306–360.
[11] A.S. Sreedhar, C. Soti, P. Csermely, Inhibition of Hsp90: a new strategy for inhibiting protein kinases, Biochim. Biophys. Acta 1697 (1-2) (2004) 233–242.
[12] W. Sullivan, B. Stensgard, G. Caucutt, B. Bartha, N. McMahon, E.S. Alnemri, G. Litwack, D. Toft, Nucleotides and two functional states of hsp90, J. Biol. Chem. 272 (12) (1997) 8007–8012.
[13] Y. Nagata, T. Anan, T. Yoshida, T. Mizukami, Y. Taya, T. Fujiwara, H. Kato, H. Saya, M. Nakao, The stabilization mechanism of mutant-type p53 by impaired ubiquitination: the loss of wild-type p53 function and the hsp90 association, Oncogene 18 (44) (1999) 6037–6049.
[14] E.G. Mimnaugh, C. Chavany, L. Neckers, Polyubiquitination and proteasomal degradation of the p185c-erbB-2 receptor protein–tyrosine kinase induced by geldanamycin, J. Biol. Chem. 271 (37) (1996) 22796–22801.
[15] M. Futai, H. Omote, Y. Sambongi, Y. Wada, Synthase (H+ ATPase): coupling between catalysis, mechanical work, and proton translocation, Biochim. Biophys Acta 1458 (2000) 276–288.
[16] R.E. Gray, D.G. Grasso, R.J. Maxwell, P.M. Finnegan, P. Nagley, R.J. Devenish, Identification of a 66 kDa protein with yeast mitochondrial ATP synthase as heat shock protein hsp60, FEBS Lett. 268 (1) (1990) 265–268.
[17] S.B. Krasnoff et al., Antifungal and insecticidal properties of efrapeptins: metabolites of the fungus *Tolypocladium niveum*, J. Invert. Pathol. 58 (1991) 180–188.
[18] R.L. Cross, W.E. Kohlbrenner, The mode of inhibition of oxidative phosphorylation by efrapeptin (A23871). Evidence for an alternating site of mechanism for ATP synthesis, J. Biol. Chem. 253 (14) (1978) 4865–4873.
[19] J.P. Abrahams, S.K. Buchanan, M.J. Van Raaij, I.M. Fearnley, A.G. Leslie, J.E. Walker, The structure of bovine F1-ATPase complexed with the peptide antibiotic efrapeptin, Proc. Natl. Acad. Sci. USA 939 (18) (1996) 9420–9424.
[20] G.L. Orriss, A.G.W. Leslie, K. Braig, J.E. Walker, Bovine F1-ATPase covalently inhibited with 4-chloro-7-nitrobenzofurazan: the structure provides further support for a rotary mechanism, Structure 6 (7) (1998) 831–837.
[21] R.J. Devenish, M. Prescott, G.M. Boyle, P. Nagley, The oligomycin axis of mitochondrial ATP synthase: OSCP and the proton channel, J. Bioenerg. Biomembr. 32 (5) (2000) 507–515.
[22] S.M. Roe, C. Prodromou, R. O'Brien, J.E. Ladbury, P.W. Piper, L.H. Pearl, Structural basis for inhibition of Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin, J. Med. Chem. 42 (1999) 260–266.
[23] X. Peng, X. Guo, S.C. Borkan, A. Bharti, Y. Kuramochi, S. Calderwood, D.B. Sawyer, Heat shock protein 90 stabilization of

ErbB2 expression is disrupted by ATP depletion in myocytes, J. Biol. Chem. 280 (13) (2005) 13148–13152.
[24] S.A. Loktionova, O.P. Ilyinskaya, V.L. Gabai, A.E. Kabakov, Distinct effects of heat shock and ATP depletion on distribution and isoform patterns of human Hsp27 in endothelial cells, FEBS Lett. 392 (1996) 100–104.
[25] H.-S. Seo, F. Journé, D. Larsimont, C. Sotiriou, G. Leclercq, Decrease of estrogen receptor expression and associated ERE-dependent transcription in MCF-7 breast cancer cells after oligomycin treatment, Steroids 68 (2003) 257–269.
[26] J.M. Cuezva, G. Chen, A.M. Alonso, A. Isidoro, D.E. Misek, S. Hanash, D. Beer, The bioenergetic signature of lung adenocarcinomas is a molecular marker of cancer diagnosis and prognosis, Carcinogenesis 25 (7) (2004) 1157–1163.
[27] A. Isidoro, E. Casado, A. Redondo, P. Acedo, E. Espinosa, A.M. Alonso, P. Cejas, D. Hardisson, J.A. Fresno Vara, C. Belba-Iniesta, M. González-Barón, J.M. Cuezva, Breast carcinomas fulfill the Warburg hypothesis and provide metabolic markers of cancer prognosis, Carcinogenesis 26 (12) (2005) 2095–2104.
[28] M. Guppy, P. Leedman, X. Zu, V. Russell, Contribution by different fuels and metabolic pathways to the total ATP turnover of proliferating MCF-7 breast cancer cells, Biochem. J. 364 (2002) 309–315.
[29] R. Bagatell, G.D. Paine-Murrieta, C.W. Taylor, E.J. Pulcini, S. Akinaga, I.J. Benjamin, L. Whitesell, Induction of a heat shock factor 1-dependent stress response alters the cytotoxic activity of Hsp90-binding agents, Clin. Cancer Res. 6 (2000) 3312–3318.
[30] E.G. Mimnaugh, W. Xu, M. Vos, X. Yuan, J.S. Isaacs, K.S. Bisht, D. Gius, L. Neckers, Simultaneous inhibition of Hsp90 and the proteasome promotes protein ubiquitination, causes endoplasmic reticulum-derived cytosolic vacuolization, and enhances antitumor activity, Mol. Cancer Ther. 3 (5) (2004) 551–566.