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Disulfiram targets cancer stem-like cells and reverses resistance and cross-resistance in acquired paclitaxel-resistant triple-negative breast cancer cells

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Background: Triple-negative breast cancer (TNBC) has significantly worse prognosis. Acquired chemoresistance remains the major cause of therapeutic failure of TNBC. In clinic, the relapsed TNBC is commonly pan-resistant to various drugs with completely different resistant mechanisms. Investigation of the mechanisms and development of new drugs to target pan-chemoresistance will potentially improve the therapeutic outcomes of TNBC patients.

Methods: In this study, 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT), combination index (CI)–isobologram, western blot, ALDEFLUOR analysis, clonogenic assay and immunocytochemistry were used.

Results: The chemoresistant MDA-MB-231$_{PAC10}$ cells are highly cross-resistant to paclitaxel (PAC), cisplatin (CDDP), docetaxel and doxorubicin. The MDA-MB-231$_{PAC10}$ cells are quiescent with significantly longer doubling time (64.9 *vs* 31.7 h). This may be caused by high expression of p21^{Waf1}. The MDA-MB-231$_{PAC10}$ cells express high aldehyde dehydrogenase (ALDH) activity and a panel of embryonic stem cell-related proteins, for example, Oct4, Sox2, Nanog and nuclealisation of HIF2α and NF-κBp65. We have previously reported that disulfiram (DS), an antialcoholism drug, targets cancer stem cells (CSCs) and enhances cytotoxicity of anticancer drugs. Disulfiram abolished CSC characters and completely reversed PAC and CDDP resistance in MDA-MB-231$_{PAC10}$ cells.

Conclusion: Cancer stem cells may be responsible for acquired pan-chemoresistance. As a drug used in clinic, DS may be repurposed as a CSC inhibitor to reverse the acquired pan-chemoresistance.

Triple-negative breast cancer (TNBC) is an aggressive variant of breast cancer. Because of lack of molecular target to be tackled, there are very few chemotherapeutic agents available for TNBC chemotherapy. Paclitaxel (PAC) is one of the first-line therapeutic agents in chemotherapy of the early-stage and metastatic TNBC. Paclitaxel targets cancer cells mainly by binding to and stabilising microtubules (Schiff *et al*, 1979), arresting cancer cells in G2/M mitotic checkpoint and subsequently inducing apoptosis via an intrinsic apoptotic pathway (Ferlini *et al*, 2009).

As with other anticancer drugs, TNBC can develop an acquired resistance after repeated exposure to PAC. The acquired chemoresistance remains a major hurdle for the PAC-based chemotherapy. The most recognised resistant mechanisms include overexpression of P-glycoprotein (Pgp/MDR1) and alterations in microtubule

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system (Trock *et al*, 1997; Kavallaris, 2010). The acquired PAC resistance can also be introduced by mutations in tubulin that modulate the binding affinity of PAC to microtubules. The following molecular mechanisms are also related to PAC resistance; for example, HER2 overexpression (Knuefermann *et al*, 2003) altered apoptotic and molecular signalling pathways (Takahashi *et al*, 2005). Chemotherapy would be benefited from identifying new compounds to target alternative chemoresistant pathways and sensitise cancer cells to classical anticancer drugs.

It has been suggested that human breast cancer contains a small population of cancer stem cells (CSCs) that can be detected by the expression of stem cell markers (aldehyde dehydrogenases (ALDHs), $CD24^{Low}/CD44^{High}$) and activation of embryonic-related pathways (Sox2, Oct4, Nanog) (Tirino *et al*, 2013). Breast cancer stem cells (BCSCs) are slow-cycling and quiescent population expressing high levels of Pgp (Dean, 2009). The TNBC cells with CSC phenotypes are resistant to a variety of conventional anticancer drugs with poor prognosis (Dean, 2009; Ohi *et al*, 2011). Targeting CSCs may improve the outcomes of TNBC chemotherapy (Deng *et al*, 2012).

Disulfiram (DS), a commercially available antialcoholism drug (Schreck *et al*, 1992), shows anticancer activity *in vitro* and *in vivo* (Chen *et al*, 2006; Yip *et al*, 2011). It also potentiates cyclophosphamide, cisplatin and radiation *in vitro* and protects normal cells in kidney, gut and bone marrow *in vivo* while increasing the therapeutic index of cytotoxic drugs (Hacker *et al*, 1982; Bodenner *et al*, 1986). Our previous studies demonstrate that DS enhances 5-fluorouracil (5-FU)-, PAC- and gemcitabine (dFdC)-induced apoptosis in colon and breast cancer cell lines (Wang *et al*, 2003; Guo *et al*, 2010; Yip *et al*, 2011). The randomised clinical trial indicates that in combination with chemotherapy, ditiocarb, the derivative of DS, significantly improves the 5-year overall survival of high-risk breast cancer patients (Dufour *et al*, 1993). The anticancer activity of DS is copper (Cu) dependent (Cen *et al*, 2004; Chen *et al*, 2006). Copper plays a crucial role in redox reactions and triggers the generation of reactive oxygen species (ROS) in human cells. The DS/Cu is a strong ROS inducer (Nobel *et al*, 1995) and proteasome–NF-κB pathway inhibitor (Chen *et al*, 2006). Disulfiram specifically inhibits the activity of ALDH, a functional CSC marker and ROS scavenger (Estey *et al*, 2007; Ginestier *et al*, 2007). A combination of DS with Cu may target cancer cells by simultaneous modulation of both ROS and NF-κB. Disulfiram and its metabolites can also covalently modify cysteine residues within the nucleotide-binding domain of Pgp and permanently inhibit Pgp activity (Loo *et al*, 2004). This will potentially reverse multidrug resistance.

In clinic, the relapsed TNBC is commonly pan-resistant to anticancer drugs with completely different resistant mechanisms. In this study, we demonstrated that MDA-MB-231_{PAC10} cells express various CSC markers and are cross-resistant to cisplatin (CDDP), docetaxel (DOC) and doxorubicin (DOX). Disulfiram eradicates CSC characters and reverses PAC and CDDP resistance in MDA-MB-231_{PAC10} cells.

MATERIALS AND METHODS

Cell lines and reagents. The PAC-resistant cell line MDA-MB-231_{PAC10} (PAC10) was generated from MDA-MB-231 (MDA) (purchased from ATCC, Middlesex, UK) by continuously cultured in medium containing PAC (Sigma, Dorset, UK) in a stepwise concentration-increasing procedure. Cisplatin, DOC, DOX, DS and copper (II) chloride ($CuCl_2$) were purchased from Sigma.

Cell culture and cytotoxicity analysis. All cell lines were cultured in DMEM (Lonza, Wokingham, UK) supplemented with 10% FCS, 50 units ml^{-1} penicillin and 50 $\mu g\,ml^{-1}$ streptomycin. The MDA-MB-231_{PAC10} cells were maintained in the medium containing 10 nM of PAC. For *in vitro* cytotoxicity assay, the overnight cultured cells (5000 per well) in 96-well flat-bottomed microtiter plates were exposed to drugs for 72 h (PAC) or 120 h (CDDP) and subjected to a standard MTT assay (Plumb *et al*, 1989).

Analysis of the combinational effect of PAC + DS/Cu and CDDP + DS/Cu by CI–isobologram. Overnight cultured cells were exposed to various concentrations of PAC, CDDP, DS/$Cu_{1\,\mu M}$ or in combination of PAC/DS/$Cu_{1\,\mu M}$ or CDDP/DS/$Cu_{1\,\mu M}$ at a constant ratio of PAC/DS (10 : 1) and CDDP/DS (500 : 1) determined by IC_{50} data generated from previous experiments. The cells were exposed to DS/Cu for 4 h and then cultured in DS/Cu-free fresh medium containing PAC or CDDP for another 72 and 120 h, respectively, and subjected to MTT analysis as described above. The combinational cytotoxicity of PAC/DS/$Cu_{1\,\mu M}$ and CDDP/DS/$Cu_{1\,\mu M}$ was analysed by combination index (CI)–isobologram analysis using CalcuSyn software (Biosoft, Cambridge, UK) (Chou and Talalay, 1984). The CI was determined by mutually exclusive equations.

Growth curves and doubling time analysis. The cells (5×10^3 cells per well) were cultured in 24-well plates in triplicate. The cells were collected by trypsinisation and cell numbers in each of three wells were counted every 24 h for 120 h. The cell doubling time was calculated using the program from the Doubling Time Online Calculator http://www.doubling-time.com/compute.php.

Clonogenic assay. Cells (5×10^4 cells per well) were cultured in six-well plates overnight and then exposed to designated concentration of DS in combination with 1 μM $CuCl_2$ (DS/$Cu_{1\,\mu M}$) for 4 h or PAC (20 nM) for 72 h. The cells were collected and further cultured for 10 days in six-well plates containing drug-free medium at a cell density of 2.5×10^3 cells per well. Clonogenic cells were determined as those able to form a colony consisting of at least 50 cells.

Western blotting analysis. The protein expression levels were determined by staining with primary antibodies and relevant HRP-conjugated secondary antibodies. The primary antibodies (Bcl2, Bax, MDR1, p53, p21, p65, CDK2, cyclin D1 and cyclin E supplied by Santa Cruz, Dallas, TX, USA; HIF2α, Sox2 and Oct4 by Cell Signaling, Herts, UK) were diluted in a ratio of 1 : 1000 in 5% fat-free milk-TBST. Anti-α-tubulin (Amersham, Buckinghamshire, UK; 1 : 8000 diluted) and nucleolin (Sigma) were used as a loading control. The signal was detected using an ECL western blotting detection kit (GeneFlow, Dallas, TX, USA, Staffordshire, UK). The strength of western blotting bands was determined by ImageJ density measurement program (http://imagej.en.softonic.com).

Immunofluorescent flow cytometry and confocal microscopy. The expression of Nanog, Oct4 and Sox2 was determined by immunofluorescent flow cytometry and confocal microscopy. For immunocytochemistry confocal microscopy analysis, the cells were grown on culturing chamber slide (Sigma) overnight and fixed by acetone/methanol and permeabilised by 0.1% Triton X-100. After being blocked with 3% BSA for 1 h, the cells were stained with primary antibodies (1 : 50 dilution) and FITC-conjugated secondary antibody for 1 h at RT. The coverslips were mounted on glass slides with VectaShield mounting media containing the nucleic acid stain, 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA, USA), and examined by laser scanning confocal microscopy using a Zeiss Axiovert 200 microscope and ZEN 2009 software (Carl Zeiss Canada Ltd, Mississauga, ON, Canada). For immunofluorescent flow cytometric analysis, the cells were cultured in T25 flasks until 80% confluence and collected by trypsinisation. The cells were stained in suspension using the same concentration of antibodies and procedure as immunocytochemistry analysis. The positively stained population was detected using

a FACSCalibur flow cytometer with 488-nm blue laser and standard FITC 530/30 nm bandpass filter.

Flow cytometric analysis of DNA content. The untreated and drug-treated cells (1×10^6) were harvested by trypsinisation. The cells were fixed in 70% ethanol and then incubated with RNase A (100 μg ml^{-1}) and propidium iodide (Sigma, 50 μg ml^{-1}) for 30 min. The data from 10 000 cells of each sample were collected by FACS Scan (Becton Dickinson, NJ, USA) and the DNA contents were analysed using CellQuest software (BD Biosciences, Oxford, UK).

Flow cytometric analysis of ALDH activity. The parental and PAC-resistant cells (2.5×10^5) were stained for 30 min at 37 °C using ALDEFLUOR kit (StemCell Tech., Durham, NC, USA) following the manufacturer's instructions. Cells treated with diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor, were used as a control to determine the specificity of ALDEFLUOR assay. The ALDH$^+$ population was detected using a FACSCalibur flow cytometer with 488-nm blue laser and standard FITC 530/30 nm bandpass filter. The ALDH$^+$ cells were determined by dot plot.

Statistical analysis. The data statistical analysis in this study was performed using Student's *t*-test.

RESULTS

MDA-MB-231$_{PAC10}$ cell line is pan-resistant to anticancer drugs. First, the cytotoxic effect of PAC on both sensitive and resistant cell lines was compared by MTT assay (Table 1 and Figure 1A). The MDA-MB-231 cells are sensitive to the cytotoxicity of PAC with an $IC_{50_72\,h}$ of 8.7 nM. In contrast, the MDA-MB-231$_{PAC10}$ cell line is highly resistant to PAC with an $IC_{50_72\,h}$ of over 1000 nM. The cytotoxic effect of CDDP, DOC and DOX on MDA-MB-231$_{PAC10}$ cell line was also evaluated. Table 1 and Figure 1B demonstrate that MDA-MB-231$_{PAC10}$ cells are also significantly cross-resistant to CDDP, DOC and DOX. In line with the MTT data, PAC (20 nM) abolished the clonogenicity of the parental cell line but had no effect on MDA-MB-231$_{PAC10}$ cells (Figure 1C and D). Because of the slower proliferation rate, the colonies developed from the resistant cell line are smaller than that from the parental cell line (Figure 1C). The overexpression of MDR1 is the most common mechanism involved in multidrug resistance that includes PAC resistance. High expression of Pgp was detected in the resistant cell line by western blot (Figure 1E). Paclitaxel induces apoptosis mainly via intrinsic apoptotic pathway (Ferlini *et al*, 2009). Therefore, the protein expression status of Bax and Bcl2, the two major components involved in intrinsic apoptotic pathway, was examined by western blot. Figure 1F shows that MDA-MB-231$_{PAC10}$ cell line expresses significantly higher background levels of Bcl2 protein than those in the parental cells. The Bcl2/Bax ratio in the resistant cell line is markedly higher than that in the parental cell line.

Table 1. Cytotoxicity of disulfiram and conventional anticancer drugs to MDA-MB-231 and MDA-MB-231$_{PAC10}$ BC cell lines

	PAC	CDDP	DOC	DOX	DS
IC_{50}					
MDA	8.7 (2.3)	256.7 (26.1)	4.6 (3.3)	27.6 (2.5)	151.9 (12.1)
MDA$_{PAC10}$	>1000**	645.4* (127.3)	>250**	1575** (169.3)	116.4 (30.0)
CI value					
IC_{50}	0.61	0.64	NA	NA	NA
IC_{75}	0.64	0.41	NA	NA	NA
IC_{90}	0.72	0.28	NA	NA	NA

Abbreviations: CDDP = cisplatin; CI = combination index; DOC = docetaxel; DOX = doxorubicin; DS = disulfiram; IC = inhibitory concentration; NA = not available; PAC = paclitaxel. The half-maximal inhibitory concentration (IC_{50}) value (nM) from three experiments (mean (s.d.)) is shown. *$P<0.05$, **$P<0.01$ ($n=3$). The CI value lower than 1.0: synergistic effect. The cells were exposed to drug for 72 or 120 h (CDDP). DS/Cu = DS in medium supplemented with 1 μM $CuCl_2$.

Resistance of MDA-MB-231$_{PAC10}$ cell line to PAC-induced apoptosis. After a 72-h exposure to 20 nM PAC, the phase-contrast microscopic images demonstrate apoptotic morphologies (cell blebbing and nuclear condensation and fragmentation) in MDA-MB-231 but not in the MDA-MB-231$_{PAC10}$-resistant cells (Figure 2A). Flow cytometry DNA content analysis manifested that PAC induced a significantly higher ($P<0.01$) apoptotic sub-G1 population (30.4%) in the parental cell line than those in the untreated cells (0.4%). Paclitaxel (20 nM, 72 h) also introduced G2/M-phase blockade leading to an increased G2/M population (untreated: 17.9%, treated: 36.4%; $P<0.01$) and a decreased G0/G1 population (dropped from 64.9 to 15.6%, $P<0.01$; Figure 2B and C) in the parental cell line. In contrast, there is no significant effect of PAC on the apoptotic status in the resistant cells. The cell cycle status in MDA-MB-231$_{PAC10}$ cell line is also not affected by PAC exposure (Figure 2D). Paclitaxel exposure induces Bax expression leading to high Bax/Bcl2 ratio in the parental cells but not the resistant cells (Figure 2E).

MDA-MB-231$_{PAC10}$ has longer doubling time. In the cell culture, the MDA-MB-231$_{PAC10}$ cells grow markedly slower than MDA-MB-231 cells. Therefore, we compared the doubling time and cell cycle parameters in these two cell lines. Figure 3A shows the growth curves of both cell lines. The doubling time of MDA-MB-231$_{PAC10}$ cells (64.9 h) is significantly longer than that of the sensitive cells (31.7 h; $P<0.01$). Flow cytometry analysis shows that in comparison with the parental cell line, the MDA-MB-231$_{PAC10}$ cells have significantly higher G0/G1 and lower S-phase population (Figure 3B and C). The expression levels of cell cycle-determinant proteins were examined by western blot. Figure 3D shows the western blotting image and relative band density analysed by ImageJ program. The relative density ((Target protein/Tubulin) $\times$ 100) of p21 protein is markedly higher in the resistant cell line. The other moderately upregulated proteins include p53, cyclin D1 and cyclin E.

MDA-MB-231$_{PAC10}$ cells demonstrate CSC characteristics. It has been widely accepted that CSCs are responsible for chemo- and radio-resistance (Dean, 2009). The resistant cell line is slow cycling with high expression of p21 protein and expresses high levels of Pgp, which are the common features in CSCs (Tirino *et al*, 2013). Therefore, we examined CSC markers in the resistant and parental cell lines. High ALDH activity is a functional marker of CSCs derived from different cancer types including breast cancer. Figures 3E and G show that in comparison with the parental cells, the MDA-MB-231$_{PAC10}$ cell line possesses higher ALDH$^+$ population that also expresses higher levels of embryonic stem cell markers (Oct4, Sox2 and Nanog). The overexpression of Oct4 and Sox2 protein was detected in nuclear protein by western blotting assay (Figure 3G). High expression of Oct4 and Sox2 in the resistant cell line was detected by immunofluorescent confocal microscopy (Figure 3H). The nuclear translocation of Oct4 was detected but for some unknown reason Sox2 nuclear translocation was not detected by immunocytochemistry. The specificity of ALDEFLUOR assay was determined by treating the cells with DEAB, a specific inhibitor of ALDH (Figure 3F). The expression of NF-κB and HIF2α protein was also examined by western blotting analysis because emerging evidence indicates that hypoxia and NF-κB are

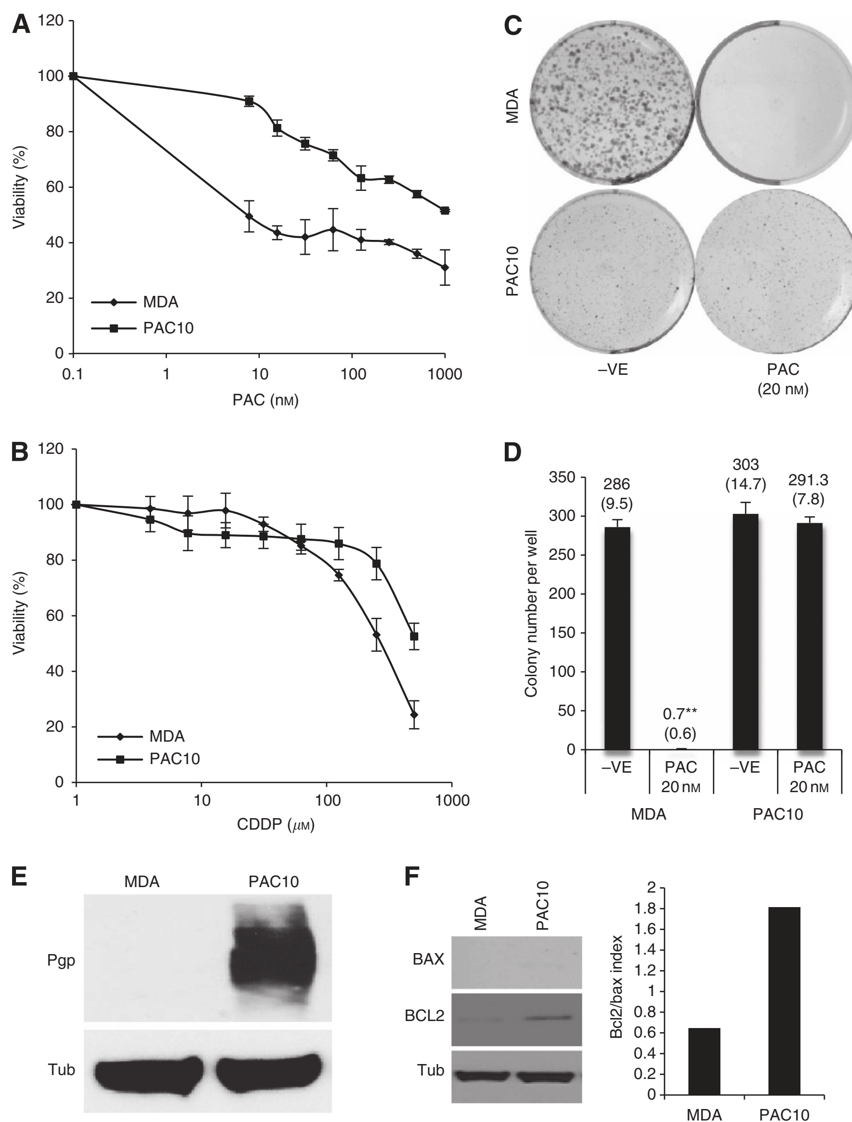

Figure 1. The MDA-MB-231$_{PAC10}$ cell line is resistant to PAC and cross-resistant to CDDP. (**A** and **B**) Surviving curves of MTT cytotoxicity assay. The MDA-MB-231$_{PAC10}$ (PAC10) and MDA-MB-231 (MDA) cell lines were exposed to PAC and CDDP for 72 and 120 h, respectively. (**C**) Clonogenic assay. The cells were exposed to PAC (20 nM) for 72 h and then subcultured in drug-free medium at a cell density of 2500 cells per well in 6-well plates for another 10 days. (**D**) The colony number of clonogenic assay. The colonies with $\geq$50 cells were counted. The number in the figure represents mean and (s.d.) from three independent experiments. –VE = without drug, $^{**}P<0.01$. (**E** and **F**) Western blotting analysis of Pgp, BAX and Bcl2 expression. The column in (**F**) represents the grey density of the western blotting band detected by the ImageJ program.

responsible for maintaining stemness in CSCs (Hinohara *et al*, 2012). In comparison with the parental cell line, higher levels of HIF2α and NF-κBp65 protein were detected in the nuclear extract of MDA-MB-231$_{PAC10}$ cells. The expression levels of NF-κBp65 in whole-cell lysate are comparable in the resistant and parental cell lines (Figure 3G).

Disulfiram is highly cytotoxic in MDA-MB-231$_{PAC10}$ cells. Our previous studies demonstrate that DS is a strong CSC inhibitor and highly cytotoxic to a wide range of cancer cell lines (Yip *et al*, 2011; Liu *et al*, 2012). In spite of resistance to PAC and CDDP, the sensitivity of MDA-MB-231$_{PAC10}$ cell line to DS is comparable to that of parental cells (Figure 4A and Table 1). The clonogenecity of both parental and resistant cell lines is completely abolished after very short exposure (4 h) to DS$_{1\,\mu M}$/Cu$_{1\,\mu M}$ (Figure 4B). The DS/Cu induces apoptosis in MDA-MB-231$_{PAC10}$ cells. After exposure to DS/Cu for 24 h, massive apoptotic cells were detected (Figure 4C and D). The DS/Cu inhibits and induces the expression of Bcl2 and Bax in MDA-MB-231$_{PAC10}$ cells, respectively, leading to significantly increased Bax/Bcl2 ratio in the resistant cell line (Figure 4E). Although DS is a specific inhibitor of MDR1 enzyme activity, the protein expression of Pgp in MDA-MB-231$_{PAC10}$ cell line was not affected by DS/Cu (Figure 4F). The effect of DS/Cu on cell cycle-regulating proteins was analysed by western blot. Figure 4G shows that DS/Cu induces the expression of p21 and p53 protein but has no effect on CDK2, Cyclin D1 and E.

Disulfiram inhibits CSC marker expression and reverses PAC and CDDP resistance in MDA-MB-231$_{PAC10}$ cells. The MDA-MB-231$_{PAC10}$ cell line is composed of high population of cells expressing stem cell markers that may play a key role in the pan-resistance. Furthermore, we examined if DS/Cu inhibits the CSCs in the resistant cell line. The ALDH activity in the resistant cell line is inhibited after 4 h of exposure to DS/Cu. In addition, DS/Cu inhibits the expression of Sox2 and Nanog in the resistant cells (Figure 5A). We also examined if DS/Cu can enhance cytotoxicity of PAC and CDDP and reverse PAC and CDDP resistance in MDA-MB-231$_{PAC10}$ cell line. In combination with DS/Cu the

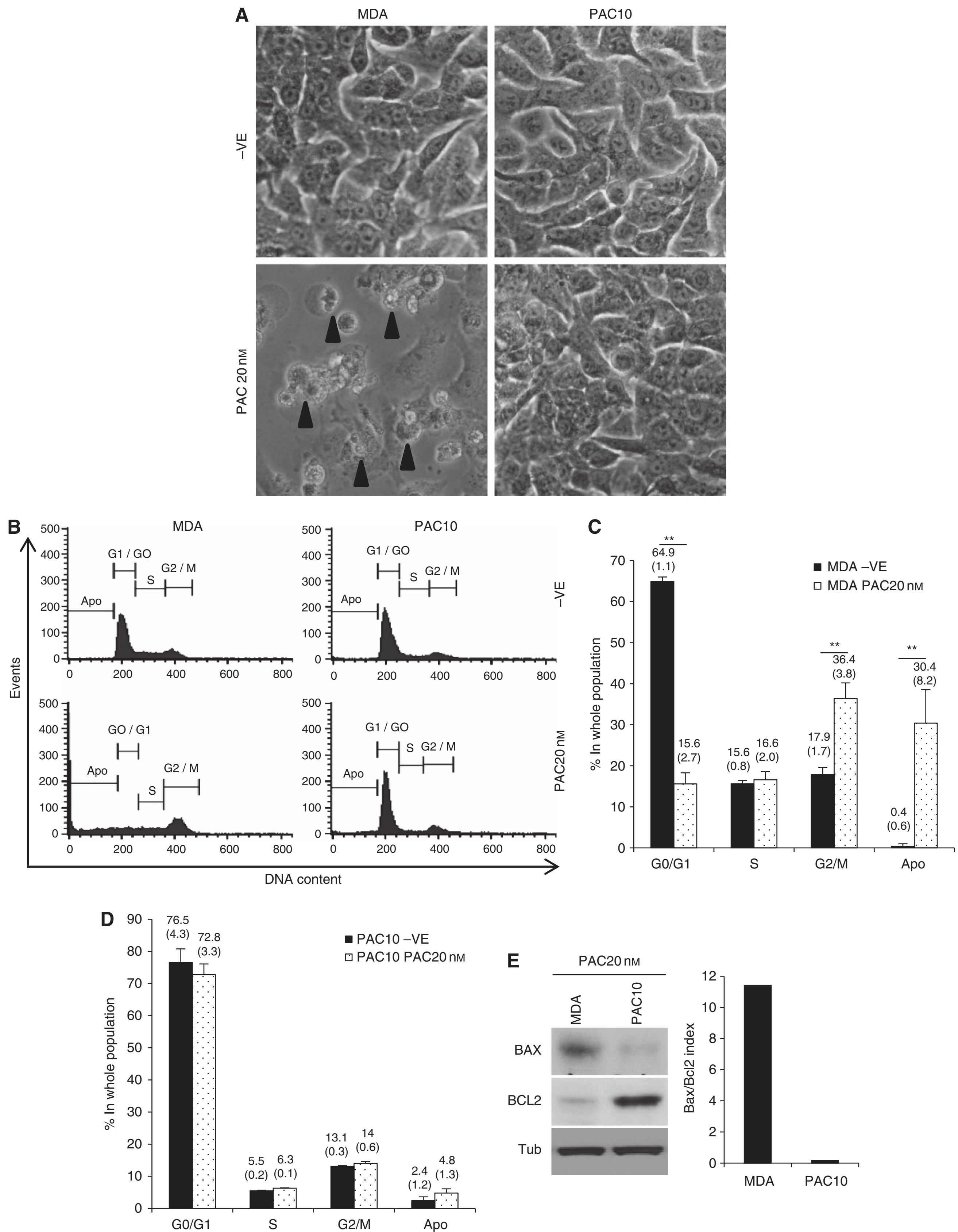

Figure 2. **The MDA-MB-231$_{PAC10}$ cell line is resistant to PAC-induced apoptosis.** (**A**) The morphology ($\times$400 magnification) of parental and resistant cells after 72 h of exposure to PAC (20 nM). (**B**) Histogram of flow cytometric DNA content analysis. (**C** and **D**) The effect of PAC (20 nM, 72 h) on cell cycle parameters in MDA-MB-231 (**C**) and MDA-MB-231$_{PAC10}$ (**D**) cell lines. Apo = apoptosis, $n=3$, $^{**}P<0.01$. (**E**) Western blotting analysis of the background Bax and Bcl2 protein expression in MDA-MB-231 and MDA-MB-231$_{PAC10}$ cell lines after exposure to PAC (20 nM) for 72 h. The column represents the grey density ratio of Bax and Bcl2 bands detected by the ImageJ program.

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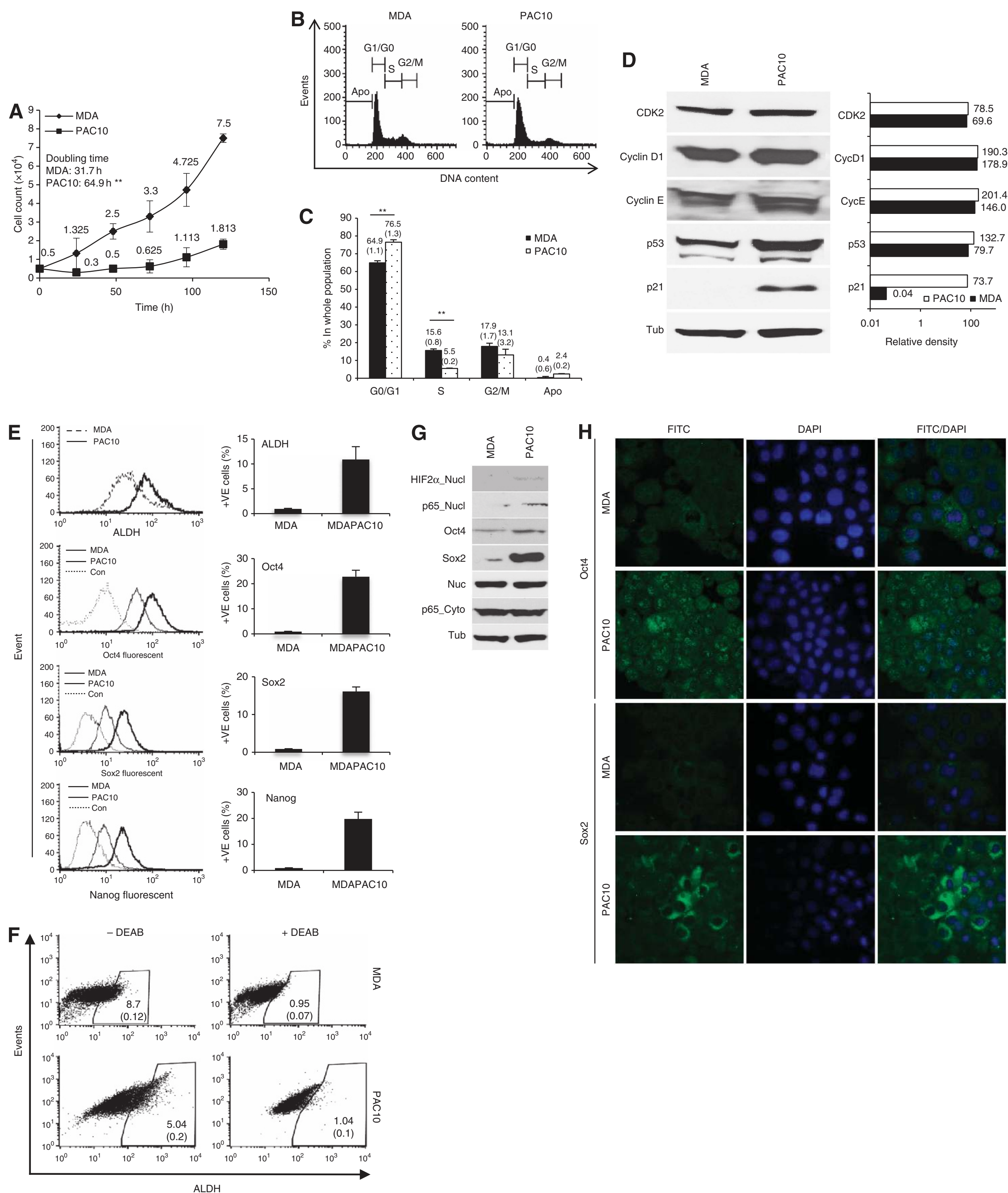

Figure 3. The MDA-MB-231$_{PAC10}$ cell line proliferates slower and expresses cancer stem cell markers. (**A**) Growth curves of MDA-MB-231 and MDA-MB-231$_{PAC10}$ cells. The doubling time (h) is presented, $n=3$, $^{**}P<0.01$. (**B** and **C**) Cell cycle parameters in MDA-MB-231 and MDA-MB-231$_{PAC10}$ cell lines, respectively. Mean and s.d. of three experiments ($^{**}P<0.01$). (**D**) Western blotting analysis of cell cycle-related proteins. The bar chart on the right represents relative density index of the bands. (**E**) Flow cytometry analysis of ALDH activity and Oct4, Sox2 and Nanog protein expression levels. Con = Isotype control of PAC10. The column represents the percentage of positive cells determined by dot plot. (**F**) The ALDH activity was measured in the cancer cell lines treated with DEAB (30 μM) at 37 °C for 30 min. (**G**) High expression of HIF2α, NF-κBp65, Sox2 and Oct4 protein was detected in nuclear extract of MDA-MB-231$_{PAC10}$ cell line. Nucleolin (Nuc) and α-tubulin (Tub) were used as loading control. (**H**) High expression of Oct4 and Sox2 was detected in MDA-MB-231$_{PAC10}$ cells by immunofluorescent confocal microscopy $\times$400 magnification.

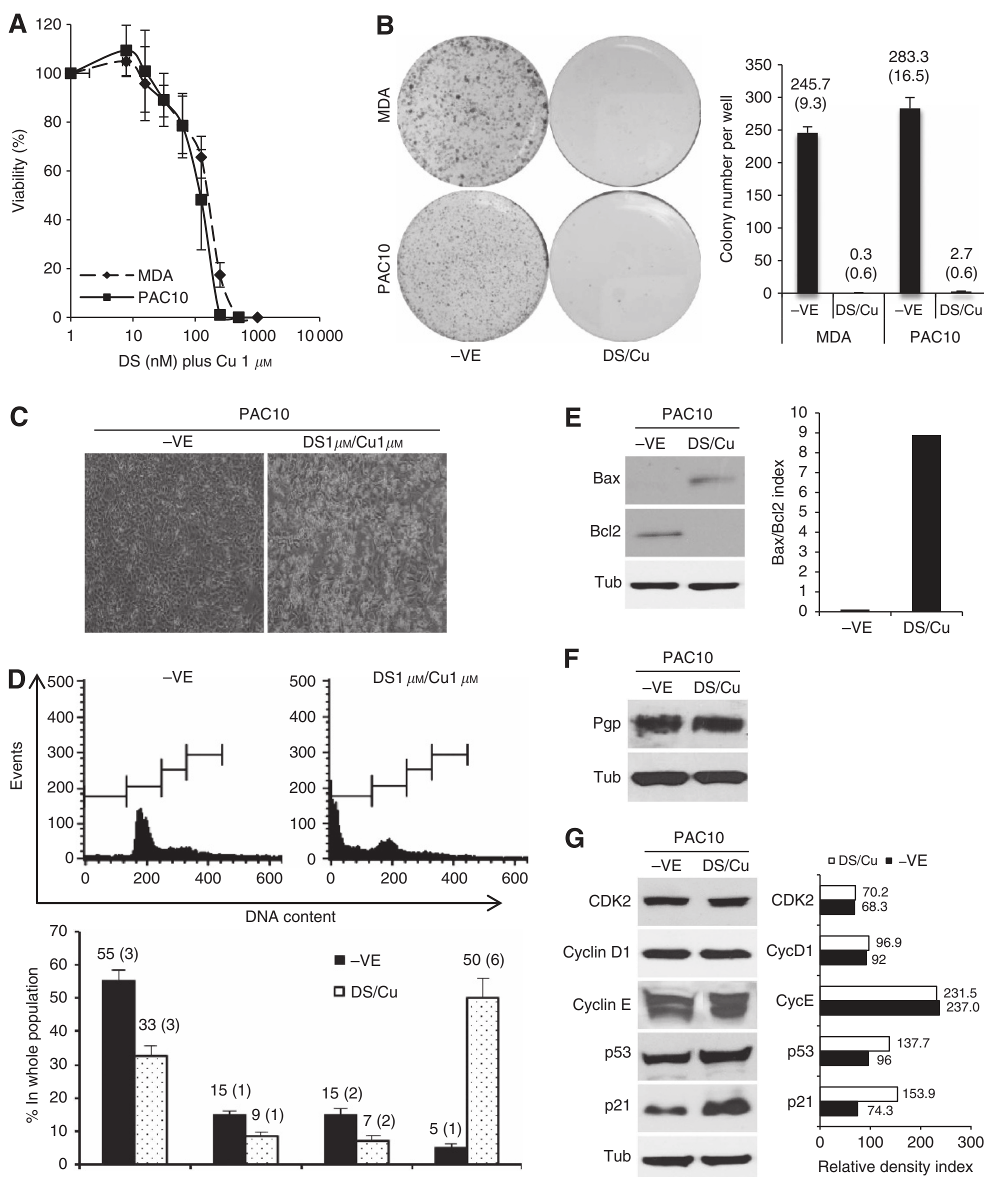

Figure 4. Disulfiram is highly cytotoxic and enhances cytotoxicity of PAC in MDA-MB-231$_{PAC10}$ cell line. (A) The MTT cytotoxicity assay. The MDA-MB-231 and MDA-MB-231$_{PAC10}$ cell lines were exposed to different concentrations of DS in combination with 1 μM of $CuCl_2$ for 4 h and then released in drug-free medium for 72 h. (B) Clonogenic assay. The cells were exposed to different concentrations of DS in combination with 1 μM of $CuCl_2$ for 4 h and then released in drug-free medium for 10 days. Mean and s.d. of colony number from three independent experiments. (C) The morphology (×200 magnification) of MDA-MB-231$_{PAC10}$ cells after drug exposure. (D) The influence of DS/Cu on cell cycle parameters in MDA-MB-231$_{PAC10}$ cells. Mean and s.d. from three independent experiments. (E and F) Western blotting analysis of DS-induced alteration in Bax, Bcl2 and Pgp expression in MDA-MB-231$_{PAC10}$ cells. (G) Western blotting examination of the effect of DS/Cu on cell cycle-related protein expression. The cells were exposed to DS (1 μM) and $CuCl_2$ (1 μM) for 4 h and then released in drug-free medium for 24 h.

cytoxicity of PAC and CDDP in MDA-MB-231$_{PAC10}$ cells is significantly higher than PAC, CDDP or DS/Cu single-drug exposure (Figure 5B–E). The CI–isobologram indicates that the cytotoxicity of DS/Cu + PAC is synergistic in a wide range of concentrations ($IC_{50} - IC_{90}$, Figure 5F and G and Table 1).

DISCUSSION

Triple-negative breast cancer has worse chemotherapeutic outcomes than other BC subtypes, with at best 12 months of median survival of advanced TNBC (Gelmon *et al*, 2012). Although in the recent years taxane- and platin-based primary chemotherapy demonstrates efficacy (Frasci *et al*, 2009), TNBC commonly acquires chemoresistance and the relapsed cancer is commonly pan-resistant to all anticancer agents (Borst, 2012).

The MDA-MB-231$_{PAC10}$ cell line is highly resistant to PAC-induced cytotoxicity (>115-fold), inhibition of clonogenicity (~400-fold) and apoptosis (~75-fold). It is also significantly cross-resistant to CDDP, DOC and DOX. The resistant cells have significantly lower proliferation rate and longer doubling time with higher proportion of cells blocked in the G0/G1 phase. It has been known for long time that classical anticancer agents primarily target cycling cancer cells. The quiescent cancer cell population located in the G0/G1 phase is resistant to chemotherapeutic agents (Shah and Schwartz, 2001; Guo *et al*, 2008). Paclitaxel is predominately an M-phase-specific drug that stabilises microtubules causing an M-phase arrest followed by apoptosis (Schiff *et al*, 1979).

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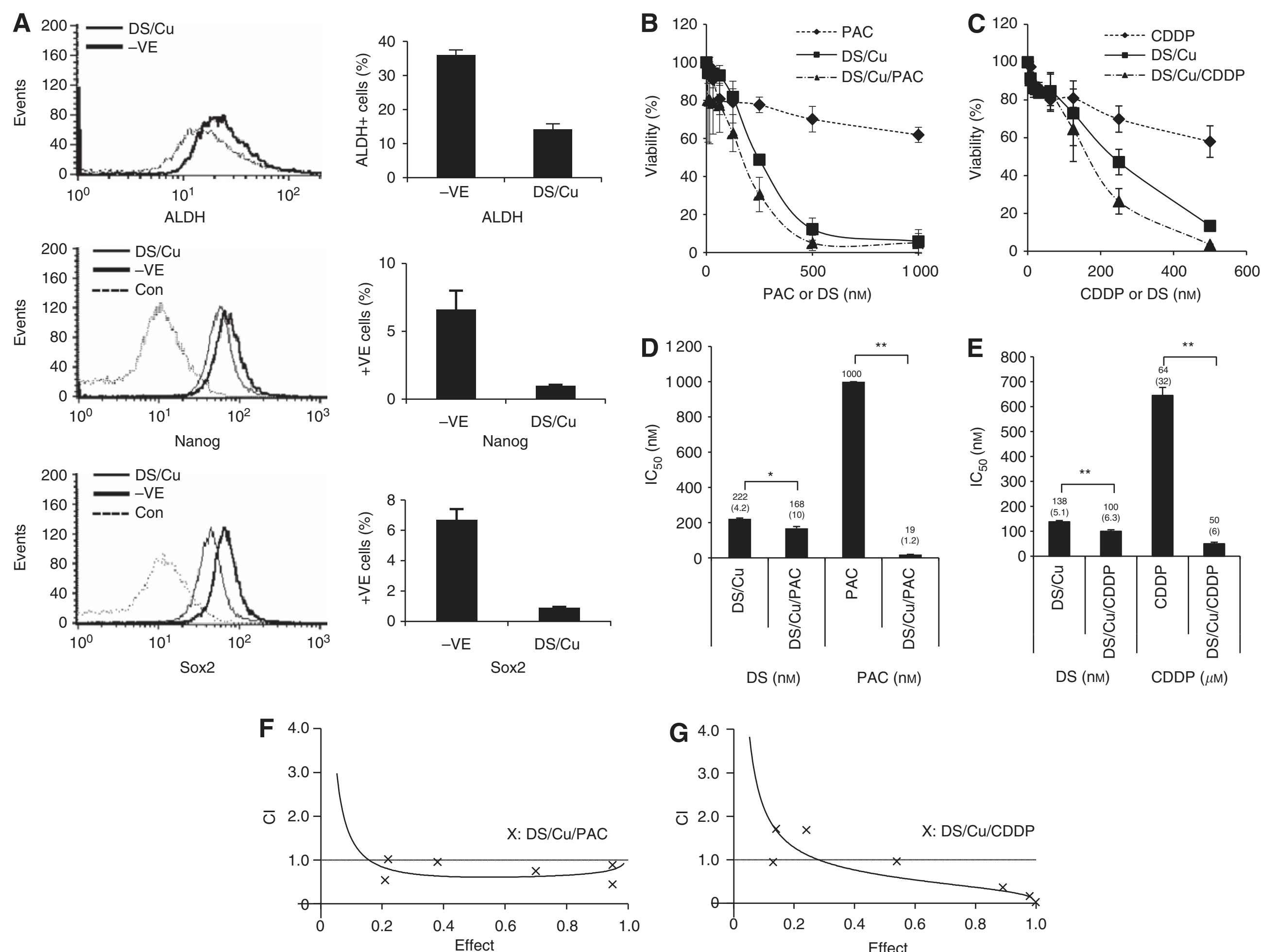

Figure 5. The DS/Cu inhibits CSC markers and synergistically enhances cytotoxicity of PAC and CDDP in MDA-MB-231$_{PAC10}$ cells. (**A**) The DS/Cu inhibits ALDH activity and the expression of Sox2 and Nanog protein in MDA-MB-231$_{PAC10}$ cell line. Con = Isotype control of PAC10. The DS/Cu enhances the cytotoxicity of PAC (**B** and **D**) and CDDP (**C** and **E**) in MDA-MB-231$_{PAC10}$ cells Mean from three independent experiments (*$P<0.05$, **$P<0.01$). (**F** and **G**) The Fa-CI plot of isobologram analysis for DS/Cu plus PAC (**F**) and DS/Cu plus CDDP (**G**). The CI value of below 1 indicates synergistic effect between DS/Cu and PAC or CDDP. The cells were exposed to DS and $CuCl_2$ (1 μM) for 4 h and then released in drug-free medium for 24 h.

Similarly, CDDP, DOC and DOX can only target the cycling and proliferating cells. Therefore, all of these drugs may lose their anticancer activity if the cancer cells are prevented from entering cell cycle by G0/G1-phase arrest. The p21^{Waf1} is a CDK inhibitor inactivating the activity of cyclin A, E and CDK2 that are essential for G1/S transition. Overexpression of p21^{Waf1} induces anticancer drug resistance (Bunz *et al*, 1999; Lazzarini *et al*, 2008). Western blot shows that p21^{Waf1} protein is massively upregulated in the resistant cell line. Because MDA-MB-231 is p53 mutant (Phalke *et al*, 2012), the upregulation of p21^{Waf1} in the resistant cell line is p53 independent. The high p21^{Waf1} expression may be responsible for the G0/G1 block in the resistant cell line. It has been demonstrated that bryostatin-1 induced PAC resistance via upregulation of p21^{Waf1} (Koutcher *et al*, 2000). Flavopiridol and bryostatin-1 are CDK inhibitors that slow down cell cycle. After pre-exposure to flavopiridol or bryostatin-1, breast cancer cells become highly resistant to PAC because of flavopiridol- and bryostatin-1-induced G0/G1 arrest. The cell cycle disturbance may be one of the determinants of PAC resistance in the MDA-MB-231$_{PAC10}$ cell line. Previous studies indicate that over-expression of p21 and cell cycle perturbations can also induce resistance to CDDP, DOC and DOX (Wilkins *et al*, 1997; Shah and Schwartz, 2001; Koster *et al*, 2010). The overexpression of p21 and cell cycle perturbation in MDA-MB-231$_{PAC10}$ cell line may be, at least partly, responsible for its pan-resistance characteristics. In line with previous report (Trock *et al*, 1997), markedly overexpressed Pgp is also detected in the resistant cell line. Although high expression of Pgp plays a role in PAC resistance, MDR1 has no influence on cancer cell sensitivity to CDDP. High expression of Bcl2 protein and Bcl2/Bax ratio was detected in MDA-MB-231$_{PAC10}$ cells that may desensitise the resistant cell line to apoptosis induced by PAC and other drugs (Ferlini *et al*, 2009).

The term of CSCs is adopted from normal stem cells. This is based on the findings that a small proportion (<1%) of cancer cells possess normal stem cell markers, for example, CD133, CD44, Nanog, Oct4, Sox2, ALDH and so on. Some studies demonstrated that this group of cancer cells is responsible for tumour initiation. However, there are many contradictory reports as well (Clevers, 2011). In contrast with normal stem cells, the CSCs and non-CSCs are reversible *in vitro* and *in vivo*. The stemness status of CSCs is highly microenvironment dependent. Recent studies suggested that hypoxia and some hypoxia-regulated transcription factors are the determinants for the stemness of CSCs (Conley *et al*, 2012). Actually, CSCs may reflect the microenvironment-dependent heterogeneity and epithelial–mesenchymal transition within tumour tissues. Although the role of CSCs in tumourigenesis is still debatable, it is widely accepted that the cancer cells expressing stem cell markers are highly resistant to radio- and chemotherapy

and are the sources of cancer recurrence (Bjerkvig *et al*, 2005; Dean *et al*, 2005; Clevers, 2011). Also, the cells with CSC markers are resistant to all different anticancer drugs. Therefore, CSCs may be the cause of pan-chemoresistance that is a common and a very serious problem faced in cancer therapeutics. Elimination of these cells may improve the outcomes of cancer chemotherapy. It has recently been reported that CSCs are involved in acquired taxane resistance (Domingo-Domenech *et al*, 2012; McAuliffe *et al*, 2013). In contrast with the fast growing cancer mass, CSCs are slow-cycling dormant cells expressing stem cell markers. High expression of Pgp is also a common feature of CSCs (Dean, 2009). Recent reports indicate that $p21^{Waf1}$ is indispensable for maintaining the quiescent status, stemness and preventing excess DNA-damage accumulation in CSCs (Viale *et al*, 2009). Our findings in MDA-MB-231_{PAC10} cell line, for example, high p21 expression, cell cycle slowing down and high expression of Pgp, indicate that the high population of CSCs in this cell line may play a crucial role for the pan-resistance. Based upon this hypothesis, we examined several other CSC phenotypes. High levels of ALDH, a functional CSC marker, were detected in the resistant cells. The resistant cell line also expresses higher levels of CD44 (data not shown). The recent publications (Landen *et al*, 2010; Schafer *et al*, 2012) and our unpublished data indicate that high ALDH activity confers chemoresistance upon cancer cells that can be reversed by targeting ALDH. High expression of the embryonic stem cell-associated genes Sox2, Oct4 and Nanog was also detected in the resistant cell line. Hypoxia-induced HIFs overexpression and NF-κB pathway activation is responsible for chemoresistance (Wang *et al*, 2004) and also the determinant factors for maintaining stemness of CSCs (Conley *et al*, 2012). Even cultured in normoxic condition, the overexpression and nuclear translocation of HIF2α and NF-κBp65 were detected in the resistant cell line. Further studies are being performed in our lab to elucidate the relationship between these factors and CSC-related chemoresistance.

Disulfiram is a very efficacious ALDH inhibitor and CSC-targeting agent, demonstrating strong chemoresistance-reversing activity (Yip *et al*, 2011; Hothi *et al*, 2012; Liu *et al*, 2012; Triscott *et al*, 2012). Previous clinical studies manifest that DS and its derivative effectively improve survival of breast and other cancer patients (Lewison, 1977; Dufour *et al*, 1993; Brar *et al*, 2004). In this study we examined its direct cytotoxicity and resistance-reversing effect on PAC and CDDP in MDA-MB-231_{PAC10} cells. Our results show that in contrast to its high resistance to PAC, DOC, DOX and CDDP, the MDA-MB-231_{PAC10} cell line remains very sensitive to DS-induced cytotoxicity. After exposure to DS for only 4 h, the clonogenicity of the resistant cell line was completely eradicated. The CI–isobologram analysis demonstrates that DS synergistically enhances the cytotoxicity of PAC and CDDP in MDA-MB-231_{PAC10} cells. In combination with DS/Cu, the PAC and CDDP resistance in MDA-MB-231_{PAC10} cell line is completely reversed. The stem cell markers, for example, ALDH activity and the expression of Sox2 and Nanog in the resistant cell line, are markedly inhibited by DS exposure. Therefore, DS may reverse pan-chemoresistance in MDA-MB-231_{PAC10} cell line by targeting BCSCs. The simultaneous inhibition and induction of Bcl2 and Bax indicates that DS may induce apoptosis in the resistant cells via an intrinsic pathway (Guo *et al*, 2010; Yip *et al*, 2011; Liu *et al*, 2012). Although DS inhibits MDR1 activity (Loo *et al*, 2004), it has no effect on the expression of Pgp. There is no effect of DS on cell cycle status in the resistant cell line. Similar to many other DNA-targeting agents, DS exposure further induces p21 expression in the resistant cells. Anticancer stem cell is a hot spot for anticancer drug development (Zhou *et al*, 2009). New drug development is a very time-consuming and costly procedure. Disulfiram has been used as an antialcoholism drug for over 60 years with preclinical and clinical safety data available. Therefore, it is relatively easier for repositioning of it into cancer indication (Cvek, 2012).

CONCLUSIONS

A newly developed PAC-resistant BC cell line, MDA-MB-231_{PAC10}, is cross-resistant to a panel of different anticancer drugs, for example, DOC, DOX and CDDP. We first reported that acquired BC cell line consists of high proportion of cells expressing CSC markers that may be, at least partly, responsible for its acquired pan-chemoresistant characteristics. We also manifested that DS, an antialcoholism drug, abolishes the cancer stem-like population and efficaciously reverses the PAC and CDDP resistance in MDA-MB-231_{PAC10} cell line.

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