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PRECLINICAL STUDY

Elesclomol, counteracted by Akt survival signaling, enhances the apoptotic effect of chemotherapy drugs in breast cancer cells

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Abstract Elesclomol is a small-molecule investigational agent that selectively induces apoptosis in cancer cells by increasing oxidative stress. Elesclomol plus paclitaxel was shown to prolong progression-free survival compared with paclitaxel alone in a phase II clinical trial in patients with metastatic melanoma. However, the therapeutic potential of elesclomol in human breast cancer is unknown, and the signaling mechanism underlying the elesclomol effect is unclear. Here, we show that elesclomol alone modestly inhibited the growth of human breast cancer cells but not normal breast epithelial cells. Elesclomol potentiated doxorubicin- or paclitaxel-induced apoptosis and suppression of breast cancer cell growth. While both c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase were activated by elesclomol, elesclomol-induced apoptosis was only in part mediated by JNK1. The additive effect of elesclomol on chemotherapy drug-induced apoptosis was associated with increases in cleaved caspase-3, $p21^{Cip1}$, and $p27^{Kip1}$ and decreases in the Inhibitor of Apoptosis Protein levels and NF-κB activity. We also found that Akt/Hsp70 survival signaling was induced by elesclomol, which may reflect a cellular feedback mechanism. Blockade of Akt activation using a small-molecule inhibitor enhanced elesclomol-elicited apoptosis, while expression of a hyperactive Akt abolished the elesclomol effect. These data suggest that elesclomol's interaction with conventional chemotherapeutic and Akt-targeting agents may be exploited to induce apoptosis in breast cancer cells, and clinical trials of combined treatment of elesclomol and chemotherapy drugs or Akt-targeting agents in breast cancer patients, especially the estrogen receptor negative subgroup, may be warranted.

Keywords Akt · Apoptosis · Chemotherapy · Elesclomol · JNK · Reactive oxygen species

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Introduction

Elesclomol [N-malonyl-bis(N′-methyl-N′-thiobenzoylhydrazide)], previously designated as STA-4783, is a novel small-molecule oxidative stress inducer that has been granted Orphan Drug and Fast Track designation from the United States Food and Drug Administration for the treatment of metastatic melanoma. It is also being actively pursued for clinical evaluation in other solid tumors [1–3]. Elesclomol can trigger a drastic increase in reactive oxygen species (ROS) levels in cancer cells. ROS such as superoxide O^{-2}, hydroxyl radical ·OH, and H_2O_2 are constantly generated during intracellular metabolism and in response

to environmental stimuli. The mitochondrial respiratory chain is the major source of ROS for the most cells, and increased production of ROS is involved in committing cells to apoptosis [4]. Generally, ROS are regarded as host-defending molecules released by neutrophils to destroy exogenous pathogens such as bacteria. They can act as secondary messengers in signal transduction. However, excess oxidative stress and weakened anti-oxidative defense can damage macromolecules vital for cellular functions [5, 6], which in turn results in physiopathological changes such as apoptosis, cell cycle disruption, and necrosis.

Cancer cells usually generate and maintain higher levels of ROS compared to normal cells partially due to their higher metabolic rate, activation of proliferative signaling pathways, and mutations in mitochondrial DNA, leading to defects in the electron transport chain. Because ROS levels are already high, cancer cells are vulnerable to agents that can further increase ROS levels to a critical point that will initiate apoptosis [7]. On the other hand, cancer cells with high ROS scavenger expression and low ROS levels are resistant to therapies [8]. Elesclomol and motexafin gadolinium are representative investigational drugs that kill cancer cells specifically through this ROS-mediated mechanism [9, 10]. Elesclomol-induced expression of heat shock and metallothionein genes reportedly matches the signature transcription profile of cells under oxidative stress [9].

Previous studies have demonstrated that total cellular antioxidant capacity influences cellular responses to paclitaxel and doxorubicin [11, 12]. It is conceivable that elesclomol may enhance the efficacy of chemotherapy drugs by generating ROS, and thus diminishing the anti-apoptotic effect of antioxidants in cancer cells [9]. A study using xenograft models of human cancers found that elesclomol significantly enhanced the anti-tumor activity of paclitaxel, in a dose-dependent manner, without increasing host toxicity [13]. In the phase II clinical trial of elesclomol for stage IV metastatic melanoma, patients who received elesclomol plus paclitaxel had longer progression-free survival than those who received paclitaxel alone [1, 2].

Targeting tumor oxidative stress represents a new category of anti-cancer therapy for solid tumors [3]. However, despite strong preclinical and phase II clinical evidence for the anti-cancer potential of elesclomol, the phase III trial in melanoma did not show a significant survival benefit for patients who received elesclomol plus paclitaxel versus paclitaxel alone [14]. Clearly, the biological basis for the interaction between elesclomol and standard chemotherapy agents requires further study. In addition, the therapeutic potential of elesclomol in human breast cancer is unknown. The present work was designed to address these issues. We found that elesclomol potentiates cytotoxic chemotherapy agents-induced apoptosis in breast cancer cells via c-Jun N-terminal kinase (JNK) signaling, downregulation of survival proteins, and inhibition of NF-κB activity. Interestingly, Akt signaling was induced by elesclomol as a cellular survival response. Blockade of Akt enhanced elesclomol-elicited apoptosis in breast cancer cells.

Materials and methods

Cell culture and chemicals

Estrogen receptor (ER)-positive MCF-7, ER-negative MDA-MB-468, MDA-MB-231, and HCC1806 human breast cancer cell lines were obtained from American Type Culture Collection. Normal human mammary epithelial cells (HMECs) were purchased from Clonetics (Walkersville, MD). MCF-7 cells expressing myr-Akt, a constitutively active Akt containing a myristoylation membrane-targeting sequence, and the control vector were gifts from Adrian Lee (Baylor College of Medicine, Houston). Breast cancer cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C humidified incubator containing 5% CO_2. HMECs were grown in Mammary Epithelial Growth Medium (Clonetics). Elesclomol was provided by Synta Pharmaceuticals, and the p38 MAPK inhibitor JX401, the JNK inhibitor SP600125, and the Akt inhibitor AI-IV (a benzimidazole compound) [11], were from Calbiochem (Gibbstown, NJ).

Cell proliferation assay

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. Cells were seeded in 24-well plates at 50% confluence, and the MTT assay was performed 1, 3, and 5 days after treatment. For each assay, 50 μl of MTT (5 mg/ml) was added to each well and cells were incubated at 37°C for an additional 4 h. After centrifugation, the supernatant was carefully aspirated, and 300 μl of DMSO (Sigma) was added to each well. Immediately after resolubilization, all plates were scanned at 575 nm on a microplate reader. The absorbance (A) value represented the number of live cells.

Analysis of synergy

MCF-7 and MDA-MB-231 cells were plated in 24-well dishes. The cells were treated with elesclomol alone, doxorubicin or paclitaxel alone, or elesclomol plus doxorubicin or paclitaxel for 3 days followed by MTT assays. We used the SAS software (SAS Institute Inc) for synergism analysis because it allows us to analyze the drug interaction when the growth inhibition effect of a drug,

such as elesclomol, is too weak to achieve an IC_{50} value [15]. The effect of the combined treatment of elesclomol and chemotherapy drugs was examined by incorporating the factors elesclomol and doxorubicin or paclitaxel in the general linear model (Proc glm, SAS 9.1.3) and cross checked by simple linear regression method (Proc Reg SAS 9.1.3). We first incorporated individual drugs along with the interaction term of both drugs in the general linear model (glm). When (1) both drugs were significant covariates in the glm, (2) the maximum likelihood estimates (mle) of the two drugs in the regression model had the same direction (negative value), and (3) the interaction term was not a significant covariate, the two drugs showed an additive effect. When (1) both drugs and the interaction term were significant covariates in the glm, (2) the mle of both covariates (drugs) in the linear regression model had negative values, and (3) the mle of the interaction term was positive, the drugs showed a partial additive effect. When (1) both drugs and the interaction term were significant covariates in the glm, and (2) the mle of all the factors in the linear regression model had negative values, the drugs displayed a synergistic effect. When (1) both drugs were significant covariates in the glm, and (2) the mle of the covariates in linear regression model had different directions (one positive and one negative), the drugs were considered to be antagonistic, regardless of the significance of the interaction term. When only one drug was a significant predictor, the combined treatment only had a single-drug effect.

JNK shRNA-expressing cells

The lentiviral construct pLKO-Puro (Sigma, St. Louis, MO) expressing JNK1 or JNK2 shRNA was stably transduced into breast cancer cells. The JNK1 shRNA-targeting sequence is CGGGACTTAAAGCCTAGTAAT and the JNK2 shRNA-targeting sequence is GCGGACTCAACT TTCACTGTTCT. JNK1 or JNK2 shRNA cells were selected with 5 μg/ml puromycin. A shRNA that does not match any known human-coding cDNA was used as an experimental control.

Apoptosis analysis

Apoptotic cells were analyzed by flow cytometry using Annexin V-fluorescent isothiocyanate (FITC) and propidium iodide (PI) staining (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. Cytometry was performed using the FACS Calibur and Cell Quest software (BD Biosciences). Four distinct cell populations are distinguishable: (1) the viable population (Annexin V and PI negative cells), (2) the early apoptotic population (Annexin V positive and PI negative cells), (3) the late apoptotic population (Annexin V and PI positive cells), and (4) the necrotic or lysed population (Annexin V negative and PI positive cells). To visualize apoptotic cells, PI (5 μg/ml) and SYTO-13 green fluorescent nucleic acid dye (1 μM; Invitrogen, Carlsbad, CA) were added to the culture medium. After 15 min, cells were examined under a fluorescent microscope using excitation at 488 nm. PI stains necrotic or late apoptotic cells (red), whereas SYTO-13 stains live cells and early apoptotic cells (green).

Western blotting

Whole cell lysates for western blotting were generated by cell lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 10% glycerol) supplemented with a protease inhibitor cocktail (Sigma, St. Louis, MO). Equal amounts of protein were separated by 10% SDS–PAGE and then transferred onto a nitrocellulose membrane. The remaining steps were conducted according to a standard immunoblotting protocol. Anti-phospho-p38 MAPK (Thr180/Tyr182), anti-phospho-SAPK/JNK (Thr183/ Tyr185), anti-phospho-Akt (Ser473), phospho-Erk1/2 (Thr202/Tyr204), and anti-PARP antibodies were from Cell Signaling (Danvers, MA) and used at 1:1000 dilutions. Anti-β actin (Sigma) and anti-heat shock protein 70 (Chemicon, Billerica, MA) were used at a 1:5000 dilution.

Human apoptosis protein array

To compare the levels of apoptosis-related proteins under different treatment conditions, a human apoptosis protein array (R&D Systems, Minneapolis, MN) was used according to manufacturer's instructions. Briefly, protein lysates (400 μg) were loaded onto an array membrane that had been blocked with PBST plus 5% non-fat milk for 1 h. The membrane was incubated overnight at 4°C, washed three times for 5 min each with PBST, and then incubated with a horseradish peroxidase (HRP)-linked secondary antibody at a dilution of 1:4000 in blocking solution. After the membrane was washed, blotting dots were visualized by chemiluminescence assays. Densitometry of protein dot signals was obtained. The average density of duplicate spots representing each apoptosis-related protein indicated its relative levels. To compare the spot density from different membranes, relative density was used (relative density = mean density of each protein/mean density of positive control × 100%). Protein expression levels in cells treated by paclitaxel plus elesclomol were compared with those in vehicle-treated or paclitaxel-treated cells.

NF-κB activity assay

The NF-κB responsive luciferase reporter construct in pGL4 was from Promega (Madison, WI). Breast cancer cells in 6-well dishes were transfected with 200 ng of the NF-κB reporter construct and 20 ng of a β-galactosidase plasmid. Then cells were treated with 0.01 μM paclitaxel alone or 0.01 μM paclitaxel plus 1 μM elesclomol. Cell lysates were harvested. The luciferase activity was measured and normalized by β-galactosidase activity.

Statistics

Values represented mean ± standard deviation (SD) of samples measured in triplicate. Each experiment was repeated twice, unless otherwise indicated. The significance of differences between experimental groups was analyzed using the Student's *t* test and two-tailed distribution.

Results

Elesclomol inhibits growth of breast cancer cells

Elesclomol is a potent inducer of oxidative stress in cancer cells [9]. To determine its effect on breast cancer cell growth, we treated HMECs and three human breast cancer cell lines MCF-7, MDA-MB-231, and HCC1806 with elesclomol. The MTT assay showed that 0.1, 1, or 10 μM of elesclomol caused a time-dependent, mild decrease in cancer cell growth, but did not inhibit HMEC growth (Fig. 1). Compared with vehicle-treated control, growth of the three breast cancer cell lines treated with 1 μM elesclomol was reduced by 10% or less on Day 1 and 20% on Day 3 after treatment. Cell growth was inhibited by 40% and 60% on Day 5 in 1 μM elesclomol-treated MCF-7 and HCC1806 cells, respectively, but only by 10% in MDA-MB-231 cells, suggesting that MDA-MB-231 cells were relatively insensitive to elesclomol treatment. Surprisingly, these cells exhibited similar responses to the three different concentrations of elesclomol. In summary, our data suggest that elesclomol alone can modestly suppress breast cancer cell growth with little or no effect on HMECs.

Elesclomol potentiates the sensitivity of breast cancer cells to chemotherapy drugs

Elesclomol extends progression-free survival in melanoma patients receiving paclitaxel [3]. We postulated that elesclomol may also render breast cancer cells more sensitive to chemotherapy drugs. To address this question, we tested the combined treatment of 0.1 or 1 μM elesclomol and 0.1 μM doxorubicin or 0.01 μM paclitaxel in MCF-7 and MDA-MB-231 breast cancer cell lines, which are commonly used cell models for ER-positive and ER-negative breast cancer, respectively. Cell growth was assessed by the MTT assay. As demonstrated in Fig. 2a, doxorubicin and paclitaxel each inhibited the growth of MCF-7 and MDA-MB-231 cells in a time-dependent manner. Elesclomol dramatically enhanced the effect of the two drugs on these cells, especially on Day 3 and 5 when the combined treatment further reduced cell growth by 50% or

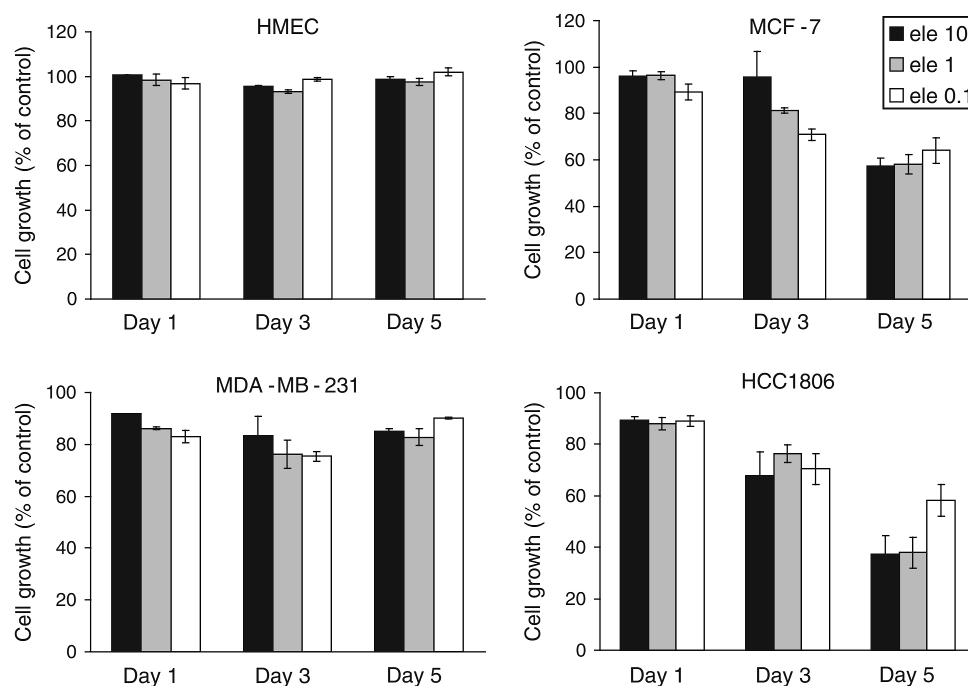

Fig. 1 Effects of elesclomol on the growth of breast cancer cells and HMEC. HMEC, MCF-7, MDA-MB-231, and HCC1806 cells were treated with different concentrations of elesclomol (10, 1, 0.1 μM) for the indicated time. The MTT assay was conducted to measure the cell growth. The values (mean ± SD) are presented as relative growth rates compared with the vehicle control. The results represent three separate experiments each performed in triplicate

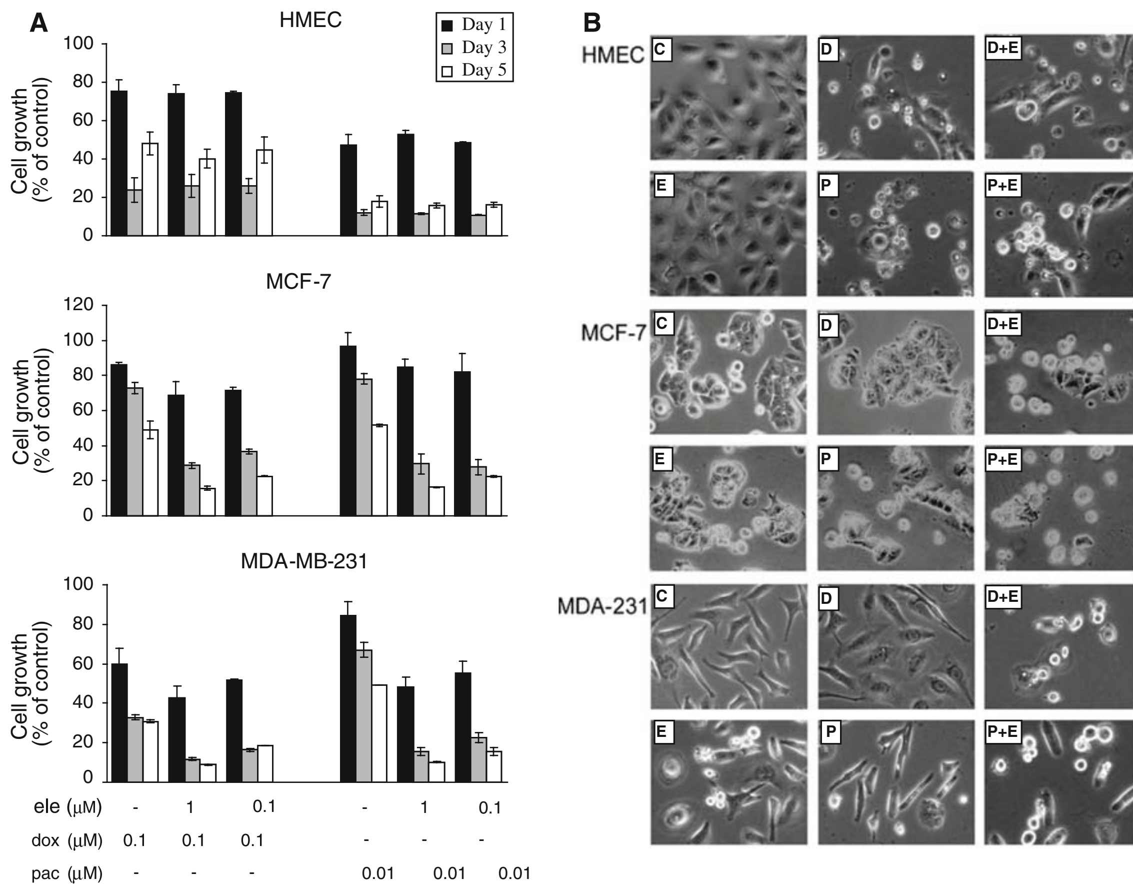

Fig. 2 Effects of elesclomol in combination with doxorubicin or paclitaxel on the growth of breast cancer cells and HMEC. (**a**) HMEC, MCF-7, and MDA-MB-231 cells were treated with 0.1 μM doxorubicin (dox), 0.1 μM doxorubicin plus 1 μM elesclomol (ele), 0.1 μM doxorubicin plus 0.1 μM elesclomol, 0.01 μM paclitaxel (pac), 0.01 μM paclitaxel plus 1 μM elesclomol, or 0.01 μM paclitaxel plus 0.1 μM elesclomol for the indicated time. The MTT assay was conducted to measure the cell growth. The values (mean ± SD) are presented as relative growth rates compared with the vehicle control. The results represent three separate experiments each performed in triplicate. (**b**) Morphologies of cells treated with elesclomol and/or the chemotherapy drugs for 24 h (magnification ×200). C, control; D, doxorubicin; E, elesclomol; P, paclitaxel

more compared with doxorubicin or paclitaxel as a single agent. For MDA-MB-231 cells, the increase in apoptosis by elesclomol and paclitaxel could be detected on Day 1 after treatment. An increased number of round and floating cells (likely apoptotic cells) were observed under the combined treatment of elesclomol and a chemotherapy agent (Fig. 2b). Similar results were also observed in HCC1806 breast cancer cells (data not shown). As expected, the two chemotherapy agents had a pronounced cytotoxicity in HMECs, and there was no additive effect of elesclomol, confirming that elesclomol was not cytotoxic to HMECs. Furthermore, even with paclitaxel at a much higher dose of 0.1 μM versus 0.01 μM, elesclomol still enhanced the paclitaxel effect (Supplementary Fig. S1). Synergy analysis of the treatment results from Day 3 using the SAS program further demonstrated that elesclomol and the chemotherapy drugs could synergistically inhibit cell growth (Supplementary Fig. S2). Taken together, our data indicate that elesclomol acts in concert with doxorubicin or paclitaxel to suppress growth of human breast cancer cells.

Because elesclomol induces apoptosis through oxidative stress [9], we used Annexin V and PI double staining to investigate the effect of elesclomol plus doxorubicin or paclitaxel on apoptosis induction in MDA-MB-231 cells [16]. Flow cytometry analysis demonstrated that elesclomol plus doxorubicin or paclitaxel led to a significantly higher apoptotic rate than each single chemotherapy agent after 48 h treatment (Supplementary Fig. S3A). Of note, the seeming paradox of a pronounced effect of doxorubicin on cell growth but a modest effect on apoptosis may be attributed to that the doxorubicin also induces G2/M cell cycle arrest and non-apoptotic cell death [17] (data not shown). As a cleavage product of caspase-3 and caspase-7, the 89 kD fragment of poly (ADP-ribose) polymerase

(PARP) was a marker of cell apoptosis [18]. Immunoblotting indicated that the 89 kD cleaved PARP band was detected after the combined treatment, but not after the single-agent treatment (Supplementary Fig. S3B). These results indicate that elesclomol acts cooperatively with cytotoxic agents to induce apoptosis in breast cancer cells.

JNK mediated the effect of elesclomol in cell apoptosis

JNK and p38 mitogen-activated protein kinase (MAPK) are well known for their essential roles in chemotherapy agent-elicited apoptosis [19]. ROS-generating agents have also been shown to utilize JNK [20] or p38 MAPK [21] to elicit apoptosis. To identify the signaling mechanism underlying the effect of elesclomol, we first performed immunoblotting of ERK, JNK, and p38 MAPK using their corresponding phospho-specific antibodies after MDA-MB-231 cells were treated with elesclomol, doxorubicin, and paclitaxel alone or in combination for 1 or 6 h. As shown in Fig. 3a, JNK and p38 MAPK were activated by elesclomol or its combination with doxorubicin or paclitaxel after 6 h treatment, as indicated by the levels of p-JNK and p-p38 MAPK. ERK was not activated by elesclomol. Interestingly, elesclomol plus doxorubicin or paclitaxel induced higher levels of p-JNK, not p-p38 MAPK, compared with each single-agent treatment.

Next, we used the small-molecule JNK inhibitor SP600125 and p38 MAPK inhibitor JX401 to block the activation of JNK and p38 MAPK by elesclomol. After 48 h or 60 h treatment, MDA-MB-231 cells were labeled by Annexin V and PI double staining. Flow cytometry analysis showed that JNK blockade partially repressed elesclomol-induced apoptosis, whereas p38 MAPK inhibition did not exhibit any effect (Fig. 3b, Supplementary Fig. S4). To further corroborate these results, shRNAs were used to inhibit JNK1 or JNK2 in these cells. As illustrated in Fig. 3c, suppression of JNK1 impeded the elesclomol induction of apoptosis, while reduction in JNK2 potentiated the elesclomol effect. This is in agreement with previous findings that JNK1 and JNK2 play opposite roles with regard to survival and apoptosis in cancer cells [22, 23]. In summary, these results suggest that JNK1 signaling is involved in the elesclomol induction of apoptosis in breast cancer cells.

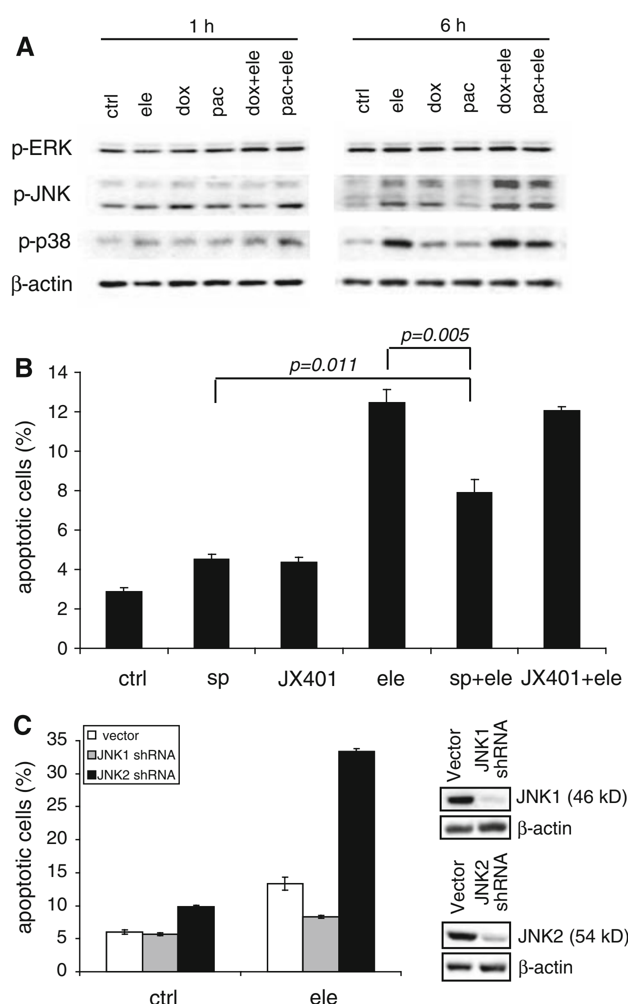

Fig. 3 JNK mediates the elesclomol effect on cell apoptosis. (**a**) MDA-MB-231 cell lysates were collected 1 or 6 h after treatment with 1 μM elesclomol and/or 0.1 μM doxorubicin or 0.01 μM paclitaxel. Western blotting was conducted using p-ERK, p-JNK and p-p38 MAPK antibodies. *β*-actin was used as a loading control. (**b**) Cells were treated for 48 h with 1 μM elesclomol in the presence of the JNK inhibitor SP100625 (sp, 5 μM) and the p38 MAPK inhibitor JX401 (1 μM). Cell apoptotic rate was assessed by Annexin V and PI double staining followed by flow cytometry analysis. The results represent three independent experiments. (**c**) Apoptosis analysis of JNK1 or JNK2-silenced cells treated with 1 μM elesclomol for 48 h. The percentage of apoptotic cells in the total cell population is plotted. Immunoblotting of JNK1 and JNK2 is shown in the right inset. Only the 46 kD isoform of JNK1 and the 54 kD isoform of JNK2 were detected

Apoptosis- or survival-related proteins are regulated by elesclomol plus paclitaxel

As the combined treatment markedly induces breast cancer cell apoptosis, we speculate that expression of proteins involved in survival or apoptosis may be dysregulated by elesclomol plus the chemotherapy agents. With this in view, we conducted an apoptosis/survival protein antibody array analysis with MDA-MB-231 cell lysates from different treatments. Immunoblotting of the array showed that the inhibitor of apoptosis protein (IAP) family, including XIAP, CIAP-1, survivin, and livin, were downregulated by elesclomol plus paclitaxel compared with paclitaxel alone (Fig. 4a). Interestingly, paclitaxel alone increased the livin protein level compared with vehicle control, but this effect was reversed by the combined treatment. Of note, other survival proteins like Bcl-2 included in the protein antibody array were not further reduced by the combined treatment

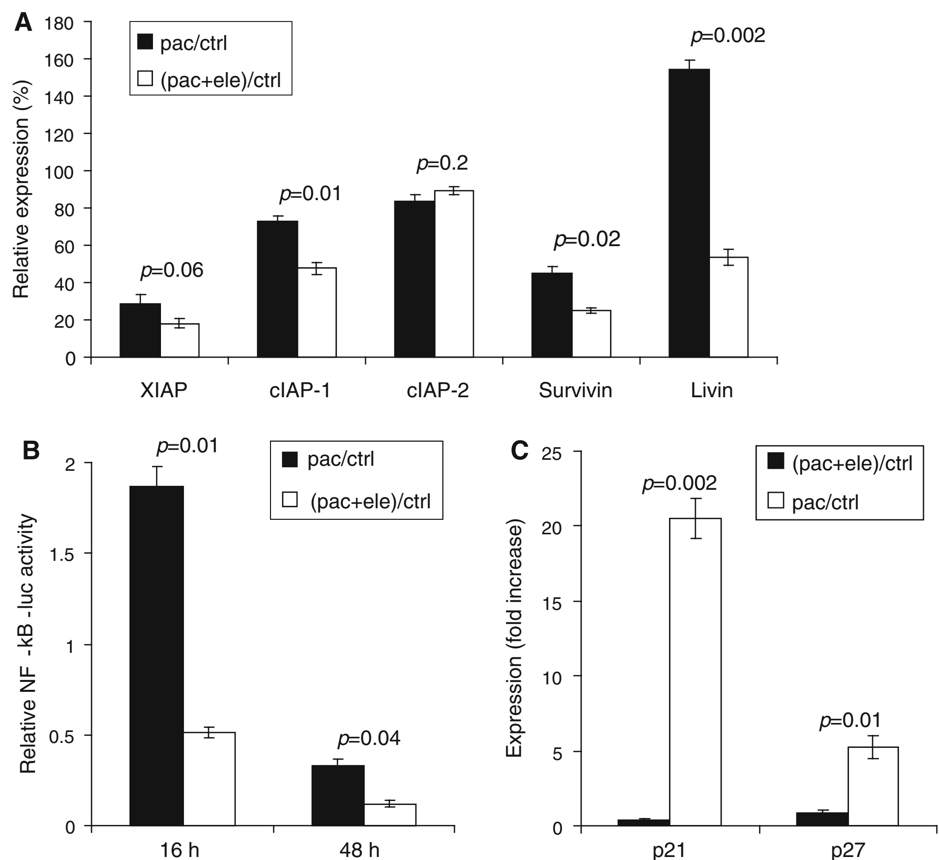

Fig. 4 Apoptosis/survival-related proteins are regulated by elesclomol plus paclitaxel. (**a**) MDA-MB-231 cells were treated by vehicle, 1 μM elesclomol, 0.01 μM paclitaxel, or 1 μM elesclomol plus 0.01 μM paclitaxel for 24 h. Cell lysates were collected. Protein antibody arrays of pro- or anti-apoptosis proteins were conducted. Relative densitometry (see section 'Materials and methods') was used to compare the levels of IAPs induced by paclitaxel alone or paclitaxel plus elesclomol compared with vehicle control. The data represent mean ± SD of two independent experiments. (**b**) Cells were transfected with a NF-κB-driven luciferase reporter plasmid or the control vector and then treated as in (**a**). Luciferase activity was measured. (**c**) Protein antibody array analysis of p21^{Cip1} and p27^{Kip1} protein levels induced by paclitaxel alone or paclitaxel plus elesclomol for 24 h

compared with single-agent paclitaxel (data not shown). As the transcription factor NF-κB is involved in ROS effects and can regulate IAP expression [24], we examined the effect of elesclomol on NF-κB activity using a NF-κB-driven reporter construct. As illustrated in Fig. 4b, elesclomol treatment dramatically reduced NF-κB-induced luciferase activity, which might be explained by the previous finding that ROS oxidizes the cysteine residues within the DNA-binding region of transcription factors, resulting in their inactivation [25].

Using the protein antibody array, we also found that well-established chemotherapy-responsive genes such as cyclin-dependent protein kinase inhibitors p21^{Cip1} and p27^{Kip1} were markedly further increased by elesclomol plus paclitaxel compared with paclitaxel alone (Fig. 4c). Taken together, these data suggest that the combined treatment of elesclomol and paclitaxel may potentiate apoptosis by regulating the expression of specific pro- or anti-apoptotic proteins in breast cancer cells.

Elesclomol induces a survival response through Akt

The heat shock protein Hsp70, which can protect cells from a variety of stress conditions, was found to be remarkably upregulated by elesclomol [9, 26]. It has been shown that Hsp70 expression is regulated by Akt in multiple myeloma cells [27] and that oxidative stress-inducing motexafin gadolinium elicits Akt phosphorylation [28]. Thus, we explored whether elesclomol treatment activated Akt. Immunoblotting showed that levels of p-Akt and Hsp70 were strongly induced by elesclomol and its combination with paclitaxel or doxorubicin in MDA-MB-231 breast cancer cells (Supplementary Fig. S5). In contrast to growth factors, which activate Akt within minutes, elesclomol elicits Akt activation over a slow time course. This suggests that Akt may mediate a survival feedback mechanism in response to elesclomol. When a small-molecule Akt inhibitor, AI-IV [11], was added in the presence of elesclomol, the induction of p-Akt and Hsp70 was severely impaired (Supplementary Fig. S5). Accordingly, immunoblotting demonstrated that Akt activation and Hsp70 expression continued to increase in the 24-h time period in a similar pattern (Fig. 5a). To confirm that Akt signaling regulates Hsp70 expression, we used MCF-7 cells overexpressing a constitutively active Akt (myr-Akt) containing a myristoylation membrane-targeting sequence [29]. Western blot analysis showed that Hsp70 levels were elevated by Akt overexpression (Fig. 5b). In conclusion, these

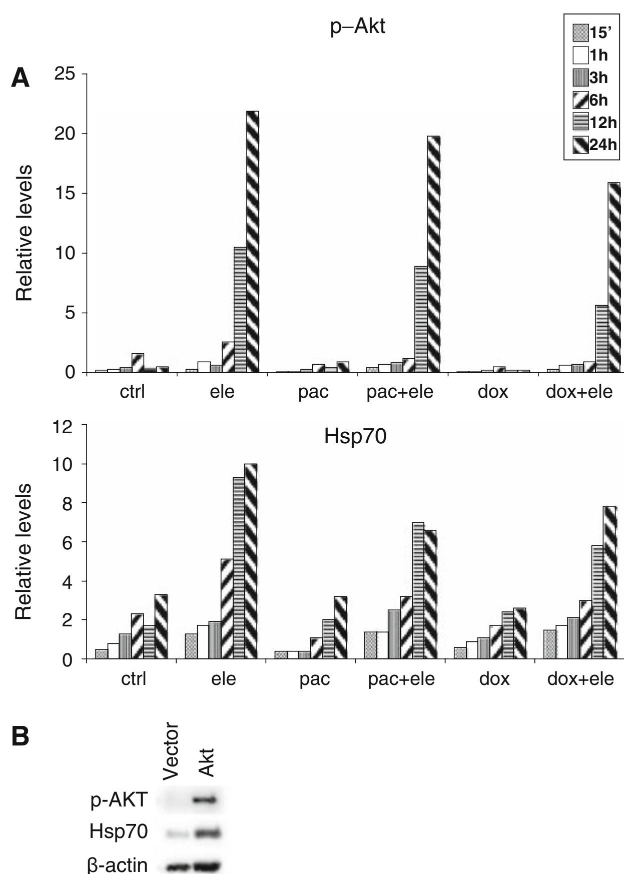

Fig. 5 Elesclomol induces Akt and Hsp70 in a time-dependent manner. (**a**) MDA-MB-231 cells were treated with vehicle, 1 μM elesclomol, 0.1 μM doxorubicin, 0.01 μM paclitaxel, 0.1 μM doxorubicin plus 1 μM elesclomol, or 0.01 μM paclitaxel plus 1 μM elesclomol. Cell lysates were collected at different time points. Immunoblotting of p-Akt and Hsp70 was performed. Relative levels of p-Akt and Hsp70 (normalized by actin levels) were measured using densitometry analysis of immunoblots. (**b**) Immunoblotting of p-Akt and Hsp70 in control and myr-Akt-overexpressing MCF-7 cells is shown. β-actin was used as a loading control

data implicate the Akt survival pathway as a cell feedback mechanism in response to elesclomol-triggered oxidative stress.

Akt blockade enhances elesclomol-induced apoptosis

Next, we tested whether blocking Akt activity increases cell sensitivity to elesclomol. As illustrated in Fig. 6a, the Akt inhibitor AI-IV at different concentrations (0.1, 0.5, 1, 2 μM) induced apoptosis in MDA-MB-231 cells, although to a different extent. However, the cell apoptosis was significantly enhanced in the presence of both elesclomol and AI-IV. This effect was more pronounced at 48 h than at 24 h (Supplemental Fig. S6 for flow cytometry data). To further consolidate this study, we examined the combined treatment with AI-IV and elesclomol in MDA-MB-468 breast cancer cells, which lack PTEN and thus possess sustained Akt activation [30]. As illustrated in Fig. 6b, AI-IV plus elesclomol markedly increased apoptosis compared with each single agent, whereas elesclomol did not induce apoptosis in MDA-MB-468 cells. Accordingly, elesclomol-induced apoptosis also diminished in MCF-7 cells overexpressing myr-Akt, as shown by flow cytometry analysis after Annexin V and PI staining (Fig. 6c) and by fluorescent staining of apoptotic and live cells with PI and SYTO-13, respectively (Supplemental Fig. S7). These data further support that Akt may mediate a feedback mechanism to overcome elesclomol-induced stress and that inhibiting Akt may improve the efficacy of elesclomol treatment in cancer cells.

Discussion

Elesclomol represents an actively pursued anti-cancer strategy based on its ability to selectively induce oxidative stress in cancer cells. In a phase I clinical trial of elesclomol in combination with paclitaxel for patients with refractory solid tumors, elesclomol was well tolerated and did not increase toxicity [31]. We showed that elesclomol had no cytotoxicity in normal breast epithelial cells, consistent with the results from clinical trials [1, 2]. Our finding that elesclomol enhanced the cell-killing activity of paclitaxel and doxorubicin in breast cancer cells but not in HMECs suggests that elesclomol might increase the efficacy of chemotherapy against breast carcinoma without increasing the toxicity to normal tissues and thereby could increase the cancer selectivity and therapeutic index of these chemotherapies.

Because ROS levels are already higher in cancer cells than normal cells, cancer cells might be more vulnerable to any stimulus that further elevates ROS levels beyond the antioxidant capacity of the cells. However, a single-pathway mechanism (from ROS generation to apoptosis induction) for the elesclomol effect may be over-simplistic because elesclomol, on its own, only mildly suppressed growth of breast cancer cells. High levels of antioxidant proteins and hormones in breast cancer cells might antagonize the action of elesclomol. Induced Akt activation may limit the effect of elesclomol in breast cancer cells. Furthermore, we speculate that limited abundance and nature of the unidentified ROS-generating target of elesclomol might also contribute to the lack of dose-dependence of the elesclomol effect in breast cancer cells.

Paclitaxel is a microtubule-targeting agent widely used in breast cancer therapy [32]. As a secondary mechanism of its action, paclitaxel regulates Bcl-2 family proteins to disrupt mitochondrial membrane potential, resulting in release of cytochrome C and generation of ROS [33, 34].

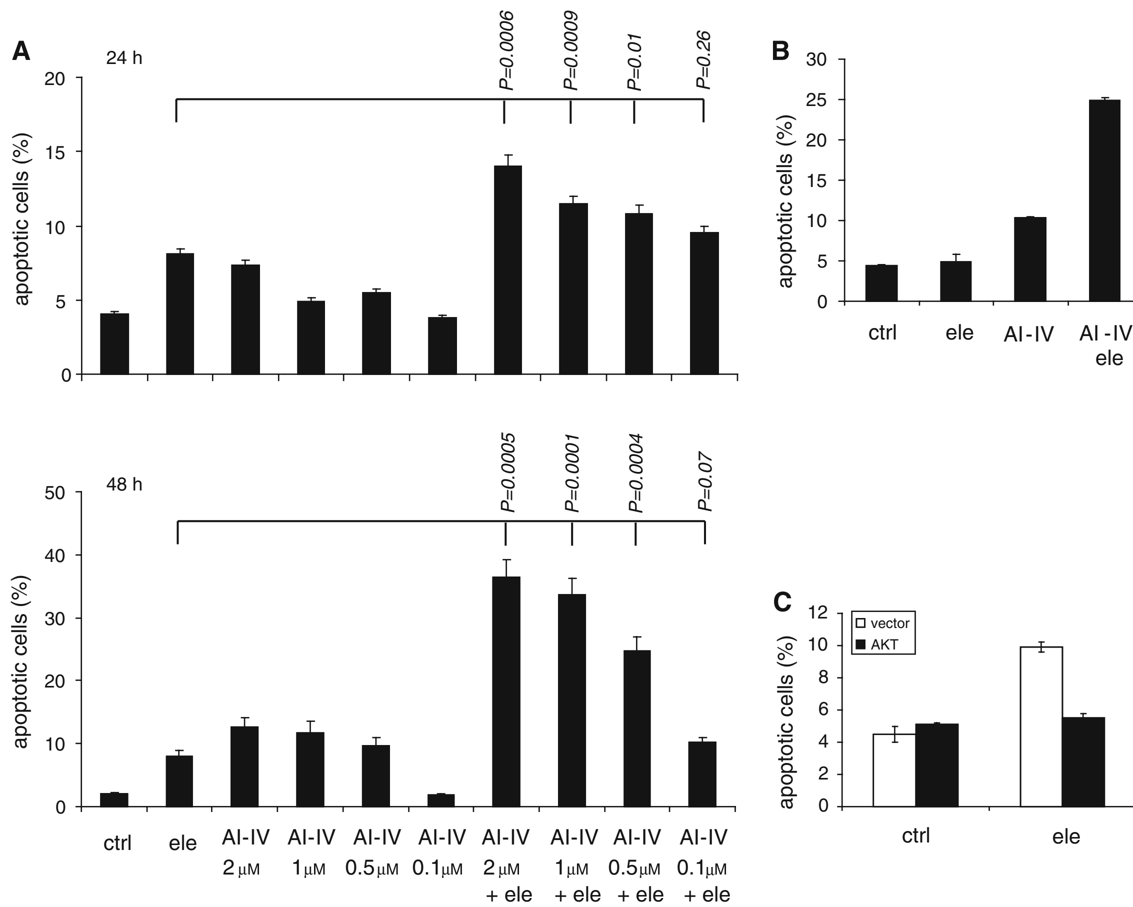

Fig. 6 Akt inhibition potentiates elesclomol-induced cell apoptosis. (**a**) Different concentrations (2, 1, 0.5, 0.1 μM) of the Akt inhibitor AI-IV were added in the presence or absence of elesclomol (1 μM). After 24 or 48 h, MDA-MB-231 cells were stained with Annexin V and PI, and analyzed by flow cytometry. The percentage of apoptotic cells in the total cell population is plotted. (**b**) MDA-MB-468 cells were treated with vehicle, 1 μM elesclomol, 1 μM AI-IV, or 1 μM elesclomol plus 1 μM AI-IV for 48 h. Apoptosis was examined. (**c**) MCF-7 cells expressing the control vector or a constitutively active Akt (myr-Akt) were treated with vehicle or 1 μM elesclomol for 48 h. Apoptosis was examined

Doxorubicin causes cell death by introducing DNA double-strand breaks and impairing DNA unwinding. Its induction of cell apoptosis has also been attributed to the production of ROS [35]. Interestingly, the pro-apoptotic activity of chemotherapy drugs is inversely correlated with the antioxidant capacity of cells. Our data indicate that elesclomol acts in cooperation with either paclitaxel or doxorubicin to induce apoptosis. This action may be the result of interaction between different signaling pathways induced by elesclomol and chemotherapy agents. This is supported by our finding that paclitaxel or doxorubicin does not increase elesclomol-induced ROS levels (unpublished data) and that elesclomol and paclitaxel, at different concentrations even with high paclitaxel concentration, maintain enhanced cytotoxicity in their combined treatment.

Cancer cells have higher levels of anti-apoptotic proteins such as IAPs compared with normal cells [36]. Our data suggest that JNK1 activation, NF-κB inactivation, downregulation of IAPs may collectively mediate the effect of elesclomol. IAPs are a family of caspase inhibitors that selectively bind and inhibit activated caspase-3, caspase-7, and caspase-9, blocking the apoptosis pathway. Some IAPs are reportedly involved in resistance to paclitaxel treatment [37]. Our present results showed specific downregulation of IAPs by paclitaxel plus elesclomol compared with control and single-agent treatment, suggesting that this group of proteins may be involved in the interaction between the two agents. In addition to downregulation of anti-apoptotic proteins, pro-apoptotic proteins $p21^{Cip1}$ and $p27^{Kip1}$ were upregulated by paclitaxel plus elesclomol. Overexpression of $p21^{Cip1}$ or $p27^{Kip1}$ promotes drug-induced apoptosis in cancer cells [38, 39]. Oxidative stress can directly activate p53 pro-apoptotic signaling [40], which is known to induce p21 and p27. However, $p21^{Cip1}$ and $p27^{Kip1}$ can also be activated via p53-independent pathways [41]. Both MDA-MB-231 and HCC1806

breast cancer cells possess mutant p53, yet elesclomol and chemotherapy drugs can synergistically induce apoptosis in these cells. This suggests that p53 may not play a major role in the elesclomol action.

One of the genes strongly induced by elesclomol is Hsp70, an oxidative stress-responsive gene. Hsp70 facilitates protein folding and translocation through the membrane, and it prevents aggregation of stress-related proteins [42]. Hsp70 inhibits JNK activity and thereby JNK-mediated apoptosis [43]; it can also prevent apoptosis by selectively inhibiting the activations of caspase-9 and caspase-3 [44]. We found that elesclomol mildly inhibited cell growth, suggesting that a survival signaling pathway counterbalances the induction of apoptosis. The Akt/Hsp70 pathway, which plays an important role in drug resistance of breast cancers [45–47], is involved in this feedback response. Akt activation by doxorubicin has been implicated in the desensitization to doxorubicin [48, 49]. We showed that Akt and Hsp70 had a similar time-course induction by elesclomol. Blocking Akt activity inhibited the upregulation of Hsp70 by elesclomol and rendered cells more sensitive to elesclomol, whereas hyperactive Akt induced Hsp70 levels and resistance to elesclomol. These results argue for clinical investigation of using Akt inhibitors to boost the efficacy of elesclomol.

In summary, our studies unveil clues for the mechanism underlying the interaction between elesclomol and two widely used chemotherapy drugs. The apoptosis enhancing effect of combined treatment, which is not accompanied by increased killing of normal cells, highlights the therapeutic potential of elesclomol as a component of more effective drug regimens for breast cancer (especially the ER-negative subgroup). The feedback survival signaling induced by elesclomol is an intriguing dichotomy that likely can be managed by Akt inhibitors.

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