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 (Kneuermann 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 sensitize 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/CD44High) and activation of embryonic-expression of stem cell markers (aldehyde dehydrogenases population of cancer stem cells (CSCs) that can be detected by the identifying new compounds to target alternative chemoresistant pathways and sensitize cancer cells to classical anticancer drugs.

The MDA-MB-231PAC10 cells were maintained in the medium containing 10 μM 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/Cu1 μM or in combination of PAC/DS/Cu1 μM or CDDP/DS/Cu1 μM at a constant ratio of PAC/DS (10:1) and CDDP/DS (500:1) determined by IC50 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/Cu1 μM and CDDP/DS/Cu1 μM 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 × 104 cells per well) were cultured in 24-well plates in triplicate. The cells were collected by trypsination 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 × 104 cells per well) were cultured in six-well plates over night and then exposed to designated concentration of DS in combination with 1 μM CuCl2 (DS/Cu1 μM) for 4 h or PAC (20 μM) 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 × 103 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.nih.gov/ij).}

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 nuclei 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 trypsination. 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 × 10⁶) 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 × 10⁶) 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-231PAC10 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 IC₅₀ of 8.7 nM. In contrast, the MDA-MB-231PAC10 cell line is highly resistant to PAC with an IC₅₀ of over 1000 nM. The cytotoxic effect of CDDP, DOC and DOX on MDA-MB-231PAC10 cell line was also evaluated. Table 1 and Figure 1B demonstrate that MDA-MB-231PAC10 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-231PAC10 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-231PAC10 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.

**Resistance of MDA-MB-231PAC10 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-231PAC10-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-231PAC10 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).

**Table 1. Cytotoxicity of disulfiram and conventional anticancer drugs to MDA-MB-231 and MDA-MB-231PAC10 BC cell lines**

|        | PAC  | CDDP | DOC  | DOX  | DS  |
|--------|------|------|------|------|-----|
| IC₅₀   |       |      |      |      |     |
| MDA    | 8.7 (2.3) | >1000** | 256.7 (26.1) | 4.6 (3.3) | 27.6 (2.5) |
| MDA-PAC10 | 645.4 (127.3) | >250** | 1575** (169.3) | 151.9 (12.1) | 116.4 (30.0) |

**CI value**

|        | PAC  | CDDP | DOC  | DOX  | DS  |
|--------|------|------|------|------|-----|
| IC₅₀   | 0.61 | 0.64 | NA   | NA   | NA  |
| IC₇₅   | 0.64 | 0.41 | NA   | NA   | NA  |
| IC₅₀   | 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 (IC₅₀) 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 120h (CDDP). DS/Cu = DS in medium supplemented with 1 µM CuCl₂.

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MDA-MB-231PAC10 has longer doubling time. In the cell culture, the MDA-MB-231PAC10 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-231PAC10 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-231PAC10 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] x 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-231PAC10 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 E and G show that in comparison with the parental cells, the MDA-MB-231PAC10 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...
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Disulfiram is highly cytotoxic in MDA-MB-231PAC10 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-231PAC10 cell line to DS is comparable to that of parental cells (Figure 4A and Table 1). The clonogenicity of both parental and resistant cell lines is completely abolished after very short exposure (4 h) to DS$_{1\, \mu M}$/C$_{1\, \mu M}$ (Figure 4B). The DS/Cu induces apoptosis in MDA-MB-231PAC10 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-231PAC10 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-231PAC10 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-231PAC10 cells. The MDA-MB-231PAC10 cell line is composed of high population of cells expressing stem cell markers that may play a key role in the panresistance. 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-231PAC10 cell line. In combination with DS/Cu the

Figure 1. The MDA-MB-231PAC10 cell line is resistant to PAC and cross-resistant to CDDP. (A and B) Surviving curves of MTT cytotoxicity assay. The MDA-MB-231PAC10 (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 ≥ 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.
Figure 2. The MDA-MB-231_{PAC10} cell line is resistant to PAC-induced apoptosis. (A) The morphology (× 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.
Figure 3. The MDA-MB-231PAC10 cell line proliferates slower and expresses cancer stem cell markers. (A) Growth curves of MDA-MB-231 and MDA-MB-231PAC10 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-231PAC10 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-231PAC10 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-231PAC10 cells by immunofluorescent confocal microscopy × 400 magnification.
cytotoxicity of PAC and CDDP in MDA-MB-231PAC10 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 (IC50/C0, IC90, 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-231PAC10 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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Similiarly, 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 p21Waf1 is a CDK inhibitor inactivating the activity of cyclin A, E and CDK2 that are essential for G1/S transition. Overexpression of p21Waf1 induces anticancer drug resistance (Bunz et al, 1999; Lazzarini et al, 2008). Western blot shows that p21Waf1 protein is massively upregulated in the resistant cell line. Because MDA-MB-231 is p53 mutant (Phalke et al, 2012), the upregulation of p21Waf1 in the resistant cell line is p53 independent. The high p21Waf1 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 p21Waf1 (Koutrcher 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-231PAC10 cell line. Previous studies indicate that overexpression 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-231PAC10 cell line may be, at least partly, responsible for its pan-resistance characteristics. In line with previous report (Troick 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-231PAC10 cells that may desensitize 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 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.

Figure 5. The DS/Cu inhibits CSC markers and synergistically enhances cytotoxicity of PAC and CDDP in MDA-MB-231PAC10 cells. (A) The DS/Cu inhibits ALDH activity and the expression of Sox2 and Nanog protein in MDA-MB-231PAC10 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-231PAC10 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 CuCl₂ (1 µM) for 4 h and then released in drug-free medium for 24 h.
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 p21Waf1 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-231PAC10 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; Hothis 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 (Leuwison, 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-231PAC10 cells. Our results show that in contrast to its high resistance to PAC, DOX, DOX and CDDP, the MDA-MB-231PAC10 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-231PAC10 cells. In combination with DS/Cu, the PAC and CDDP resistance in MDA-MB-231PAC10 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-231PAC10 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 (Zhao 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-231PAC10 is cross-resistant to a panel of different anticancer drugs, for example, DOX, 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-231PAC10 cell line.

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