Antitumor activity of efrapeptins, alone or in combination with 2-deoxyglucose, in breast cancer in vitro and in vivo

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Abstract Efrapeptins (EF), a family of fungal peptides, inhibit proteasomal enzymatic activities and the in vitro and in vivo growth of HT-29 cells. They are also known inhibitors of F_1F_0 -ATPase, a mitochondrial enzyme that functions as an Hsp90 co-chaperone. We have previously shown that treatment of cancer cells with EF results in disruption of the Hsp90: F_1F_0 -ATPase complex and inhibition of Hsp90 chaperone activity. The present study examines the effect of EF on breast cancer growth in vitro and in vivo. As a monotherapy, EF inhibited cell prolifer-

ation in vitro with an IC_{50} value ranging from 6 nM to 3.4 μ M. Inhibition of Hsp90 chaperone function appeared to be the dominant mechanism of action and the factor determining cellular sensitivity to EF. In vitro inhibition of proteasome became prominent in the absence of adequate levels of Hsp90 and F_1F_0 -ATPase as in the case of the relatively EF-resistant MDA-MB-231 cell line. In vivo, EF inhibited MCF-7 and MDA-MB-231 xenograft growth with a maximal inhibition of 60% after administration of 0.15 and 0.3 mg/kg EF, respectively. 2-Deoxyglucose (2DG), a known inhibitor of glycolysis, acted synergistically with EF in vitro and antagonistically in vivo. In vitro, the synergistic effect was attributed to a prolonged endoplasmic reticulum (ER) stress. In vivo, the antagonistic effect was ascribed to the downregulation of tumoral and/or stromal F_1F_0 -ATPase by 2DG.

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Introduction

Hsp90 is a molecular chaperone responsible for the stable expression and functional integrity of numerous proteins, many of which participate in signaling pathways promoting cancer cell growth and survival. Hsp90 client proteins include oncogenes such as mutated p53 (m.p53), steroid hormone receptors such as estrogen receptor α (ER α), and soluble or transmembrane protein kinases such as ErbB2/HER2. Inhibition of the chaperone function of Hsp90 leads to destabilization and degradation of Hsp90 client proteins and, concurrently, to suppression of multiple cancer supporting pathways (Xu and Neckers 2007). Several Hsp90 inhibitors are presently under investigation in various human clinical trials involving cancer (Kim et al. 2009).

Hsp90 functions in consort with 26S proteasome. Misfolded, damaged, or improperly assembled proteins, which cannot be rescued by Hsp90, become ubiquitinated and are degraded by proteasome, a multicatalytic protease. In addition, proteasome is responsible for the spatial and temporal destruction of regulatory proteins involved in cell cycle progression, activation of transcription factors, and apoptosis (Hochstrasser 1995). Inhibition of proteasome leads to accumulation of ubiquitinated proteins and inhibition of a number of cellular pathways. Cancer cells appear to be far more sensitive to inhibition of proteasome than normal cells partly due to their rapid cycling and partly because they are exposed to unique environmental stresses, which favor the accumulation of mutated and misfolded proteins (Adams 2002). A number of proteasome inhibitors exist. The best known, bortezomib, has been approved for the treatment of relapsed and refractory multiple myeloma (Adams 2001; Kane et al. 2003).

EF are a class of naturally occurring peptides isolated from fungal cultures. They are previously known as inhibitors of F_1F_0 -ATPase, the enzyme responsible for the mitochondrial synthesis of ATP (Krasnoff et al. 1991). In 2006, we reported that F_1F_0 -ATPase co-immunoprecipitated with Hsp90 and Hsp90 client proteins from cell lysates obtained from various cancer cells including MCF-7, T47D, and MDA-MB-453 breast tumor cells. In that study, exposure of the cells to low concentrations (0.001–0.1 μ M) of EF led to: (a) disruption of the Hsp90: F_1F_0 -ATPase complex, (b) dissociation of Hsp90 from its client proteins, and (c) degradation of Hsp90 and Hsp90 client proteins. Hsp90 substrates affected by EF included m.p53, ER α , Hsp70, and Hsp27 but not Raf-1. Inhibition of Hsp90 chaperone activities was also observed after treatment of cancer cells with other F_1F_0 -ATPase inhibitors such as oligomycin and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole but not after treatment with rotenone, an inhibitor of the oxidative phosphorylation complex I. The data collectively suggested that F_1F_0 -ATPase was an important partner in stabilizing Hsp90 substrates and that inhibition of F_1F_0 -ATPase was an alternative pathway for suppressing Hsp90 chaperone activities.

More recently, we identified EF as reversible inhibitors of the chymotrypsin-like (Ch-L) and caspase-like (C-L) activities of 26S proteasome (data submitted for publication). Specifically, we reported that EF inhibited the Ch-L and C-L activities of purified 20S proteasome in a dose-dependent fashion and with an IC_{50} value of ~ 0.5 μ M. Because 20S proteasome is the catalytic core of 26S and devoid of any ATPase sites, its inhibition by EF is indicative of a direct interaction of the drug with the catalytic sites of the protein. Furthermore, we showed that brief exposure of HT-29 colon cancer cells to low concentrations of EF led to rapid accumulation of ubiquitinated proteins.

Here, we examined the ability of EF to inhibit breast cancer growth in vitro and in vivo alone or in combination with 2DG. In light of the fact that EF inhibit Hsp90 and proteasome, two seemingly opposing mechanisms of actions, we also investigated the predominant pathway of EF activity and explored the factors that govern sensitivity of a cell line to the inhibitory properties of EF.

Materials and methods

Materials

EF used here were isolated as a mixture of EF D and E from cultures of fungus *Tolypocladium niveum*. The structure of EF can be found in the report by Krasnoff et al. (1991). Antibodies (abs) against Hsp90, Grp78, and Grp94 were from Lab Vision. An ab against the β -catalytic subunit of F_1F_0 -ATPase was from Invitrogen. 2DG, abs against p53 and ubiquitin, and a lactate dehydrogenase (LDH) toxicology assay kit were from Sigma Aldrich. MCF-7, T47D, MDA-MB-231, MDA-MB-453, Hs578T, and MDA-MB-435 cells were from ATCC. NCI/ADR-RES cells were from the National Cancer Institute. A Cyquant proliferation kit was from Invitrogen. An MTT cell growth assay kit was from Millipore. A cell death ELISA assay kit was from Roche Diagnostics.

Cell proliferation

Cancer cells were plated onto 96-well plates at a density of 2,000 to 5,000 cells/well. They were allowed to adhere overnight before they were subjected to various treatments for 48 to 72 h. Cell proliferation was measured using Cyquant or MTT assay kits according to the manufacturers' instructions.

ATPase assay

Cancer cells were plated onto duplicate 96-well plates at a cell density of 2,000 to 5,000 cells/well. One set of plates was used to determine intracellular ATP concentrations as previously described (Papathanassiou et al. 2006) with the notable exception that cells were treated with ATPase inhibitors for 24 h. The other set of plates was used to estimate cell numbers by Cyquant. At the end of the experiment, ATP levels were normalized per cell number and expressed as percent of normalized ATP values found in control (untreated) cells.

Co-immunoprecipitation and Western immunoblotting experiments

These experiments were performed essentially as previously described (Papathanassiou et al. 2006). Experiments were

performed at least twice. Western immunoblots shown here are representative examples. Expression level of proteins was analyzed by Image J [National Institutes of Health (NIH)] and normalized over tubulin.

LDH cytotoxicity assay

Cancer cells were plated onto 96-well plates (10,000 to 20,000 cells/well) and allowed to adhere overnight prior to EF exposure for 24 h. At the end of the treatment, LDH concentrations found in the conditioned media (CM) and in the cell lysates of equal numbers of cells were determined using the LDH cytotoxicity assay kit according to the manufacturer's instructions. Data are presented as %LDH released into CM over total LDH present in the cell cultures.

Apoptotic assay

Cancer cells were plated onto duplicate 24-well plates (25,000 cells/well) and allowed to adhere overnight before treatment with various concentrations of EF for 24 h. At the end of the experiment, apoptotic death was determined using the cell death ELISA assay kit according to manufacturer's instructions.

MCF-7 xenograft assay

Athymic nude female mice (5–6 weeks old from Harlan Sprague Dawley) were implanted s.c. with 1×10^7 MCF-7 cells per flank in both flanks 24 h after their implantation with a 17- β -estradiol pellet (Innovative Research of America, 0.72 mg; 60-day release). The tumors were allowed to grow to an average tumor volume of 100 mm³. The mice were then sorted and randomly assigned to two groups ($n=8$ per group): one group received i.p. administrations of 0.15 mg/kg EF twice a week for 4 weeks, while the other group received vehicle control [phosphate buffered saline (PBS)]. Tumor growth was monitored by serial two-dimensional caliper measurements twice a week, and tumor volumes were estimated according to the formula: $(\text{length} \times \text{width}^2)/2$. Body weights were also recorded twice a week. The experiment was terminated approximately 2 months after the 17- β -estradiol pellet implantation.

MDA-MB-231 xenograft assay

Athymic nude female mice were implanted with 5×10^6 MDA-MB-231 cells into the mammary fat pads. The tumors were allowed to grow to an average tumor volume of 70 mm³. The mice were then sorted and randomly assigned to five groups ($n=6$ per group): the first group (animals #0–5) received vehicle control, the second group

(animals #7–12) received i.p. administrations of 0.3 mg/kg EF thrice (Monday, Wednesday, and Friday or MWF) a week every other week, the third group (animals #13–18) received i.p. administrations of 0.1 mg/kg EF MWF, the fourth group (animals #19–24) received i.p. administrations of 125 mg/kg 2DG twice a week (Tuesday and Thursday or TTh), and the fifth group (animals #25–30) received combination treatment of the 0.1 mg/kg EF and 125 mg/kg 2DG dose regimens. Tumor growth was monitored as previously described. The experiment was terminated 3 weeks after initiation of treatment, when the average tumor burden of control animals reached $\sim 2 \text{ cm}^3$.

Results

Inhibition of proliferation

The effect of EF on the growth of breast cancer cells was examined using the MCF-7, T47D, Hs578T, MDA-MB-453, and MDA-MB-231 cell lines. The group also included two cell lines, NCI/ADR-RES and MDA-MB-435, which were studied as breast cancer cells for an extended period of time in the past, but were recently shown to be identical to the ovarian cancer cell line OVCAR8 and to the melanoma cell line M14, respectively. Figure 1 shows significant inhibition of cancer cell proliferation following exposure of the cells to EF. Table 1 compares IC₅₀ values obtained here for EF with those reported for the best known inhibitors of Hsp90 and proteasome. The data suggest that the anti-proliferative profile of EF has more similarities with the one reported for the Hsp90 inhibitor 17-AAG rather with the

Fig. 1 Percent control cancer cell proliferation after treatment of MCF-7 (closed circle), MDA-MB-231 (open circle), T47D (closed triangle), MDA-MB-453 (open triangle), Hs578T (closed square), NCI/ADR-RES (open square), and MDA-MB-435 cells (closed diamond) with various concentrations EF for 48–72 h. Cell proliferation assays were performed in triplicate and repeated at least three times. Values shown here are averages of similar experiments

Table 1 Comparison of IC₅₀ values of the antiproliferative activity of EF obtained presently with those reported for proteasome inhibitor bortezomib and the Hsp90 inhibitors 17-(allylamino)-17-

demethoxygeldanamycin (17-AAG) and 17-dimethylamino-ethylamino-17-demethoxygeldanamycin (17-DMAG)

Cancer cell line	Inhibition of cell proliferation [IC ₅₀ (μM)]				Phenotype
	EF	17-AAG ^a	17-DMAG ^a	Bortezomib ^b	
Hs578T	0.006±0.006 ^c	0.63	3.2	N/A	ERα(-); m.p53
MCF-7	0.027±0.016	0.013	0.01	0.100±0.008	ERα(+); wt.p53
T47D	0.057±0.022	0.79	0.063	N/A	ERα(+); m.p53
MDA-MB-453	0.132±0.038	N/A	N/A	0.100±0.012	ERα(-); m.p53
MDA-MB-231	3.430±1.197	1.0	0.016	0.007±0.003	ERα(-); m.p53
NCI/ADR-RES	0.062±0.067	6.3	20	N/A	ERα(-); m.p53
MDA-MB-435	1.892±0.633	0.04	0.01	N/A	ERα(-); m.p53

N/A: Not available

^a Published by the Developmental Therapeutics Program of NCI^b Codony-Servat et al. (2006)^c Average±stdev

one reported for the proteasomal inhibitor bortezomib. Furthermore, Table 1 shows no correlation between the sensitivity of a specific cell line to EF and the ERα or p53 status of the cell line. While ERα and m.p53 are known Hsp90 clients, wild-type p53 (wt.p53) is not. In an attempt to elucidate the factors that govern sensitivity to EF, we investigated ATP depletion, inhibition of proteasome, and suppression of Hsp90 function following exposure of breast cancer cells to EF.

ATP depletion

We have previously shown that inhibition of Hsp90 function by EF is independent of the intracellular concentration of ATP (Papathanassiou et al. 2006). To evaluate if inhibition of cell proliferation by EF is also ATP-independent, the levels of ATP following exposure of the MCF-7, T47D, MDA-MB-453, and MDA-MB-231 cells to EF for 24 h were assessed (Fig. 2). Sweet and Singh (1995) have shown that a 12% reduction in mitochondrial ATP halts cell cycling of HL-60 leukemia cells at G1/S checkpoint, while further reduction (20–30%) leads to the accumulation of the cells at the G₂/M checkpoint. The data shown in Fig. 2 suggest that T47D and MDA-MB-453 cells experience no reduction in the intracellular concentration of ATP in the presence of EF, albeit the fact that the cells are sensitive to the antiproliferative actions of EF. On the other hand, MCF-7, and, to a lesser extent, MDA-MB-231, cells appear sensitive to inhibition of oxidative phosphorylation (OXPHOS) by EF.

To further investigate how inhibition of OXPHOS may affect growth of MCF-7, T47D, MDA-MB-453, and MDA-MB-231 cells, we measured ATP concentration and cell proliferation following exposure of the cells to OXPHOS

inhibitors rotenone, thenoyltrifluoroacetone (TFFA), antimycin, and oligomycin. Rotenone inhibits OXPHOS at complex I, TFFA at complex II, and antimycin at complex III (Ramsay et al. 1981; Teeter et al. 1969). Oligomycin is another inhibitor of F₁F₀-ATPase, which also suppresses Hsp90 chaperone function through dissociation of the Hsp90:F₁F₀-ATPase complex (Papathanassiou et al. 2006). Unlike EF, oligomycin is not a proteasome inhibitor. Table 2 shows concentrations of the OXPHOS inhibitors required to deplete the intracellular concentration of ATP by 20% and to reduce proliferation by 50%, respectively. The table suggests no universal correlation between inhibition of OXPHOS and suppression of proliferation in these cell lines. It is apparent that mechanisms other than inhibition of OXPHOS are at play during treatment of the cells with EF, likely inhibition of Hsp90 and/or

Fig. 2 Percent control intracellular ATP levels after treatment of MCF-7 (closed circle), MDA-MB-231 (open circle), T47D (closed triangle), and MDA-MB-453 (open triangle) with various concentrations of EF for 24 h. The assays were performed in triplicate and repeated at least two times. Values shown here are average±stdev

Table 2 Concentrations of OXPHOS inhibitors EF, oligomycin, rotenone, antimycin and TFFA required to decrease the intracellular levels of ATP of various breast cancer cells lines by 20% (IC₂₀) afterovernight treatment and to inhibit cell growth of various breast cancer cells lines by 50% (IC₅₀) after treatment of the cells for 48 hrs

Cancer Cell Line	ATP Depletion IC ₂₀ (μM)					Inhibition of Cell Proliferation IC ₅₀ (μM)				
	EF	Oligomycin	Rotenone	Antimycin	TFFA	EF	Oligomycin	Rotenone	Antimycin	TFFA
MCF-7	1.74	0.05	0.76	0.02	14.80	0.027	0.05	0.05	>10	>10
T47D	ND ^a	ND	3.08	0.21	ND	0.057	0.023	4.17	>10	>>10
MDA-MB-453	ND	ND	4.22	0.56	26.20	0.132	>10	1.53	>>10	>>10
MDA-MB-231	20.80	27.50	0.51	3.61	13.60	3.430	>>10	0.54	>>10	>10

^a ND Not done

proteasome. To study these mechanisms, we concentrated on two cell lines that exhibit differential sensitivity to EF: the sensitive MCF-7 cells and the relatively resistant MDA-MB-231 cells.

Inhibition of Hsp90 and proteasome

In MCF-7 cells, inhibition of F₁F₀-ATPase by EF leads to dissociation of the Hsp90:F₁F₀-ATPase complex and inhibition of the Hsp90 chaperone activity (Papathanassiou et al. 2006). To examine if the mechanism is also functional in the MDA-MB-231 cells, the cells were exposed briefly (10 min) to various concentrations of EF. As in the case of MCF-7 cells, disruption of the Hsp90:F₁F₀-ATPase association was observed (Fig. 3a).

Inhibition of proteasome was investigated in MCF-7 and MDA-MB-231 cells by assessing the build-up of ubiquitinated proteins in these cells following their brief (30 min) exposure to various concentrations of EF. Figure 3b shows accumulation of ubiquitinated proteins, in general, and Hsp70, in particular, in MDA-MB-231 cells but not in MCF-7 cells. Accumulation of Hsp70 in MDA-MB-231 cells is somewhat surprising since EF treatment is known to result in dissociation of the Hsp90:Hsp70 complex and degradation of Hsp70 (Papathanassiou et al. 2006). This discrepancy was reconciled, when treatment of the cells was extended to 24 h, in which case the protein level of Hsp70 decreased in both cell lines (Fig. 3c).

Accumulation of p53 is observed following overnight treatment of both cell lines with EF (Fig. 3c). Since MDA-MB-231 cells possess m.p53, which is subject to degradation following dissociation of the Hsp90:F₁F₀-ATPase complex, the data implicate inhibition of proteasome as the predominant mechanism of EF action in these cells. On the other hand, accumulation of wt.p53 in EF-treated MCF-7 cells suggests that targeted inhibition of proteasome may take place in the cells despite the failure of EF to broadly induce accumulation of ubiquitinated proteins.

Although inhibition of Hsp90 and proteasome seems functional in both cell lines, inhibition of Hsp90 appears

predominant in MCF-7 cells, while inhibition of proteasome dominates in MDA-MB-231 cells. To understand what determines the dominant mechanism of EF action, the intracellular levels of Hsp90, F₁F₀-ATPase, and Glut-1 expressed by 1 × 10⁵ MDA-MB-231 cells were compared to those expressed by the same number of MCF-7 cells (Fig. 3d). Figure 3d suggests that MCF-7 cells possess approximately twofold more Hsp90 and eightfold more F₁F₀-ATPase than MDA-MB-231 cells. Figure 3d also suggests that, in contrast to MDA-MB-231 cells, MCF-7 cells do not possess Glut-1. These observations are in agreement with the idea that MDA-MB-231 cells rely on aerobic glycolysis to fulfill their energetic demands, while MCF-7 cells utilize OXPHOS (Guppy et al. 2002). Here, it should be noted that, while we did not observe Glut-1 expression in MCF-7 cells, other investigators reported that MCF-7 cells did express Glut-1, even though the expression was greatly reduced compared to MDA-MB-231 cells (Grover-McKay et al. 1998).

Finally, the fate of F₁F₀-ATPase and Hsp90 following treatment of the cells with EF was examined. The levels of Hsp90 decreased in both cell lines, although the decrease was more dramatic and happened at lower concentrations in the case of MCF-7 cells (Fig. 3c). This is in agreement with published studies suggesting that inhibitors of Hsp90 deplete not only Hsp90 client proteins but also Hsp90 itself (Smith et al. 2005). The levels of F₁F₀-ATPase were increased in both cell lines, likely due to ubiquitination of the protein. The increase was moderate in the case of MCF-7 cells and was followed by a downregulation of the protein at concentrations >0.01 μM, while, in the case of MDA-MB-231 cells, the increase was remarkable and sustained even at increased EF concentrations.

ErbB2/HER2 increases sensitivity of breast cancer cells to EF

ErbB2/HER2 is overexpressed in 30% of the breast cancer patients and associated with poor prognosis (Jorda et al. 2002). To examine how the presence of ErbB2/HER2 may

Fig. 3 (a) Inhibition of the co-immunoprecipitation of Hsp90 and F₁F₀-ATPase following treatment of the MDA-MB-231 cells with various concentrations of EF for 10 min. Expression ratios are reported below the protein bands. *IP* Immunoprecipitation, *IB* immunoblotting. (b) Expression level of ubiquitinated proteins and Hsp70 after treatment of MDA-MB-231 and MCF-7 cells with EF. Protein ratios have been

normalized over tubulin. *Ub* Ubiquitin. (c) Expression level of F₁F₀-ATPase, Hsp90, Hsp70, and p53 in MDA-MB-231 and MCF-7 cells after overnight treatment of the cells with reported concentrations of EF. (d) Intracellular levels of F₁F₀-ATPase, Hsp90, Glut-1, Grp78, and Grp94 in 1×10^5 untreated MDA-MB-231 and MCF-7 cells

affect sensitivity of cells to EF, MCF7 cells transfected with moderate levels of ErbB2/HER2 (NH-29 cells) were treated with various concentrations of EF (Emlet et al. 2007). Brief (10 min) treatment of the cells with EF led to the disruption of the association of Hsp90 with ErbB2/HER2 in a concentration-dependent manner (Fig. 4a). Overnight treatment of NH-29 cells with EF resulted in accumulation of ErbB2/HER2 protein (Fig. 4b). Cell proliferation assays revealed that EF had an identical inhibitory effect on NH-29 and parent MCF-7 cells (Fig. 4c), despite the fact that, visually, NH-29 cells seem subject to cell death at EF concentrations $>0.01 \mu\text{M}$. Cytotoxicity assays that measure release of LDH into the CM of cells confirmed that EF treatment was more cytotoxic to NH-29 cells than to MCF7 cells (Korzeniewski and Callewart 1983). Figure 4d shows a dramatic increase in the content of LDH found in the CM of NH-29 cells at EF concentration $\geq 0.01 \mu\text{M}$. Previous

studies have suggested that treatment of various tumor cell lines with submaximal inhibitory concentrations of EF leads to cytostasis with apoptosis becoming relevant at concentrations that are usually 1,000-fold more than the IC₅₀ value of the drug (unpublished data). Taking together, data from Fig. 4 imply that the presence of ErbB2/HER2 in a cell transforms the cytostatic effect of EF into a cytotoxic one. ATP bioluminescence assays revealed no reduction in the intracellular levels of ATP following treatment of NH-29 cells with EF (data not shown) confirming that ATP depletion is not responsible for the cytotoxic effect of EF treatment.

Combination treatment of EF with 2DG

2DG, a structural analogue of glucose, is known to prevent protein glycosylation, deplete ATP, induce unfolded protein

Fig. 4 (a) Inhibition of the Hsp90:HER2 association following treatment of ErbB2/HER2 transfected MCF-7 (NH-29) cells with various concentrations of EF for 10 min. (b) Expression levels of ErbB2/HER2 following overnight treatment of NH-29 cells with various concentrations of EF. (c) Comparison of the antiproliferative effect of EF in NH-29 cells with that of the parent MCF-7 cells. (d) EF-induced cytotoxicity in NH-29 and parent MCF-7 cells

response (UPR) and inhibit cancer growth (Kang and Hwang 2006; Maschek et al. 2004; Park et al. 2004). Recent studies suggest that 2DG may have a special role in sensitizing tumor cells to a number of chemotherapeutic drugs (Maschek et al. 2004). Here, we examine if inhibition of glycolysis in MDA-MB-231 cells reverses the resistance of the cells to EF.

MDA-MB-231 and MCF-7 cells were subjected to combination treatment with various concentrations of 2DG and EF. The cells were pretreated with 2DG for 30 min before addition of EF. Visually, the cells appear to experience massive necrotic death 48 h after drug addition. Cell proliferation was measured at 72 h using the MTT assay. The combined effect of treatment was studied using the multidrug analysis of Chou and Talalay (1984, 1987). Combination indices (CI) were calculated for both cell lines. Figure 5a and b shows the isobolograms for MDA-MB-231 and MCF-7 cells, respectively, and suggest a strong synergistic effect of the combination treatment of

2DG and EF in both cell lines. However, the synergistic effect was more pronounced in the MDA-MB-231 cells. In these cells, the IC_{50} value of EF decreased 30-fold from 3.90 ± 0.44 μ M to 0.13 ± 0.06 μ M in the presence of 2DG ($p < 0.00006$, Student's t test). In contrast, in the MCF-7 cells, the IC_{50} value of EF decreased eightfold from 0.025 ± 0.014 μ M to 0.0030 ± 0.0005 μ M ($p < 0.01$).

To investigate the underlying mechanism of the synergistic effect of the combined EF and 2DG treatment, we determined the intracellular ATP levels following treatment of the cells with 2DG alone or in combination with EF (data not shown). As expected, 2DG reduced ATP concentrations in both cell lines although the reduction was far more dramatic in MDA-MB-231 cells. For example, 10 mM 2DG depleted ATP levels by 80% in MDA-MB-231 cells and by 25% in MCF-7 cells. Combined treatment of 2DG with EF resulted in no further reduction in ATP levels in either cell line with the notable exception of MCF-7 cells, when they were treated with a combination of the

Fig. 5 Isobolograms of the combination treatment of MDA-MB-231 (a) and MCF-7 (b) cells with EF and 2DG

two drugs that included concentrations of $EF \geq 1 \mu M$. The data suggest that ATP depletion plays no major role in mediating the synergistic effect of 2DG and EF.

Because glucose deprivation by 2DG is known to disrupt protein folding in the endoplasmic reticulum (ER) and to activate UPR, we then examined induction of glucose regulated protein 78 (Grp78) and Grp94 following single and combined treatment of MDA-MB-231 and MCF-7 cells with EF and 2DG (Park et al. 2004). For this experiment, we employed EF and 2DG concentrations that resulted in 30–40% cell killing, when alone, and in 80–90% cell killing, when combined. Figure 6 shows that 2DG treatment leads to upregulation of Grp78 in MDA-MB-231 cells and Grp94 in both cell lines. A similar pattern of increased Grp expression was observed after single treatment of both cell lines with EF. Combination treatment led to additional upregulation of Grp expression and, for the first time, to increased levels of Grp78 in MCF-7 cells. Upregulation of Grps after treatment with EF and 2DG alone or in combination was far more pronounced in MDA-MB-231 cells than in MCF-7 cells likely reflecting the dependency of the former cell line on glycolysis.

2DG is known to utilize glucose transporters for entry into the cells. Aft et al. have shown that treatment of breast cancer cells with 2DG results in increased expression of Glut-1 and accelerated glucose uptake (2002). The investigators hypothesized that cancer cells facilitate their own demise by increasing the amount of 2DG entering the cells as a response to initial treatment with 2DG. Figure 6 demonstrates that EF has a similar effect with 2DG on the expression of Glut-1 in MDA-MB-231 cells. Combination treatment further increased the expression of Glut-1. Glut-1 appears as a diffused band in Western immunoblotting due to differential glycosylation patterns. The appearance of

low molecular weight species after treatment with 2DG and EF, alone or in combination, suggests inhibition of glycosylation.

In vivo effect of EF alone or in combination with 2DG

Tumor growth of MCF-7 xenografts in nude mice treated i.p. with vehicle control or 0.15 mg/kg EF twice a week for 4 weeks is shown in Fig. 7a. At the end of the treatment, mice treated with EF experienced approximately ~60% inhibition of tumor growth (Treated/Control or T/C=0.43; $p=0.05$). The mice tolerated the treatment without visible signs of distress. A weight loss of 9% was recorded in the treatment group at the end of the experiment.

Figure 7b shows tumor growth of MDA-MB-231 xenografts in nude mice treated i.p. with vehicle control, 0.1 mg/kg, or 0.3 mg/kg EF three times a week for 3 weeks. From the group of mice receiving 0.3 mg/kg EF, three out of six animals suffered a severe weight loss (>15%) after 1 week of treatment. Subsequently, the entire group was given a week off. The weight loss was reversible and administration of EF was resumed for the final (third) week. No weight loss was observed for the mice treated with 0.1 mg/kg EF. At the end of the treatment, mice receiving 0.3 mg/kg EF experienced ~60% inhibition of tumor growth (T/C=0.37; $p=0.00003$), while mice receiving 0.1 mg/kg EF experienced ~50% inhibition (T/C=0.54; $p=0.00004$).

The experiment shown in Fig. 7b also includes combination i.p. treatment of 0.1 mg/kg EF with 125 mg/kg 2DG. The two agents were given as described in the Materials and Methods. Treatment with 125 mg/kg 2DG alone led to inhibition of tumor growth by ~30% (T/C=0.71; $p=0.004$). Interestingly, combined treatment of 125 mg/kg 2DG with

Fig. 6 Expression levels of Grp78, Grp94, Glut-1, and F_1F_0 -ATPase after single or combined treatment of MDA-MB-231 and MCF-7 cells with EF and 2DG

Fig. 7 (a) Inhibition of MCF-7 xenograft growth following EF treatment. (b) Inhibition of MDA-MB-231 xenograft growth following EF treatment alone or in combination with 2DG

0.1 mg/kg EF showed no greater inhibition than single 2DG treatment and lesser inhibition than single EF treatment. This suggests that 2DG antagonizes the antitumor activity of EF *in vivo*, despite the observed *in vitro* synergistic effect.

To elucidate the reasons behind the surprising antagonism between EF and 2DG, we compared the protein expression of Grp78, Grp94, and Glut-1 found in select tumor homogenates from the various treatment groups. Tumor homogenates were also examined for the presence of F_1F_0 -ATPase and Hsp90 and the accumulation of ubiquitinated proteins. Figure 8a shows a trend towards an increased expression of Grp78, Grp94, and Glut-1 within MDA-MB-231 tumors treated with the combined EF/2DG treatment, thus, confirming activation of a sustained UPR response in these tumors. Figure 8b shows a modest

increase in the ubiquitination level of proteins from homogenates of tumors treated with 0.1 mg/kg EF, an increase that did not rise to the level of statistical significance. Interestingly, tumors receiving 125 mg/kg 2DG exhibited a modest decrease in protein ubiquitination compared to control tumors, an event that was statistically significant ($T/C=0.75 \pm 0.07$, $p=0.04$). Tumors receiving combined treatment had similar ubiquitination levels as control tumors and statistically similar to the ones observed with tumors receiving single treatments. Treatment of MDA-MB-231 xenografts with EF did not result in any significant changes in the expression of Hsp90 and F_1F_0 -ATPase, which was in contrast to *in vitro* observations. Tumor treatment with 2DG led to downregulation of F_1F_0 -ATPase. The protein appeared especially depleted in the xenografts of animals receiving combined treatment.

Fig. 8 (a) Expression levels of Grp78, Grp94, and Glut-1 found in tumors excised from the indicated animals and treatment groups. (b) Expression levels of total ubiquitinated proteins, Hsp90 and F_1F_0 -ATPase, found in tumors excised from the indicated animals and treatment groups. (c) Co-immunoprecipitation of Hsp90: F_1F_0 -ATPase complexes from equal amounts of tumor homogenates obtained from control animals and animals treated with 0.3 mg/kg EF

The Western immunoblotting of Fig. 8b utilizes an antibody against the β -subunit of the protein and detects F_1F_0 -ATPase as uncomplexed β -subunits (MW~45 kDa) or β -subunits complexed with α -subunits. (MW~90 kDa). The experiment suggests a tendency toward decreased F_1F_0 -ATPase levels following treatment with 125 mg/kg 2DG (T/C=0.61±0.12; *p*=0.11). The decrease is attributed to the downregulation of uncomplexed β -subunits, an event

that was statistically significant (T/C=0.41±0.10; *p*=0.03). Combined treatment of 2DG with EF leads to down-regulation of complexed and uncomplexed β -subunits with an overall decrease in the expression of F_1F_0 -ATPase of ~80% (*p*=0.003).

If the antagonistic effect of 2DG is due to down-regulation of F_1F_0 -ATPase within the tumor site, then the in vivo activity of EF should be ascribed to their ability to

inhibit Hsp90 through binding of the peptides to F_1F_0 -ATPase and dissociation of the Hsp90: F_1F_0 -ATPase complex. To examine this hypothesis, Hsp90 protein was immunoprecipitated from 200 μ g of tumor homogenates from MDA-MB-231 xenografts treated either with vehicle control or 0.3 mg/kg EF. Following SDS-PAGE electrophoresis, F_1F_0 -ATPase protein complexed with Hsp90 was detected by Western immunoblotting (Fig. 8c). Figure 8c also shows the amount of immunoprecipitated Hsp90 and the total expression of F_1F_0 -ATPase found in 200 μ g of the same tumor homogenates. Subsequently, we estimated the percentage of F_1F_0 -ATPase bound to Hsp90 over the total F_1F_0 -ATPase and expressed it as per unit of immunoprecipitated Hsp90. The data suggest that treatment with 0.3 mg/kg EF suppressed the association of F_1F_0 -ATPase with Hsp90 by 60%. The inhibition reached statistical significance ($p=0.05$).

Discussion

Evidence presented here suggests that EF exert a strong antitumor activity in breast cancer in vitro and in vivo, which appears to be mediated by two opposing mechanisms: inhibition of proteasome and suppression of Hsp90 chaperone action. Although EF are known inhibitors of OXPHOS, cellular energy is not found to play an active role in determining the ability of EF to inhibit tumor cell proliferation. In contrast, EF-induced inhibition of proteasome and suppression of Hsp90 function appears to be active and to compete for dominance. In cells, which express robust intracellular levels of F_1F_0 -ATPase, the target of EF, and Hsp90, inhibition of Hsp90 chaperone activity is the predominant mechanism of action of EF, while proteasomal inhibition becomes prominent only when the intracellular levels of the two proteins are reduced. This hypothesis is supported by our experimental work involving MCF-7 and MDA-MB-231 cells. MCF-7 cells possess approximately twofold more Hsp90 and eightfold more F_1F_0 -ATPase than MDA-MB-231 cells. As a result, MCF-7 cells are more sensitive to Hsp90 inhibition than to proteasomal suppression compared to MDA-MB-231 cells, an idea that is supported by the differential sensitivity of the two cell lines toward the Hsp90 inhibitor 17-AAG and the proteasomal inhibitor bortezomib (Table 1). In MCF-7 cells, EF-induced inhibition of cell proliferation appears to be the outcome of Hsp90 suppression, while in MDA-MB-231 cells, inhibition of proteasome by EF appears to be responsible for inhibition of cell growth. Because MDA-MB-231 cells are far more resistant to EF than MCF-7 cells, intracellular levels of Hsp90 and F_1F_0 -ATPase may determine not only the mechanism of EF action but also the in vitro sensitivity of a cancer cell line to EF. Here, it is worth

noting that correlation between cellular expression of Hsp90 and sensitivity to 17-AAG activity has previously been observed in thyroid cancer cells (Braga-Basaria et al. 2004).

In addition to Hsp90 and F_1F_0 -ATPase levels, sensitivity of a breast cancer cell line to EF may be determined by secondary factors such as the presence of the Hsp90 client protein ErbB2/HER2. ErbB2/HER2 appears to transform the cytostatic effect that EF has on cell growth to a cytotoxic one. This transformation may be related to the ability of EF to destabilize the interaction of ErbB2/HER2 with Hsp90 (Fig. 4a), while suppressing the degradation of ubiquitinated ErbB2/HER2 through inhibition of proteasome (Fig. 4b). It is well known that ErbB2/HER2 is a plasma protein that resists internalization and exhibits a long half-life on the surface of the cells. Because it relies on Hsp90 for stable expression, exposure of ErbB2/HER2-expressing cells to an Hsp90 inhibitor such as geldanamycin leads to rapid internalization, ubiquitination, and degradation of the protein (Mimnaugh et al. 1996; Lerdrup et al. 2006). Pedersen et al. (2008) have argued that ubiquitination of ErbB2/HER2 following inhibition of Hsp90 is essential for presentation of the internalized protein to the lysosomes for degradation. Concurrent treatment of ErbB2/HER2-expressing cells with geldanamycin and lactacystin, a well known proteasomal inhibitor, rescues ErbB2/HER2 from degradation and results in the accumulation of internalized, ubiquitinated protein (Lerdrup et al. 2006). We hypothesize that accumulation of internalized and thus inactive, ubiquitinated ErbB2/HER2 protein in MCF-7 cells transfected to express ErbB2/HER2 and treated with EF leads to such an increase in the cellular stress that cells prefer to die than enter a cytostatic phase following treatment with EF.

Despite their differential in vitro sensitivity, MCF-7 and MDA-MB-231 cells appear to respond to EF therapy equally well in vivo. Data from the MDA-MB-231 xenograft experiment show a robust expression of the Hsp90 and F_1F_0 -ATPase proteins within the tumor site and a sustaining dissociation of their complex following EF treatment. These data are in contrast with the in vitro observations. It is not clear if orthotopic implantation of the MDA-MB-231 cells leads to upregulation of tumoral Hsp90 and F_1F_0 -ATPase. The proteins detected in tumor homogenates by Western immunoblotting could be derived not only from tumor cells but also from stromal or other invading nontumorigenic cells. Regardless of the source of the detected Hsp90 and F_1F_0 -ATPase and the possible role of tumor stroma in mediating the EF antitumor effect, inhibition of Hsp90 by EF seems to be the dominant mechanism of action in the MDA-MB-231 orthotopic tumor model.

The possibility that tumor cells acquire resistance to EF as they switch to a more glycolytic phenotype implies that sensitivity to EF can be restored through inhibition of

glycolysis. 2DG, a well-known inhibitor of glycolysis, synergized with EF in inhibiting breast cancer cell proliferation. Reflecting the dependency of the cells on glycolysis, the effect was far more potent in MDA-MB-231 than in MCF-7 cells. We have attributed the synergistic activity of 2DG and EF to the induction of a prolonged ER stress as illustrated by the increased expression of Grp78 and Grp94. Grp78 and Grp94 were upregulated by single treatment of both drugs, while combined treatment induced an additive effect on the expression of the proteins. This suggests that 2DG and EF provoke ER stress by utilizing parallel pathways. Kim et al. (1999) have reported that, in an attempt to maintain ER homeostasis, cells undergoing glycolytic stress elevate their level of nuclear proteasomal activities raising the possibility that EF synergize with 2DG in increasing ER stress by inhibiting 2DG-induced proteasomal function. This may also explain why combination treatment is more effective in MDA-MB-231 cells than in MCF-7 cells, since the former are more sensitive to proteasomal inhibition. Finally, deregulation of the Glut-1 expression was observed by single and combined treatment of the drugs. It is well documented that exposure of breast cancer cells to 2DG results in upregulation of Glut-1 and the appearance of a partially glycosylated protein (Aft et al. 2002). Exposure of MDA-MB-231 cells to EF has a similar with 2DG effect on Glut-1 expression, suggesting that the EF interferes with protein glycosylation possibly by inducing ER stress through inhibition of proteasome (Fribley et al. 2004).

Combination treatment of 2DG and EF yielded unexpected results in vivo. 2DG antagonized the in vivo activity of EF, an event that was independent on the expression of Grps or Glut-1 or the accumulation of ubiquitinated proteins within the tumor site. Further experimentation showed that 2DG downregulated the uncomplexed β -subunits of F_1F_0 -ATPase in vitro as well as in vivo (Figs. 6 and 8). 2DG is known to stabilize Sp1, a ubiquitous transcription factor (Kang and Hwang 2006). Zaid et al. (1999) have reported the possible suppression of the β -subunit of F_1F_0 -ATPase by Sp1. Combined treatment of 2DG and EF further decreased uncomplexed as well as complexed β -subunits. Why a 2DG-induced decrease in the levels of F_1F_0 -ATPase will affect the in vivo but not the in vitro EF/2DG combination treatment of MDA-MB-231 cells can be explained only in terms of the dominant mechanism of EF action. If EF inhibits MDA-MB-231 cells in vitro by suppressing proteasomal activities, this suppression will increase ER stress associated with the presence of 2DG, and the combined effect of the two drugs will be synergistic. On the other hand, if EF inhibits MDA-MB-231 cells in vivo by binding to F_1F_0 -ATPase and disrupting its association with Hsp90, then downregulation of F_1F_0 -ATPase by 2DG will antagonize the inhibitory effect of EF. Therefore, the antagonistic effect of

the combined treatment strengthens the hypothesis that the in vivo activity of EF is the result of their ability to inhibit the chaperone activity of Hsp90 and cautions against extrapolating in vitro observations to in vivo settings.

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