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RESEARCH PAPER

Salinomycin sensitizes cancer cells to the effects of doxorubicin and etoposide treatment by increasing DNA damage and reducing p21 protein

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BACKGROUND AND PURPOSE

Salinomycin (Sal) has recently been shown to inhibit various cancer stem cells. Here, we investigated whether Sal could sensitize cancer cells to the effects of doxorubicin (DOX) or etoposide (ETO).

EXPERIMENTAL APPROACH

Using the Comet assay, immunocytochemistry and Western blot analysis, we assessed the ability of Sal to increase DNA breakage. We performed a cell proliferation assay to determine cell viability, cellular detachment, increased pre-G1 region, Annexin V staining and TUNEL assay to measure the ability of Sal to increase apoptosis.

KEY RESULTS

Sal increased DNA breakage and phosphorylated levels of p53 and H2AX. Sal also induced the formation of DNA foci with pH2AX and 53BP1. Furthermore, Sal increased the sensitivity of cancer cells to the apoptotic effects of DOX or ETO. We found that pH2AX, pBRCA1, p53BP1 and pChk1 levels were greatly increased after co-treatment of Sal with DOX or ETO. The level of anti-apoptotic p21 protein was increased by DOX or ETO but decreased by Sal, which increased proteasome activity.

CONCLUSIONS AND IMPLICATIONS

This is the first study to report that Sal increases DNA damage, and this effect plays an important role in the increased apoptosis caused by Sal. Overall, we demonstrated that the ability of Sal to sensitize cancer cells to the effects of DOX or ETO is associated with an increase in DNA damage and a decrease in anti-apoptotic protein p21 levels. These results may contribute to the development of Sal-based chemotherapy for cancer patients receiving DOX or ETO treatment.

Abbreviations

DOX, doxorubicin; ETO, etoposide; FACS, fluorescence-activated cell sorting; PI, propidium iodide; Sal, salinomycin

Introduction

Both doxorubicin (DOX) and etoposide (ETO) are widely used for the treatment of numerous types of cancers (Biganzoli *et al.*, 2004; Dillman *et al.*, 2005; Llovet, 2005). They cause DNA damage through the inhibition of topoisomerases, which results in apoptosis (Nitiss, 2002; Bidwell and Raucher, 2006; Hsiao *et al.*, 2008). Because patients develop resistance to these drugs, research is being undertaken to increase the apoptosis associated with DOX and ETO treatment with the aim of providing better treatment of patients (Glück, 2005; Humber *et al.*, 2007; Thomadaki and Scorilas, 2008; Chakraborty *et al.*, 2009;). One approach has been to involve the combined use of both drugs with tumour necrosis

factor-α-related apoptosis-inducing ligand, aromatase inhibitors, Herceptin or radiation (Van Valen *et al.*, 2003; Kaklamani and Gradishar, 2005; Crivellari *et al.*, 2007; Inskip *et al.*, 2009). Identifying the mechanism(s) of cell sensitization to DOX and ETO is an important step in the development of new pharmacological treatments of cancers.

Salinomycin (Sal) was originally used to kill bacteria, fungi and parasites (Miyazaki *et al.*, 1974; Mahmoudi *et al.*, 2006). However, more recently it has been shown to inhibit tumour stem cells, and is currently considered a cancer chemopreventative agent (Gupta *et al.*, 2009). Furthermore, Sal can overcome drug resistance in human cancer cells (Fuchs *et al.*, 2009; 2010). Thus, these characteristics of Sal have the potential to be exploited to increasingly sensitize cells to anticancer drugs as part of combination chemotherapy.

Hence, we investigated the effect of Sal on the reduced viability of cancer cells induced by DOX or ETO treatment. Because the anticancer drugs DOX and ETO are DNA-damaging agents, we determined whether Sal can sensitize cancer cells to the effects of these drugs by increasing DNA damage or by another mechanism.

Methods

Reagents

An aqueous solution of DOX (Boryung Pharmacy, Seoul, Korea) was obtained from the National Cancer Center in South Korea. Sal and ETO were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethylsulphoxide (DMSO). The diluent did not induce DNA damage or cytotoxic effects in a cancer cell line (Figure S1A, B and C). The proteasome inhibitors MG132 and epoxomicin were purchased from Calbiochem (La Jolla, CA, USA).

Antibodies

Antibodies against X-linked inhibitor of apoptosis protein (IAP), Bcl-X_L, p53, c-IAP1, p21, 53BP1, pBRCA1, phosphorylated-serine 20-p53, cleaved poly ADP ribose polymerase (C-PARP) and Bax were from Cell Signaling Technology (Danvers, MA, USA). Antibodies against Survivin, c-IAP2, Cyclin B1, Bcl-2, Cyclin A, pRb, Cyclin E, Cdk4, Rb, p27, p16, p18 and glyceraldehyde 3-phosphate dehydrogenase were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against Cyclin D1 was from Biosource (Camarillo, CA, USA) and that against β-actin was from Sigma-Aldrich. Antibodies against Cdk2, p53BP1 and pH2AX were obtained from Abcam (Cambridge, UK).

Cell culture

Previously described human cancer cell lines (Kim *et al.*, 2009; 2010) were used. MCF7 and HepG2 were obtained from the American Type Culture Collection (Manassas, VA, USA). The uterine sarcoma cell line, MES-SA and its multidrug-resistant subline, MES-SA/Dx5 (Wang and Arriaga, 2008) were acquired from Dr Kim Seong Hwan (Korea Research Institute of Chemical Technology). MDA-MB231 was acquired from Dr Ye Sang-Kyu at Seoul National University. The Hs578T breast cancer cell line was obtained from the Korean Cell Line Bank (Seoul, Korea). All cell lines were cultured in Dulbecco's Modified Eagle's Medium or RPMI1640 containing 10% fetal bovine serum, 100 $U \cdot mL^{-1}$ penicillin, and 100 $\mu g \cdot mL^{-1}$ streptomycin (WelGENE, Daegu, Korea).

Cell proliferation assay

Cell proliferation was determined in a colorimetric assay using 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium kit (Promega, Madison, WI, USA) as previously described (Kim *et al.*, 2009; 2010). The assay is a method for determining the number of viable cells in proliferation or cytotoxicity. All assays were performed at least in triplicate with two independent experiments.

Fluorescence-activated cell sorting (FACS) analysis

Cells grown in 60 mm-diameter dishes were treated for the prescribed times. The cells were dislodged by trypsin, and the entire volume was centrifuged to recover the dislodged cells. The pelleted cells were washed thoroughly with phosphate-buffered saline (PBS), suspended in 75% ethanol for at least 1 h at 4°C, washed with PBS, and resuspended in cold propidium iodide (PI) staining solution (100 $\mu g \cdot mL^{-1}$ Rnase A and 10 $\mu g \cdot mL^{-1}$ PI in PBS) for 40 min at 4°C. The stained cells were analysed for relative DNA content using a FACSCalibur flow cytometry system (BD, Franklin Lakes, NJ, USA).

Annexin V analysis

Annexin V analysis was conducted by Annexin V-fluorescein isothiocynate (FITC) staining using a commercial Annexin V-FITC kit (BD Bioscience, San Diego, CA, USA). Briefly, cells were harvested as described for FACS analysis. Cells (1×10^5) in 100 μL binding buffer received 5 μL of Annexin V-FITC and 5 μL of PI (50 $\mu g \cdot mL^{-1}$), and they were incubated for 15 min at room temperature. The stained cells were analysed using a FACSCalibur flow cytometry system.

Terminal transferase dUTP nick end labelling (TUNEL) assay

Terminal transferase dUTP nick end labelling analysis was conducted with FITC-anti-BrdU staining using a commercial APO-BRDU kit (Phoenix Flow Systems, San Diego, CA, USA). Briefly, cells were harvested as described above for FACS analysis. Cells (1×10^6) were fixed in 1% paraformaldehyde in PBS (pH 7.4). They were added to 5 mL of 70% ethanol and stored for 20 h at –20°C. The cells were harvested by centrifugation; washed in buffer; resuspended in DNA labelling solution consisting of TdT Reaction Buffer, TdT Enzyme and BrdUTP; and incubated for 60 min at 37°C. The cells were rinsed with 1.0 mL of rinse buffer, treated with FITC-anti-BrdU antibody solution and incubated for 30 min at RT. They were added to 0.5 mL of PI/RNase A staining solution and incubated for 15 min at RT. The stained cells were analysed using a FACSCalibur flow cytometry system.

Western blot analysis

All the proteins were extracted using a previously described trichloroacetic acid method (Hwang *et al.*, 2007). Briefly, proteins were pelleted by centrifugation after addition of 20% trichloroacetic acid and were resuspended in 1 M Tris-HCl

(pH 8.0). The proteins were subjected to Western blot analysis as described previously (Hwang *et al.*, 2007; Kim *et al.*, 2009).

Comet assay

Residual DNA damage following DOX or Sal treatment was detected using the CometAssay single gel electrophoresis assay following the manufacturer's protocol (Trevigen, Gaithersburg, MD, USA). Briefly, cells grown in 6-well plates to approximately 30% confluence were treated for 4 h with DOX or Sal. The treated cells were trypsinized and pelleted by centrifugation. The cells were washed and resuspended in 200 µL of ice-cold 1X PBS. The cell suspension was mixed 1:10 ($v \cdot v^{-1}$) with low melting point agar at 42°C and immediately pipetted onto the CometSlide™ area. Slides were incubated at 4°C in the dark for 30 min, immersed in chilled lysis solution and incubated again at 4°C for 30 min. Slides were placed in a horizontal electrophoresis chamber and electrophoresed with buffer (0.3 N NaOH, 1 mM EDTA) at 1 $V \cdot cm^{-1}$ for 20 min. Samples were dried and stained with ethidium bromide. Comet tails were imaged using a model DM 2500 fluorescent microscope (Leica, Wetzlar, Germany) and quantified by Komet 5.5 software (Andor Technology, Belfast, UK). A minimum of 50 cells were scored per treatment. All experiments were performed independently at least three times.

Immunocytochemistry

Immunocytochemistry was performed as previously described (Hwang *et al.*, 2007; Kim *et al.*, 2010). Briefly, permeabilized cells were incubated with anti-phosphorylated H2AX or 53BP1 primary antibody overnight at 4°C. They were re-incubated with FITC-conjugated secondary antibody (Zymed, South San Francisco, CA, USA), fluorescently labelled Alexa Fluor 568 secondary antibody (Molecular Probes, Carlsbad, CA, USA), and 4′-6-diamidino-2-phenylindole for 1 h at 37°C. The stained cells were subsequently examined using an inverted fluorescence microscope (Hwang *et al.*, 2007; Kim *et al.*, 2010).

Statistical analysis

The data are presented as mean ± SD. Statistical analysis was conducted using Student's *t*-test and one-way analysis of variance (ANOVA) followed by multiple-comparison test. Results were considered to be statistically significant compared with the control (*) for $P < 0.05$.

Results

Sal increases DNA breakages and damage-response proteins

To identify the mechanism of Sal sensitization of cancer cells, we investigated whether Sal increases DNA breakage in cancer cells using a Comet assay (Devlin *et al.*, 2008). As seen after DOX treatment (positive control), we found that Sal also increased DNA breakage in a dose-dependent manner (Figure 1A and B). Further, we investigated the ability of Sal to increase levels of DNA damage response proteins (Kawabe, 2004; Solier *et al.*, 2009). We found that Sal increased pH2AX levels and p53 levels with phosphorylated serine 20 (Figure 1C) and reduced Cyclin D1 (Figure 1C), suggesting a positive correlation between cellular arrest and increased DNA damage. In addition, using immunocytochemistry, we observed that Sal increased DNA foci for both pH2AX and 53BP1 in (Figure 1D), indicating DNA damage and breakage (Kawabe, 2004; Solier *et al.*, 2009). Collectively, our results indicated that Sal has the ability to increase DNA damage.

Co-treatment with Sal reduces viability of DOX-treated cells

Cell viability of Hs578T breast cancer cells was determined by use of a cell proliferation assay. Use of low (5 µM; Sal-5), moderate (10 µM; Sal-10) and high (20 µM; Sal-20) concentrations of Sal revealed that the viability of cells decreased in a concentration-dependent manner (Figure 2A), consistent with an effective sensitization of Hs578T cells by Sal. Western blot analysis was performed to determine levels of cell cycle-related proteins, which have been shown to be important for cellular proliferation and growth (Malumbres and Barbacid, 2009). Of the cell cycle proteins analysed, Sal effectively reduced Cyclin D1 in both Hs578T and MDA-MB231 cells (Figure 2B), suggesting that Sal commonly targets Cyclin D1 to reduce cell viability.

Also we tested whether co-treatment with Sal reduces the viability of the DOX-treated cancer cells. The DOX concentration was chosen based on a previous *in vitro* analysis (Kim *et al.*, 2010). Reduced viability was evident for DOX-treated cells in the presence of all the concentrations of Sal tested compared to corresponding cells treated solely with either DOX or Sal (Figure 2A). Cancer cells can display drug resistance in confluence-dependent cultures (Fang *et al.*, 2007). Consistent with this result, viability was less affected in high-density cell populations treated with DOX than in low-density cell populations (Figure 2C). In contrast, co-treatment with DOX and Sal or treatment with Sal alone reduced viability in high- and low-density cell populations in a similar manner (Figure 2C). These results implicated Sal in overcoming confluence-dependent resistance.

Additional experiments were conducted to determine if the findings in HS578T breast cancer cells extended to other cell lines originating from different organs. Four cell lines (HepG2, MES-SA/Dx5, MCF7 and MDA-MB231) were tested. MES-SA/Dx5 and MCF7 cells showed a marked reduction of viability upon co-treatment with DOX and Sal, but HepG2 and MDA-MB231 cells only exhibited a slight reduction (Figure 2D). Hence, the results obtained by use of a cell proliferation assay suggest that the role of Sal in DOX sensitization is cell line-dependent.

Co-treatment with Sal increases the apoptosis of DOX- or ETO-treated cells

Co-treatment of DOX with Sal markedly increased the pre-G1 period at 48 h in Hs578T cells (Figure 3A), suggesting that apoptosis was increased compared with treatment with DOX or Sal alone. A higher Sal concentration (10 µM) increased the apoptotic pre-G1 phase even more in the DOX-treated cells (Figure 3A). Microscopic examination of Hs578T cells showed that untreated cells grew and divided in a side-by-side fashion; whereas, cells treated with drugs for 24 h failed to grow (Figure S2A). The degree of cellular separation or

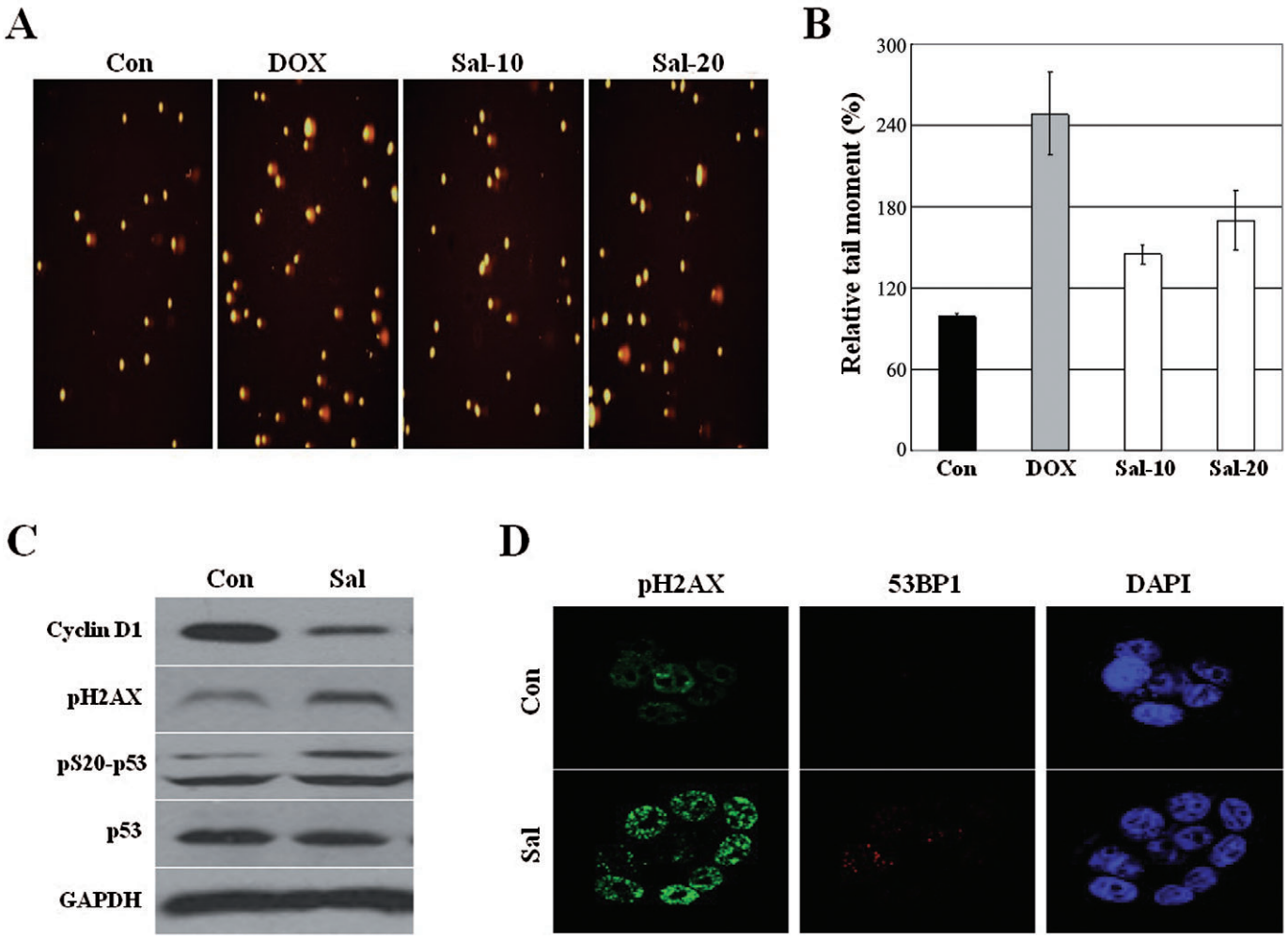

Figure 1

Salinomycin (Sal) increases DNA breakage and damage. (A–B) Hs578T cells were grown and stimulated for 4 h with 3.5 μM doxorubicin (DOX), 10 μM Sal (Sal-10), 20 μM Sal (Sal-20), or were untreated (Con). The Comet assay was performed as described in *Methods*. (A) Representative picture of Comet assay results in (B). (C) Hs578T cell extracts were collected 6 h after treatment with 10 μM Sal or no treatment (Con). The cells were used for Western blot analyses using antibodies against Cyclin D1, pH2AX, phosphorylated-serine 20 (pS20)-p53, p53 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (D) Hs578T cells were grown to 50% confluence and stimulated for 3 h with 10 μM Sal. Untreated cells served as control (Con). Immunocytochemistry was performed as described in *Methods*. Immunocytochemical staining for pH2AX is shown by green fluorescence and 53BP1 is shown by rhodamin fluorescence. 4′-6-diamidino-2-phenylindole (DAPI) nuclear staining (blue) is also shown. As a positive control, etoposide (ETO) treatment produced foci formation (Figure S2B).

detachment increased in cells co-treated with DOX and Sal relative to cells treated with either DOX or Sal alone (Figure S2A). Collectively, these observations are consistent with the suggestion that Sal increases the apoptosis of DOX-treated cells.

Etoposide is an anticancer drug that, similar to DOX, has the ability to induce DNA breakage (Nitiss, 2002; Hsiao *et al.*, 2008). The effects of ETO on the cell lines (Figure 2C and D) used in the previous experiments for co-treatment of DOX with Sal (Hs578T, HepG2, MES-SA/Dx5, MCF7 and MDA-MB231) were investigated. An ETO concentration of 50 μM was selected based on the results of a cell proliferation assay (Figure S1D), which showed approximately 50% decreased cellular viability using a linear concentration range of ETO. In all five cancer cell lines tested, the viability was decreased after treatment with ETO plus Sal compared with corresponding cells treated with either ETO or Sal alone (Figure 3B). Because HepG2 cells had higher viability than other cell lines after co-treatment with Sal and DOX (Figure 2D), it is notable that the ability of Sal to reduce the viability of ETO-treated cancer cells was also cell line-dependent (Figure 3B).

In addition, we used Annexin V staining to investigate the ability of co-treatment with ETO and Sal to increase Hs578T cell apoptosis. Comparison of single treatments to co-treatment revealed increased Annexin V staining in Sal plus ETO-treated cells (Figure 3C). Also, because both ETO and Sal cause DNA damage, we investigated the ability of Sal to increase DNA damage in ETO-treated cells. Immunocytochemistry demonstrated that Sal increased the number of pH2AX foci in ETO-treated cells (Figure S2B), indicating that

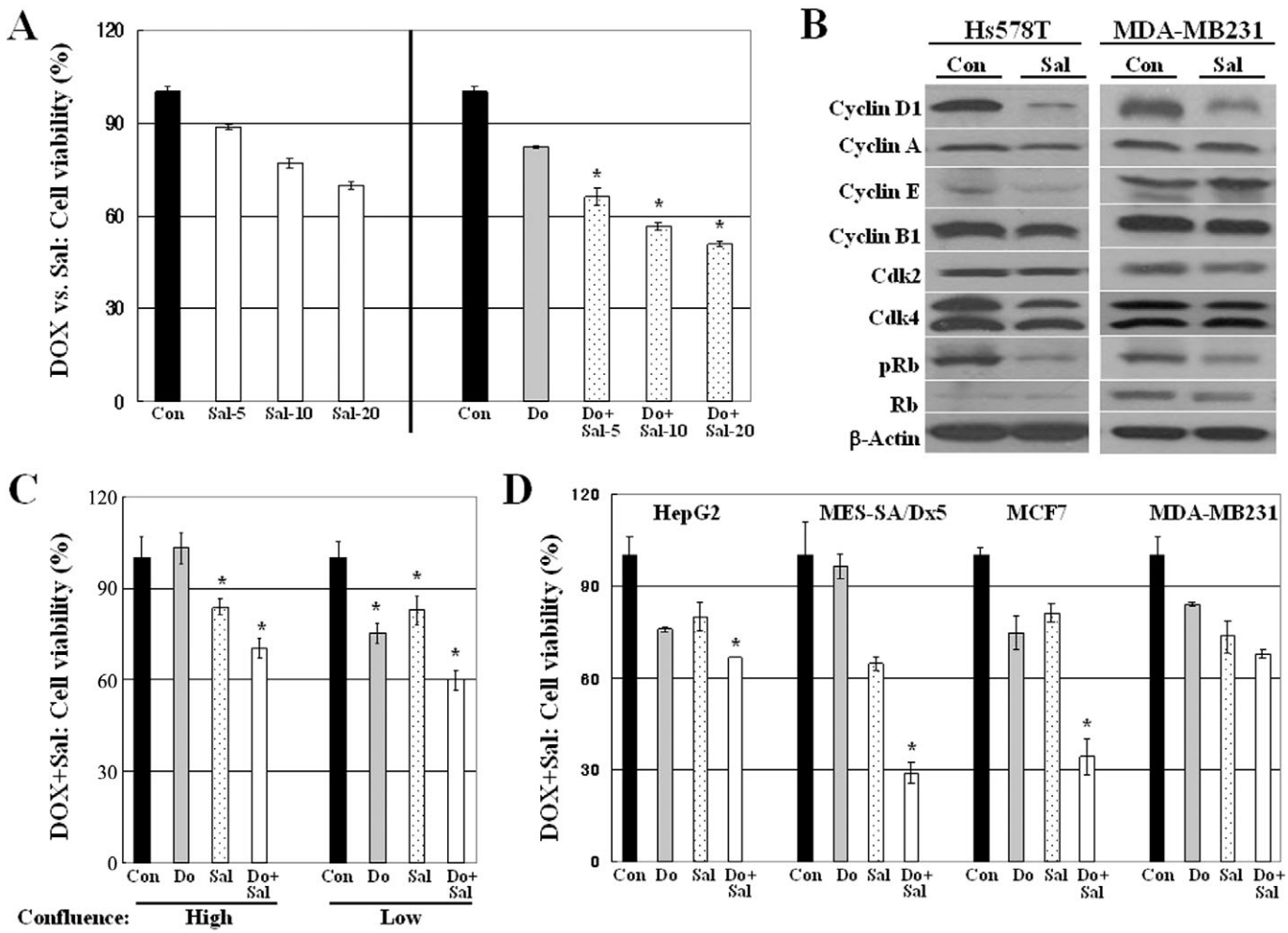

Figure 2

Salinomycin (Sal) sensitizes doxorubicin (DOX)-treated cancer cells. (A) Hs578T cells were plated in 96-well plates and grown to 30%–40% confluence. Left panel: cells were stimulated for 48 h with 5 μM Sal (Sal-5), 10 μM Sal (Sal-10), 20 μM Sal (Sal-20) or remained untreated (Con). Right panel: cells were stimulated for 48 h with 3.5 μM DOX (Do), 5 μM Sal with 3.5 μM DOX (Do + Sal-5), 10 μM Sal with 3.5 μM DOX (Do + Sal-10), 20 μM Sal with 3.5 μM DOX (Do + Sal-20), or remained untreated (Con). The cell proliferation assay was performed as described in *Methods*. (B) Hs578T and MDA-MB231 cell extracts were collected 20 h after treatment with 10 μM Sal, or they remained untreated (Con). The cells were used for Western blot analyses using antibodies against Cyclin D1, Cyclin A, Cyclin E, Cyclin B1, Cdk2, Cdk4, pRb, Rb and β-actin. (C) Hs578T cells were plated in 96-well plates and grown to 30–40% confluence for low-density (Low) or 70%–80% for high-density (High) populations. The cells were stimulated for 48 h with 3.5 μM DOX (Do), 10 μM Sal (Sal), 10 μM Sal with 3.5 μM DOX (Do + Sal), or remained untreated (Con). Viability was assessed using a cell proliferation assay. (D) HepG2, MES-SA/Dx5, MCF7, and MDA-MB231 cells were plated in 96-well plates and grown to 30%–40% confluence. The cells were stimulated for 48 h with 3.5 μM DOX (Do), 10 μM Sal (Sal), 10 μM Sal with 3.5 μM DOX (Do + Sal), or they remained untreated (Con). Viability was assessed as described in (A). The data represent mean ± SD of at least 3 experiments; $*P < 0.05$ compared with the corresponding control.

Sal increases the cytotoxic effects of DNA damage in ETO-treated cells.

Co-treatment of drug-resistant MES-SA/Dx5 cells with Sal plus DOX or ETO increases apoptosis with DNA damage

To further analyse the reduced viability of MES-SA/Dx5 cells after co-treatment with Sal and either DOX or ETO (Figures 2D and 3B), apoptosis of the resistant cell line was assessed by FACS analysis, C-PARP, Annexin V staining and TUNEL assay (Chetty *et al.*, 2007). Co-treatment of DOX or ETO with Sal greatly increased the pre-G1 region in MES-SA/Dx5 cells (Figure 4A). Comparison of the pre-G1 cell cycle interval in cells treated with Sal and DOX or ETO revealed increased apoptosis in the presence of DOX. In addition, co-treatment of DOX or ETO with Sal increased C-PARP production (Figure 4B). Further, Sal increased both Annexin V and TUNEL staining in ETO-treated cells (Figure 4C and D), consistent with the idea that co-treatment with Sal increased both early and late apoptotic events for ETO-treated MES-SA/Dx5 cells. Because the red colour of DOX overlapped the

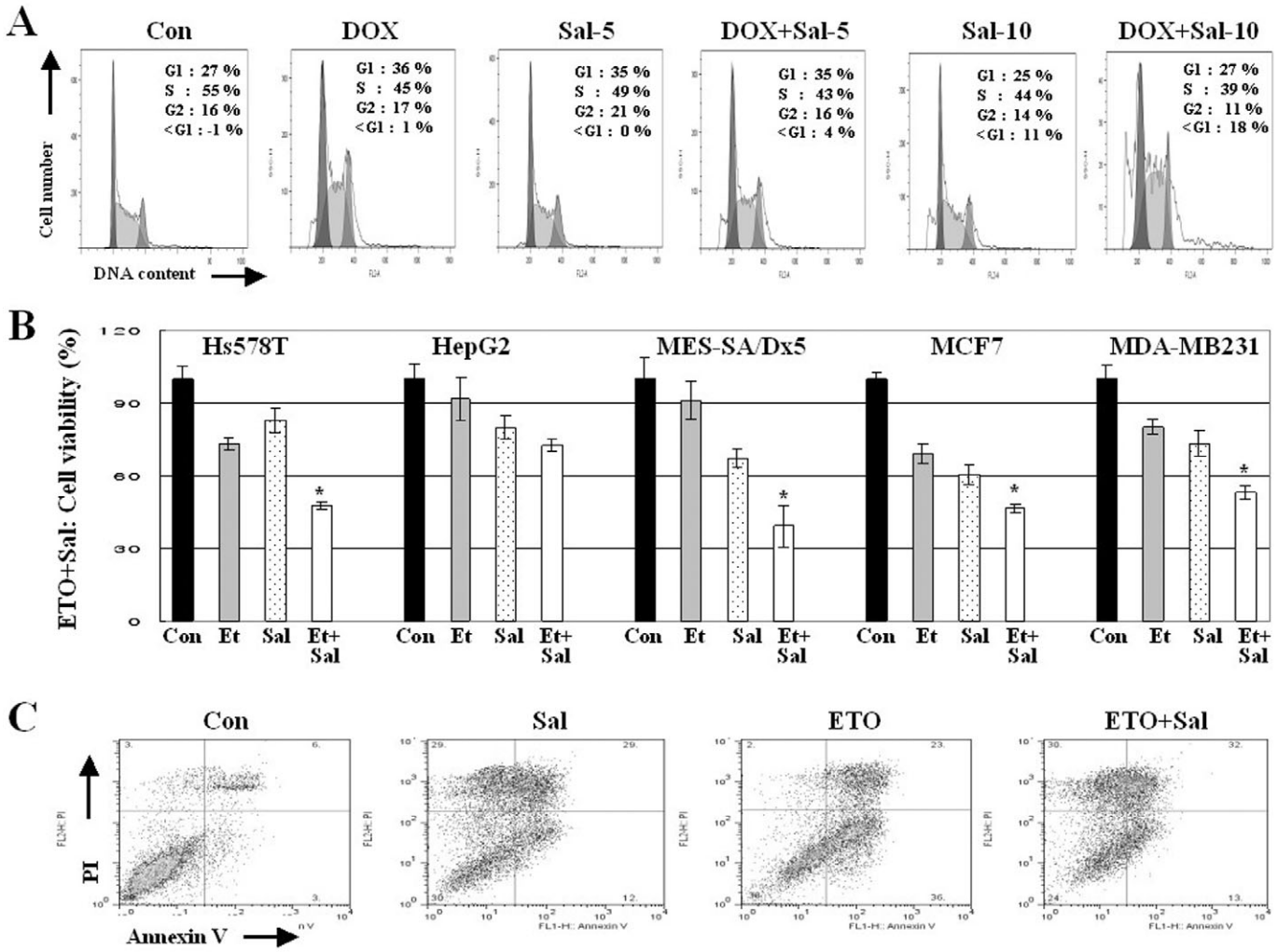

Figure 3

Co-treatment with Sal increases apoptosis of doxorubicin (DOX)- or etoposide (ETO)-treated cells. (A) Hs578T cells were stimulated for 48 h with 3.5 μM DOX, 5 μM salinomycin (Sal) (Sal-5), 10 μM Sal (Sal-10), 5 μM Sal with 3.5 μM DOX (DOX + Sal-5), 10 μM Sal with 3.5 μM DOX (DOX + Sal-10), or they remained untreated (Con). Fluorescence-activated cell sorting (FACS) analysis was performed with all extracts 48 h after each treatment. (B) Hs578T, HepG2, MES-SA/Dx5, MCF7, and MDA-MB231 cells (grown as described in Figure 2A) were stimulated for 48 h with 50 μM ETO (Et), 10 μM Sal (Sal), 10 μM Sal with 50 μM ETO (Et + Sal), or remained untreated (Con). Viability was assessed using a cell proliferation assay, as described in Figure 2A. (C) Hs578T cells were stimulated for 48 h with 50 μM ETO, 10 μM Sal, 10 μM Sal with 50 μM ETO (ETO + Sal), or remained untreated (Con). Annexin V analyses was performed. The data represent mean ± SD of at least 3 experiments; *$P < 0.05$ compared with the corresponding control.

wavelength of Annexin V and TUNEL staining during the analyses, this approach was not suitable for analysis of DOX-treated cells.

Because Sal, DOX and ETO cause DNA damage, we tested the ability of Sal to increase DNA damage-related proteins in cells treated with DOX or ETO. We assessed the influence of co-treatment with DOX or ETO and Sal on the activation status of DNA damage-related proteins (e.g. pH2AX, p53BP1, pBRCA1, pChk1). These factors have been shown to be important representative proteins for characterizing the degree of DNA damage (Kawabe, 2004; Devlin *et al.*, 2008; Solier *et al.*, 2009). Increased phosphorylation of these proteins indicates greater DNA breakage and damage (Kawabe, 2004; Solier *et al.*, 2009). For example, NU7026 is an inhibitor of DNA-PK, which is involved in double strand break repair (Willmore *et al.*, 2004). The NU7026 treatment increases double strand break in ETO-treated cells (Willmore *et al.*, 2004). We confirmed that co-treatment of cells with NU7026 plus DOX or ETO increased pH2AX, p53BP1, pBRCA1 and pChk1 protein level after 3 h (Figure S3A). Co-treatment of cells with DOX or ETO and Sal for 3 or 6 h was found to increase their pH2AX, pBRCA1, pChk1 and p53BP1 levels (Figure S3B). The combination of DOX with Sal appeared to have a greater effect than ETO with Sal. Increased protein levels were found after both 3 and 6 h with the Sal plus DOX-treated cells, but with the Sal plus ETO-treated cells the increases in protein were delayed; most were observed after 6 h. Because the damage protein levels were increased after

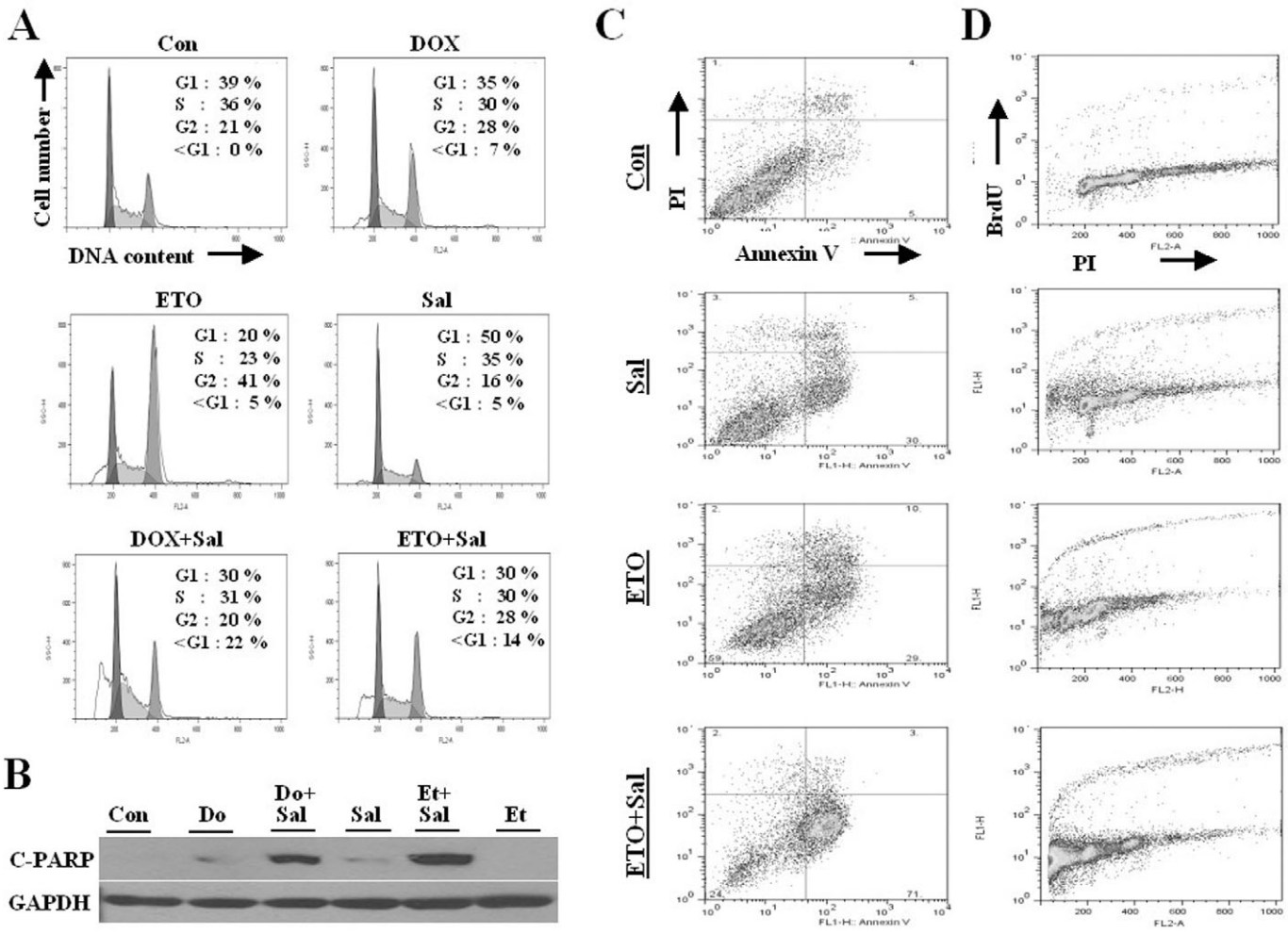

Figure 4

Co-treatment of doxorubicin (DOX) or etoposide (ETO) with Sal increases apoptosis of drug-resistant MES-SA/Dx5 cells. (A) MES-SA/Dx5 cells were stimulated for 48 h with 3.5 μM DOX, 50 μM ETO, 10 μM Sal, 10 μM Sal with 3.5 μM DOX (DOX + Sal), 10 μM Sal with 50 μM ETO (ETO + Sal), or they remained untreated (Con). Fluorescence-activated cell sorting analysis was performed for all extracts 48 h after the particular treatment. (B) MES-SA/Dx5 cell extracts were collected 6 h after treatment with 3.5 μM DOX (Do), 10 μM Sal with 3.5 μM DOX (Do + Sal), 50 μM ETO (Et), 10 μM Sal (Sal), 10 μM Sal with 50 μM ETO (Et + Sal), or they remained untreated (Con). The cells were used for Western blot analyses using antibodies against cleaved poly ADP ribose polymerase (C-PARP) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (C–D) MES-SA/Dx5 cells were stimulated for 48 h with 50 μM ETO, 10 μM Sal, 10 μM Sal with 50 μM ETO (ETO + Sal), or they remained untreated (Con). Annexin V (C) and TUNEL analyses (D) were performed. PI, propidium iodide.

3 h when cells were co-treated with NU7026 and either ETO or DOX (Figure S3A), we assumed that Sal has different DNA damage and response pathways than NU7026. Collectively, the results indicate that Sal increased apoptosis with increased DNA damage.

Co-treatment with Sal reduces p21 levels by increasing proteasome activity in DOX- or ETO-treated cell lines

We assessed whether co-treatment of DOX or ETO with Sal influenced the levels of anti-apoptotic proteins, cell cycle proteins or tumour suppressors, all of which are important for the response to anticancer drugs (Sherr and McCormick, 2002; Danson *et al.*, 2007; Maddika *et al.*, 2007; Abukhdeir and Park, 2008; Mita *et al.*, 2008). This experiment was based on the assumption that the identified proteins could be potential targets for reducing viability after co-treatment of DOX or ETO with Sal. We tried to determine if co-treatment of Hs578T cells with Sal plus DOX or ETO for 20 h substantially reduced protein levels compared with treatment with DOX or ETO alone. Cyclin D1, p16, p21, p27 and p53 proteins were reduced in cells co-treated with Sal and either DOX or ETO (Figure 5A). Anti-apoptotic proteins (Bcl-2 and IAP families) were not reduced by the co-treatment. Treatment with Sal (alone) reduced Cyclin D1 among the cell cycle-related proteins in both Hs578T and MDA-MB231 cells (Figure 2B), and it reduced the levels of p16, p21 and p27 (Figure 5A). The same experiment performed in MDA-MB231 cells demonstrated that, although Cyclin D1, p16, p27 and

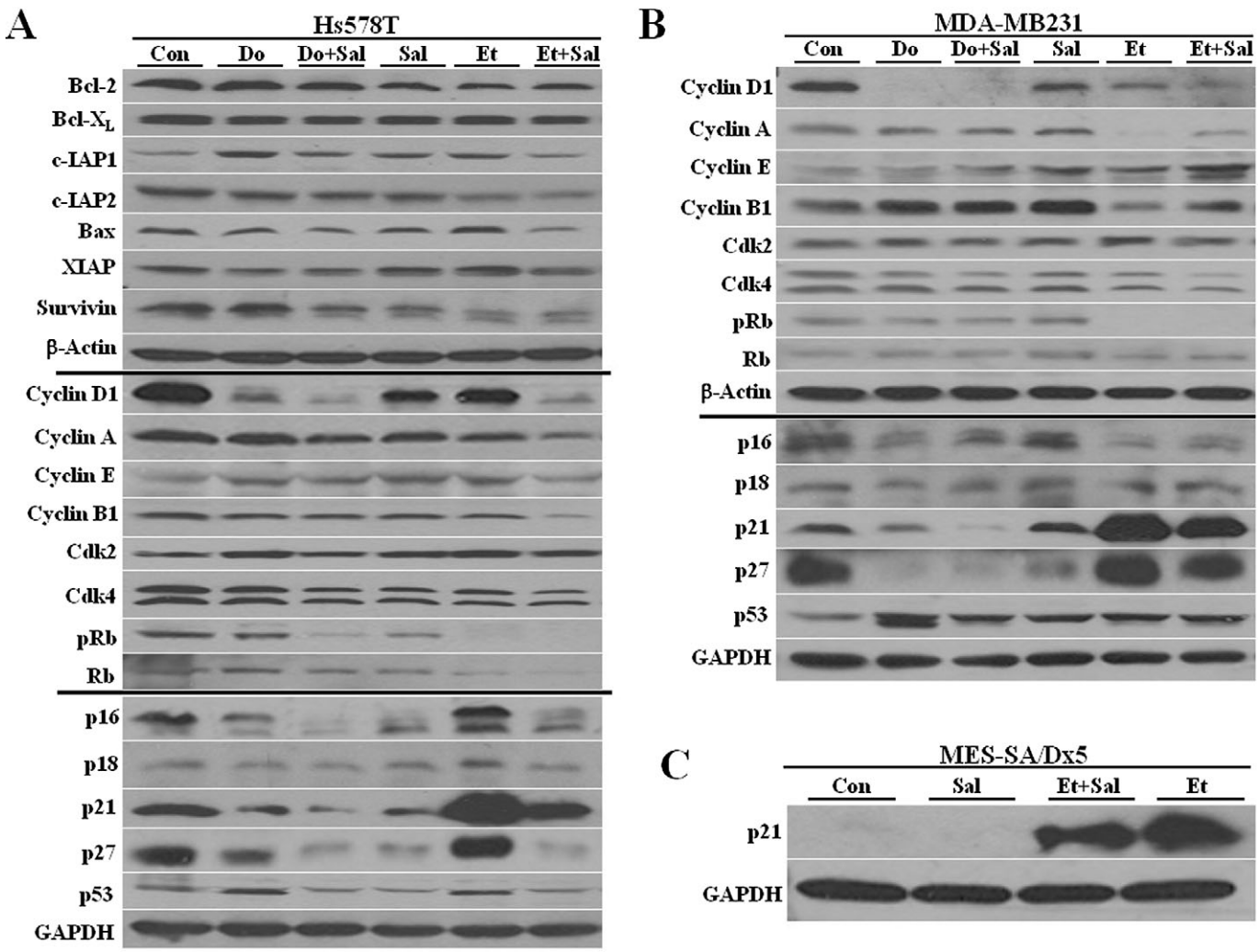

Figure 5

Co-treatment with salinomycin (Sal) reduces p21 levels in doxorubicin (DOX)- or etoposide (ETO)-treated cell lines. (A–C) Hs578T, MDA-MB231 and MES-SA/Dx5 cell extracts were collected 20 h after treatment with 3.5 μM DOX (Do), 10 μM Sal with 3.5 μM DOX (Do + Sal), 50 μM ETO (Et), 10 μM Sal (Sal), 10 μM Sal with 50 μM ETO (Et + Sal), or they remained untreated (Con). The cells were used for Western blot analyses using antibodies against Bcl-2, Bcl-X_L, c-IAP1, c-IAP2, Bax, X-linked inhibitor of apoptosis protein (XIAP), Survivin, β-actin, Cyclin D1, Cyclin A, Cyclin E, Cyclin B1, Cdk2, Cdk4, pRb, Rb, p16, p18, p21, p27, p53, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

p53 proteins were not reduced by the co-treatment, the level of p21 was effectively reduced by co-treatment of Sal with either DOX or ETO (Figure 5B).

The increased p21 level in the presence of ETO was effectively reduced by Sal in Hs578T (Figure 5A), MDA-MB231 (Figure 5B) and MES-SA/Dx5 cells (Figure 5C) at 20 h. Hence, further experiments were carried out to determine whether Sal reduces the DOX-mediated increase of p21 levels and whether the increased p21 levels observed in the presence of DOX or ETO could be reduced at relatively early time after Sal treatment. After 3 h or 6 h of treatment, the increased p21 levels observed in the presence of DOX or ETO were almost completely diminished by co-treatment with Sal in the three cell lines (Figure 6A and B). These observations are consistent with the suggestion that the effects of Sal begin early and persist. In addition, we analysed the manner by which Sal decreases p21 protein levels in ETO-treated cells. We found that the p21 protein levels reduced by Sal were recovered by co-treatment with proteasome inhibitors (e.g. MG132 or epoxomicin) (Figure 6C), suggesting that Sal increases proteasome activity to reduce p21 levels.

Discussion

The aim of the present study was to identify and improve conditions for increasing sensitivity of cancer cells to DOX or ETO. Sal has recently been shown to be a potential anticancer drug for cancer stem cells (Gupta *et al.*, 2009). Furthermore, Sal has been found to overcome the resistance of human cancer cells to apoptosis, including leukaemia stem cell-like cells (Fuchs *et al.*, 2009; 2010). However, the mechanism by

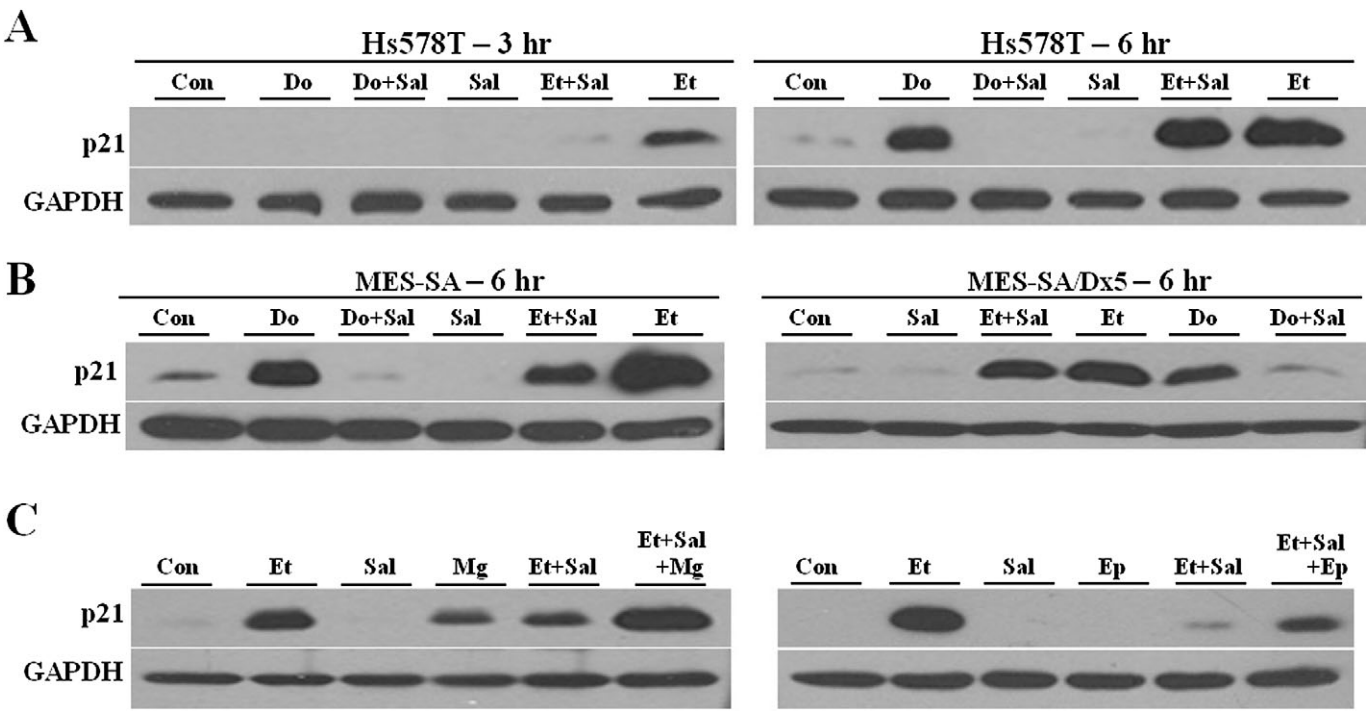

Figure 6

Co-treatment with Sal reduces increased p21 levels through increased proteasome activity in doxorubicin (DOX)- or etoposide (ETO)-treated cells. (A–B) Hs578T, MES-SA, and MES-SA/Dx5 cell extracts were collected 3 h or 6 h after treatment with 3.5 μM DOX (Do), 10 μM salinomycin (Sal) with 3.5 μM DOX (Do + Sal), 50 μM ETO (Et), 10 μM Sal (Sal), 10 μM Sal with 50 μM ETO (Et + Sal), or they remained untreated (Con). The cells were used for Western blot analyses using antibodies against p21 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (C) Hs578T cell extracts were collected 6 h after treatment with 50 μM ETO (Et), 10 μM Sal (Sal), 10 μM Sal with 50 μM ETO (Et + Sal), 5 μM MG132 after 5 h treatment of 10 μM Sal with 50 μM ETO (Et + Sal + Mg), 0.1 μM epoxomicin with 10 μM Sal and 50 μM ETO (Et + Sal + Ep), 5 μM MG132 (Mg), 0.1 μM epoxomicin (Ep), or they remained untreated (Con). The cells were used for Western blot analyses using antibodies against p21and GAPDH.

which it increases apoptosis has not been investigated in detail. Here, we investigated the manner by which Sal increases apoptosis in cancer cells and whether or not Sal sensitizes cells to the effects of DOX or ETO treatment. These results demonstrate that Sal can be used effectively for combination chemotherapy with DOX or ETO to reduce the viability of cancer cells. We also demonstrated for the first time that Sal induces sensitization of cancer cells by increasing DNA damage. Because both DOX and ETO have a similar mechanism and cause DNA breakage (Nitiss, 2002), Sal may well be capable of sensitizing cells when administered with a drug that is capable of inducing DNA breakage. Our findings also suggest several mechanisms by which Sal sensitizes cells to the effects of treatment with DOX or ETO.

Most importantly, we demonstrated that DNA breakage, pH2AX protein levels and DNA foci formation increased greatly after a single treatment with Sal, indicating that Sal increases DNA breakage. The ability of Sal to damage DNA provides important clues for its sensitization mechanism. We found that DNA foci formation of pH2AX increased with co-treatment of ETO with Sal, suggesting that the sensitization of cells to the effects of ETO induced by Sal positively correlates with increased DNA damage. Previously, it was shown that the increase in cellular death induced by co-treatment of NU7026 with DOX or ETO correlated an increase in DNA damage or a delay in the DNA repair systems (Willmore *et al.*, 2004). We found that co-treatment with NU7026 up-regulates DNA damaging proteins (e.g. pBRCA1, p53BP1, pChk1). These proteins also increased with Sal co-treatment, suggesting that Sal might also inhibit DNA-PK, thus increasing DNA damage and inducing apoptosis. It appears that DOX has a greater ability to damage DNA than ETO when combined with Sal, because the DOX combination increased proteins earlier than the ETO combination. Because NU7026 increased damage-related protein levels at 3 h and Sal at 6 h in ETO-treated cells, we suggest that Sal operates via a different mechanism than NU7026 to increase DNA damage. The results also demonstrate that in combination chemotherapy DOX is more efficiently combined with Sal than is ETO, as shown by the increased pre-G1 interval in DOX co-treated cells. Therefore, we conclude that DNA damaging ability of Sal can be more effectively combined with DOX than ETO. Also, these results indicate that Sal sensitizes DOX- or ETO-treated cells via different pathways of increased DNA damage, although both DOX and ETO act similarly as DNA breaking agents. Collectively, the study supports the belief that Sal sensitizes DOX- or ETO-treated cancer cells by mechanisms that generally target DNA breakages and increase DNA damage-related proteins (e.g. pH2AX, p53BP1, pBRCA1, pChk1). Because Sal is an efflux pump

p-glycoprotein inhibitor (Fuchs *et al.*, 2009; 2010; Riccioni *et al.*, 2010), it is possible that inhibition of p-glycoprotein by Sal increased apoptosis with increased duration of DOX and ETO in the cells. Sal reduced viability in p-glycoprotein inhibitor-treated cells (Figure S4A and B) and has been shown to increase DNA damage in radiation-treated cells (manuscript in preparation), suggesting that Sal sensitizes cancer cells by direct DNA damage. But, considering that Sal did not increase DNA damage-related protein in p-glycoprotein inhibitor-treated cells (data not shown), Sal sensitization of DOX- or ETO-treated cells may involve both DNA damage and p-glycoprotein inhibition.

Salinomycin reduces the viability of breast, liver and uterine cancer cells treated with DOX or ETO, suggesting that Sal is capable of targeting cancers originating from various organs when combined with DOX or ETO. However, some cell lines were not affected by the co-treatment with Sal, suggesting that the ability of Sal to sensitize cancer cells to treatment with DOX and ETO depends on the cell type. In a previous study (Gupta *et al.*, 2009), Sal was shown to reduce the viability, by sensitizing them, of cancer stem cells. It is possible that cell lines that are highly sensitized by Sal co-treatment exhibit more stem cell properties. Elucidating these characteristics could be used to optimize Sal co-treatments in cancer patients. In addition, drug-resistant MES-SA/Dx5 cells were affected more in the pre-G1 region of apoptosis than the relatively drug-sensitive Hs578T cell line. This suggests that Sal co-treatment also effectively increases apoptosis in drug-resistant cells. Increased cell density in cell culture model systems causes resistance to anticancer drugs; likewise, *in vivo* high-density, rapidly growing solid tumours exhibit resistance to anticancer drugs (Fang *et al.*, 2007). However, we found that the reduced viability induced by co-treatment of DOX with Sal or Sal alone was not affected by increased cell density, suggesting that Sal can overcome the resistance to DOX treatment of cancer patients with high-density solid tumours.

In our Western blot analysis for cell cycle proteins, tumour suppressors, and anti-apoptotic proteins we found that treatment with Sal positively correlated with reduced Cyclin D1, p16, p21, and p27, indicating that Sal targets cell cycle-related proteins. We did not detect major changes in anti-apoptotic proteins by Sal. In addition, we tried to identify changes in protein levels after co-treatment with Sal and either DOX or ETO. Our observations that co-treatment reduced levels of Cyclin D1, p16, p21, p27 and p53 suggest that cell-cycle regulators are also primary targets for Sal co-treatments. Among those proteins, we found that p21 was the only protein increased by treatment solely with DOX or ETO. Previously, the p21 tumour suppressor protein was shown to exert an anti-apoptotic function, enabling survival from DNA-damaging agents, and it has been studied for screening of a drug sensitizer (Weiss, 2003; Park *et al.*, 2008; Radhakrishnan *et al.*, 2008; Idogawa *et al.*, 2009). This DOX- or ETO-mediated increase in p21 was reduced by co-treatment with Sal in all cell lines studied, suggesting that reduced p21 levels might positively correlate with increased sensitization by Sal. Previously, it was shown that the ubiquitination related pathway involves the degradation and stability of regulation of the p21 protein (Lee *et al.*, 2009). In addition, we demonstrated that Sal increased proteasome activity, as shown by increased p21 levels after co-treatment with proteasome inhibitors. Hence, the increased proteasome activity induced by Sal co-treatment is the probable mechanism for its reducing effect on p21 levels.

Further analyses of the sensitizing effect of Sal will require *in vivo* mouse models, sensitization in DOX- or ETO-resistant cancer cells, or a combination with non-DNA damaging agents such as paclitaxcel.

Overall, the results demonstrate that Sal can sensitize cancer cells to the effects of DOX or ETO treatment by enhancing apoptosis via increased DNA damage and reduced p21 protein levels through increased proteasome activity. Sal could sensitize them with two different pathways simultaneously; one is through increased DNA damage and the other is through reduced p21 levels via proteasome activity. Consequently, it may be possible to direct DNA damaging agents to both increase DNA breakage and reduce p21 proteins in the treatment of cancer patients. Results of this study may be used to improve various combination-chemotherapeutic treatments of cancer patients treated with DOX or ETO for increasing apoptosis via DNA damage.

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Conflicts of interest

We do not have any conflict of interest.

References

Abukhdeir AM, Park BH (2008). P21 and p27: roles in carcinogenesis and drug resistance. Expert Rev Mol Med 10: e19.

Bidwell GL 3rd, Raucher D (2006). Enhancing the antiproliferative effect of topoisomerase II inhibitors using a polypeptide inhibitor of c-Myc. Biochem Pharmacol 71: 248–256.

Biganzoli L, Minisini A, Aapro M, Di Leo A (2004). Chemotherapy for metastatic breast cancer. Curr Opin Obstet Gynecol 16: 37–41.

Chakraborty P, Sk UH, Bhattacharya S (2009). Chemoprotection and enhancement of cancer chemotherapeutic efficacy of cyclophosphamide in mice bearing Ehrlich ascites carcinoma by diphenylmethyl selenocyanate. Cancer Chemother Pharmacol 64: 971–980.

Chetty C, Bhoopathi P, Lakka SS, Rao JS (2007). MMP-2 siRNA induced Fas/CD95-mediated extrinsic II apoptotic pathway in the A549 lung adenocarcinoma cell line. Oncogene 26: 7675–7783.

Crivellari D, Aapro M, Leonard R, von Minckwitz G, Brain E, Goldhirsch A *et al.* (2007). Breast cancer in the elderly. J Clin Oncol 25: 1882–1890.

Danson S, Dean E, Dive C, Ranson M (2007). IAPs as a target for anticancer therapy. Curr Cancer Drug Targets 7: 785–794.

Devlin HL, Mack PC, Burich RA, Gumerlock PH, Kung HJ, Mudryj M *et al*. (2008). Impairment of the DNA repair and growth arrest pathways by p53R2 silencing enhances DNA damage-induced apoptosis in a p53-dependent manner in prostate cancer cells. Mol Cancer Res 6: 808–818.

Dillman RO, Barth NM, VanderMolen LA, Allen K, Beutel LD, Chico S (2005). High-dose chemotherapy and autologous stem cell rescue for metastatic breast cancer: superior survival for tandem compared with single transplants. Am J Clin Oncol 28: 281–288.

Fang Y, Sullivan R, Graham CH (2007). Confluence-dependent resistance to doxorubicin in human MDA-MB-231 breast carcinoma cells requires hypoxia-inducible factor-1 activity. Exp Cell Res 313: 867–877.

Fuchs D, Heinold A, Opelz G, Daniel V, Naujokat C (2009). Salinomycin induces apoptosis and overcomes apoptosis resistance in human cancer cells. Biochem Biophys Res Commun 390: 743–749.

Fuchs D, Daniel V, Sadeghi M, Opelz G, Naujokat C (2010). Salinomycin overcomes ABC transporter-mediated multidrug and apoptosis resistance in human leukemia stem cell-like KG-1a cells. Biochem Biophys Res Commun 394: 1098–1104.

Glück S (2005). Adjuvant chemotherapy for early breast cancer: optimal use of epirubicin. Oncologist 10: 780–791.

Gupta PB, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA *et al*. (2009). Identification of selective inhibitors of cancer stem cells by high-throughput screening. Cell 138: 645–659.

Hsiao CJ, Li TK, Chan YL, Hsin LW, Liao CH, Lee CH *et al*. (2008). WRC-213, an l-methionine-conjugated mitoxantrone derivative, displays anticancer activity with reduced cardiotoxicity and drug resistance: identification of topoisomerase ? inhibition and apoptotic machinery in prostate cancers. Biochem Pharmacol 75: 847–856.

Humber CE, Tierney JF, Symonds RP, Collingwood M, Kirwan J, Williams C *et al*. (2007). Chemotherapy for advanced, recurrent or metastatic endometrial cancer: a systematic review of Cochrane collaboration. Ann Oncol 18: 409–420.

Hwang MN, Ha TH, Park J, Shim J, Lee H, Kim YN *et al*. (2007). Increased SOCS6 stability with PMA requires its N-terminal region and the Erk pathway via Pkcdelta activation. Biochem Biophys Res Commun 354: 184–189.

Idogawa M, Sasaki Y, Suzuki H, Mita H, Imai K, Shinomura Y *et al*. (2009). A single recombinant adenovirus expressing p53 and p21-targeting artificial microRNAs efficiently induces apoptosis in human cancer cells. Clin Cancer Res 15: 3725–3732.

Inskip PD, Robison LL, Stovall M, Smith SA, Hammond S, Mertens AC *et al*. (2009). Radiation dose and breast cancer risk in the childhood cancer survivor study. J Clin Oncol 27: 3901–3907.

Kaklamani VG, Gradishar WJ (2005). Adjuvant therapy of breast cancer. Cancer Invest 23: 548–560.

Kawabe T (2004). G2 checkpoint abrogators as anticancer drugs. Mol Cancer Ther 3: 513–519.

Kim JH, Kim TH, Kang HS, Ro J, Kim HS, Yoon S (2009). SP600125, an inhibitor of Jnk pathway, reduces viability of relatively resistant cancer cells to doxorubicin. Biochem Biophys Res Commun 387: 450–455.

Kim JH, Lee SC, Ro J, Kang HS, Kim HS, Yoon S (2010). Jnk signaling pathway-mediated regulation of Stat3 activation is linked to the development of doxorubicin resistance in cancer cell lines. Biochem Pharmacol 79: 373–380.

Lee EW, Lee MS, Camus S, Ghim J, Yang MR, Oh W *et al*. (2009). Differential regulation of p53 and p21 by MKRN1 E3 ligase controls cell cycle arrest and apoptosis. EMBO J 28: 2100–2113.

Llovet JM (2005). Updated treatment approach to hepatocellular carcinoma. J Gastroenterol 40: 225–235.

Maddika S, Ande SR, Panigrahi S, Paranjothy T, Weglarczyk K, Zuse A *et al*. (2007). Cell survival, cell death and cell cycle pathways are interconnected: implications for cancer therapy. Drug Resist Updat 10: 13–29.

Mahmoudi N, de Julián-Ortiz JV, Ciceron L, Gálvez J, Mazier D, Danis M (2006). Identification of new antimalarial drugs by linear discriminant analysis and topological virtual screening. J Antimicrob Chemother 57: 489–497.

Malumbres M, Barbacid M (2009). Cell cycle, CDKs and cancer: a changing paradigm. Nat Rev Cancer 9: 153–166.

Mita AC, Mita MM, Nawrocki ST, Giles FJ (2008). Survivin: key regulator of mitosis and apoptosis and novel target for cancer therapeutics. Clin Cancer Res 14: 5000–5005.

Miyazaki Y, Shibuya M, Sugawara H, Kawaguchi O, Hirsoe C (1974). Salinomycin, a new polyether antibiotic. J Antibiot 27: 814–821.

Nitiss JL (2002). DNA topoisomerases in cancer chemotherapy: using enzymes to generate selective DNA damage. Curr Opin Investig Drugs 3: 1512–1516.

Park SH, Wang X, Liu R, Lam KS, Weiss RH (2008). High throughput screening of a small molecule one-bead-one-compound combinatorial library to identify attenuators of p21 as chemotherapy sensitizers. Cancer Biol Ther 7: 2015–2022.

Radhakrishnan SK, Bhat UG, Halasi M, Gartel AL (2008). P-TEFb inhibitors interfere with activation of p53 by DNA-damaging agents. Oncogene 27: 1306–1309.

Riccioni R, Dupuis ML, Bernabei M, Petrucci E, Pasquini L, Mariani G *et al*. (2010). The cancer stem cell selective inhibitor salinomycin is a p-glycoprotein inhibitor. Blood Cells Mol Dis 45: 86–92.

Sherr CJ, McCormick F (2002). The RB and p53 pathways in cancer. Cancer Cell 2: 103–112.

Solier S, Sordet O, Kohn KW, Pommier Y (2009). Death receptor-induced activation of the Chk2- and histone H2AX-associated DNA damage response pathways. Mol Cell Biol 29: 68–82.

Thomadaki H, Scorilas A (2008). Molecular profile of breast versus ovarian cancer cells in response to treatment with the anticancer drugs cisplatin, carboplatin, doxorubicin, etoposide and taxol. Biol Chem 389: 1427–1434.

Van Valen F, Fulda S, Schäfer KL, Truckenbrod B, Hotfilder M, Poremba C *et al*. (2003). Selective and nonselective toxicity of TRAIL/Apo2L combined with chemotherapy in human bone tumour cells vs. normal human cells. Int J Cancer 107: 929–940.

Wang Y, Arriaga EA (2008). Monitoring incorporation, transformation and subcellular distribution of N-l-leucyl-doxorubicin in uterine sarcoma cells using capillary electrophoretic techniques. Cancer Lett 262: 123–132.

Weiss RH (2003). p21Waf1/Cip1 as a therapeutic target in breast and other cancers. Cancer Cell 4: 425–429.

Willmore E, de Caux S, Sunter NJ, Tilby MJ, Jackson GH, Austin CA *et al*. (2004). A novel DNA-dependent protein kinase inhibitor, NU7026, potentiates the cytotoxicity of topoisomerase II poisons used in the treatment of leukemia. Blood 103: 4659–4665.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 DMSO did not damage DNA in the cells or have a cytotoxic effect, whereas ETO dose-dependently decreased the viability of the cells. (A) Hs578T cell extracts were collected 6 h after treatment with 1 μL DMSO of 1 mL medium (DM1), 2 μL DMSO of 1 mL medium (DM2), 1 μL of 50 mM ETO (Et; 50 μM), DM1 with 1 μL of 50 mM ETO (Et + DM1), or they remained untreated (Con). The cells were used for Western blot analyses using antibodies against pH2AX and GAPDH. (B-C) Hs578T cells were plated on 96-well plates and grown to 30–40% confluence. The cells were stimulated for 48 h with DM1, DM2, 50 μM ETO (Et), DM1 with 50 μM ETO (Et + DM1), 3.5 μM DOX (Do), DM1 with 3.5 μM DOX (Do + DM1), or they remained untreated (Con). The cell proliferation assay was performed as described in *Methods*. (D) Hs578T cells were plated on 96-well plates and grown to 30–40% confluence. The cells were either untreated (0 μM) or stimulated by treatment with 20, 40 50, 100 or 200 μM ETO for 20 h or 40 h. The cell proliferation assay was performed at each time point. All experiments were carried out in triplicate; the percentage of viable cells quoted was calculated as the mean ± SD with respect to the controls set to 100%.

Figure S2 Co-treatment with Sal increases apoptosis of DOX- or ETO-treated Hs578T cells. (A) Hs578T cells were plated on 60 mm-diameter dishes and grown to 80%–90% confluence. A scratch was made in the middle of the culture to enable detection of cellular growth. The cells were stimulated for 24 h with 3.5 μM DOX, 10 μM Sal, 10 μM Sal with 3.5 μM DOX (DOX + Sal), or they remained untreated (Con). They were subsequently observed using an inverted microscope. (B) Hs578T cells were grown to 50% confluence and stimulated with 25 μM ETO, 10 μM Sal, or 25 μM ETO in the presence of 10 μM Sal (ETO + Sal). Untreated cells served as a control (Con). Immunocytochemistry was performed as described in Methods. Immunocytochemical staining for pH2AX is shown by green fluorescence. DAPI nuclear staining (blue) is also shown.

Figure S3 Co-treatment with NU7026 or Sal increases DNA damage-related proteins by DOX- or ETO-treated cells. (A) Hs578T cell extracts were collected 3 h or 6 h after treatment with 3.5 μM DOX (Do), 5 μM NU7026 with 3.5 μM DOX (Do + Nu), 50 μM ETO (Et), 5 μM NU7026 (Nu), 5 μM NU7026 with 50 μM ETO (Et + Nu), or they remained untreated (Con). The cells were used for Western blot analyses using antibodies against pH2AX, p53BP1, pBRCA1, pChk1, and GAPDH. (B) Hs578T cell extracts were collected 3 h or 6 h after treatment with 3.5 μM DOX (Do), 10 μM Sal with 3.5 μM DOX (Do + Sal), 50 μM ETO (Et), 10 μM Sal, 10 μM Sal with 50 μM ETO (Et + Sal), or they remained untreated (Con). The cells were used for Western blot analyses using antibodies against pH2AX, p53BP1, pBRCA1, pChk1 and GAPDH.

Figure S4 Co-treatment with Sal reduces viability for p-glycoprotein inhibitor-treated cells. (A–B) Hs578T cells were plated on 96-well plates and grown to 30–40% confluence. The cells were stimulated for 48 h with 20 μM verapamil (Ve), 10 μM Sal (Sal), 10 μM Sal with 20 μM verapamil (Ve + Sal), 10 μM C-4, 10 μM Sal with 10 μM C-4 (C4 + Sal), or remained untreated (Con). The cell proliferation assay was performed as described in Methods.

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