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Activation of ALDH1A1 in MDA-MB-468 breast cancer cells that over-express CYP2J2 protects against paclitaxel-dependent cell death mediated by reactive oxygen species

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Author contribution statement

SE Allison, Y Chen, J Zhang, K Bourget and N Petrovic performed the research, SE Allison and M Murray designed the study, N Petrovic contributed essential reagents, SE Allison, Y Chen, J Zhang, K Bourget, PI Mackenzie and M Murray analysed the data, SE Allison, Y Chen, N Petrovic, PI Mackenzie and M Murray wrote the paper
Abstract

Cytochrome P450 2J2 (CYP2J2) expression is elevated in breast and other tumors, and is known to be protective against cytotoxic agents that may be used in cancer chemotherapy. This study evaluated the mechanisms by which MDA-MB-468 breast cancer cells that stably expressed CYP2J2 (MDA-2J2 cells) were protected against killing by the anti-cancer agent paclitaxel. Compared to control cells caspase-3/7 activation by paclitaxel was lower in MDA-2J2 cells, while cell proliferation and colony formation following paclitaxel treatment were increased. Basal lipid peroxidation was lower in MDA-2J2 cells than in control cells, and the paclitaxel-mediated increase in peroxidation was attenuated. The mitochondrial complex III inhibitor antimycin A modulated basal and paclitaxel-activated reactive oxygen species (ROS) formation in control cells; paclitaxel-activated ROS production was also modulated by the NADPH oxidase inhibitor diphenyleneiodonium. Paclitaxel increased the formation of protein adducts by the reactive aldehyde 4-hydroxynonenal that is produced by lipid peroxidation; adduct formation was attenuated in MDA-2J2 cells. ALDH1A1 expression and activity was strongly upregulated in MDA-2J2 cells that was attributed to CYP2J2-derived 14,15-epoxyeicosatrienoic acid (14,15-EET); the 8,9- and 11,12-EET regioisomers did not activate ALDH1A1 expression. Silencing of ALDH1A1 restored the sensitivity of MDA-2J2 cells to paclitaxel, as indicated by a more pronounced decrease in proliferation, and greater increases in caspase activity and formation of ROS to levels comparable with control cells. Similar findings were observed with doxorubicin, sorafenib and staurosporine, that also promoted ROS-mediated cell death that was attenuated in MDA-2J2 cells and reversed by ALDH1A1 gene silencing. These findings implicate ALDH1A1 as an important gene that is activated in MDA-MB-468-derived cells that contain high levels of CYP2J2. ALDH1A1 modulates the production of ROS by anti-cancer agents such as paclitaxel and diminishes their efficacy. Future approaches could adapt this information to facilitate the targeting of
ALDH1A1 to promote the efficacy of ROS-generating cytotoxic agents and enhance the treatment of breast cancer.

**Key Words**

Cytochrome P450 2J2; breast cancer drug resistance; reactive oxygen species; aldehyde dehydrogenase 1A1; 14,15-EET; cell survival

**Compounds**


**Abbreviations**

ALDH1A1, aldehyde dehydrogenase 1A1; CYP2J2, cytochrome P450 2J2; DCFDA, 2′,7′-dichlorofluorescein diacetate; DMEM, Dulbecco’s modified eagle medium; DMSO, dimethylsulfoxide; EET, epoxyeicosatrienoic acid; 4-HNE, 4-hydroxynonenal; MDA-CTL, MDA-MB-468 cells stably transfected with green fluorescent protein; MDA-2J2, MDA-MB-468 cells stably transfected with CYP2J2 and green fluorescent protein; MTT, 3-(4,5-
dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; ROS, reactive oxygen species; RT-PCR, real time-PCR; TBARs, thiobarbituric acid-reactive substances; TBS, Tris-buffered saline; UGT, UDP-glucuronosyltransferase.
1. Introduction

The cytochrome P450 2J2 (CYP2J2) epoxygenase is upregulated in a number of cancer types (Jiang et al., 2005), including breast tumours (Wei et al., 2014). CYP2J2 converts arachidonic acid to four regioisomeric epoxyeicosatrienoic acids (5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET) that promote tumourigenesis and tumour cell survival by increasing proliferation and decreasing apoptosis (Chen et al., 2011; Jiang et al., 2007). Although the activation of prosurvival signalling pathways has been demonstrated in cells that over-express CYP2J2, or that produce high quantities of EETs (Yang et al., 2007), there is less information on the underlying protection mechanisms.

Reactive oxygen species (ROS) have bimodal actions in the regulation of tumour viability. ROS are present in breast tumours (Matsui et al., 2000; Trachootham et al., 2009), which promotes cell proliferation and metastatic progression (Nishikawa, 2008). In contrast, enhanced production of ROS is implicated in tumour cell killing by a range of chemotherapeutic agents (Alexandre et al., 2006; 2007; Coriat et al., 2012; Lown, 1985; Mimnaugh et al., 1989). Mitochondrial electron transport activity is a major site of ROS generation with other potentially important sites including the enzymes xanthine oxidase and NADPH oxidase (Bedard and Krause, 2007; McNally et al., 2003; St Pierre et al., 2002). Antioxidant enzymes, such as glutathione peroxide and catalase, and biotransformation enzymes, such as the aldehyde dehydrogenases (ALDHs) and UGT-glucuronosyltransferases (UGTs), are potentially important in protecting cells against ROS and ROS-derived reactive aldehydes that mediate lipid peroxidation (Singh et al., 2013).

The present study was undertaken to investigate the mechanism by which CYP2J2 overexpression protects triple-negative MDA-MB-468 breast cancer cells (MDA-2J2 cells) against the cytotoxic chemotherapeutic agent paclitaxel. The principal finding was that
expression of ALDH1A1 was markedly enhanced in MDA-2J2 cells and that this enzyme was a major determinant of the efficacy of paclitaxel by conferring protection against ROS-mediated cell death.
2. Materials and methods

2.1. Materials

Paclitaxel, doxorubicin, staurosporine, clotrimazole, dimethylsulfoxide (DMSO), 2′,7′-dichlorofluorescein diacetate (DCFDA), 2-thiobarbituric acid, 1,1,3,3-tetramethoxypropane, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), malondialdehyde tetrabutylammonium, trans-2-hexenal, PD98059 (ERK inhibitor) the protease inhibitor cocktail and Dulbecco’s modified eagle medium (DMEM, containing 1 g glucose/L) were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). Sorafenib was from Toronto Research Chemicals (Toronto, ON, Canada). Fetal bovine serum was purchased from Thermo Scientific (Scoresby, VIC, Australia) and trypan blue, 0.5% trypsin-EDTA and penicillin-streptomycin (5000 U/mL) were from Life Technologies (Mulgrave, VIC, Australia). Phosphate buffered saline (PBS) was from Amresco (Solon, OH, USA). The ALDH activity assay kit, thiobarbituric acid-reactive substances (TBARs) lipid peroxidation kit, the JC-1 Mitochondrial Membrane Potential Assay Kit, 4-hydroxynonenal (4-HNE), diphenyleneiodonium chloride (DPI), antimycin A1 (antimycin) and allopurinol were purchased from Cayman Chemical (Ann Arbor, MI, USA). SB203580 (p38 mitogen-activated protein kinase; p38 MAP kinase inhibitor), and LY294002 (phosphoinositide 3-kinase/Akt inhibitor) were purchased from Sapphire Biosciences (Waterloo, NSW, Australia).

Murine monoclonal antibodies used to detect human ALDH1A1 and β-actin were from BD Bioscience (cat 611194; Heidelberg, Germany) and Santa Cruz Biotechnology (sc-47778; Santa Cruz, CA), respectively. The mouse monoclonal 4-HNE antibody (MAB3249) and recombinant human ALDH1A1 enzyme were from R&D Systems Inc. (Minneapolis, MN, USA). The rabbit anti-human glyceraldehyde 6-phosphate dehydrogenase antibody
(14C10, #2118) was from Cell Signaling Technology (Arundel, QLD, Australia) and the goat anti-mouse secondary antibody (LCR 926-68020) was purchased from Millennium Science (Mulgrave, VIC, Australia).

2.2. Cell lines

The human MDA-MB-468 cell line was obtained from American Type Culture Collection (Manassas, VA, USA). MDA-MB-231, A549 and PC3 cell lines were obtained from ATCC, while the SK-BR-3 and MCF10A cell lines were generous gifts from A/Prof Jenny Byrne, Children’s Hospital, Westmead, and Prof Christine Clarke, The Westmead Institute, Westmead Hospital, NSW, Australia, respectively. MDA-MB-468 cells were stably transfected with CYP2J2 and green fluorescent protein vectors under the control of the CMV promoter (MDA-2J2 cells) or green fluorescent protein vector alone (MDA-CTL cells), as described previously (Allison et al., 2016). Three other clonal cell lines were obtained during the preparation of MDA-2J2 cells (MDA-2J2-I, MDA-2J2-II and MDA-2J2-III). CYP2J2 mRNA was over-expressed to 0.12-, 0.19- and 0.30-fold, respectively, of that in MDA-2J2 cells. Corresponding ALDH1A1 mRNA expression was 0.21-, 0.33- and 0.39-fold, respectively, of that in MDA-2J2 cells. The MDA-2J2 cell line was used in all of the experiments described. CYP2J2 over-expression was confirmed by real time-PCR (RT-PCR) and immunoblotting. All cell lines were maintained in DMEM medium containing 10% fetal bovine serum and 100 U/mL penicillin-streptomycin, were cultured at 37°C in an atmosphere of 5% CO₂ and used between passage 12-20.

2.3. Western blotting

Whole cell lysates were prepared in Laemmli sample buffer containing 50 mM dithiothreitol, sonicated for 5 sec (Sonifier 250; Branson Ultrasonics Co., Danbury, CT) and incubated at 95°C for 5 min. Lysates were electrophoresed on 7.5% sodium dodecyl sulfate-
polyacrylamide gels (Murray et al., 1991), and subjected to western immunoblotting essentially as described previously (Chen et al., 1995). Proteins were transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany), washed with 5% milk in Tris-buffered saline (TBS; pH 8.8) and then incubated overnight at 4°C with primary antibody (1:1000 dilution; 1% bovine serum albumin in TBS containing 0.1% Tween). After washing and incubation with the secondary antibody, signals on immunoblots were detected using an Odyssey IP imaging system (LI-COR Biosciences, Lincoln, NE, USA).

2.4. RNA extraction and real-time PCR

Cells were seeded on six-well plates (3 x 10^5 cells/well), allowed to adhere for 24 h and then incubated in minimal DMEM medium for 18 h. Cells were washed with PBS and total RNA was extracted (Purelink RNA mini kit; Life Technologies) and quantified spectrophotometrically (NanoDrop Technologies; BioLab Pty Ltd., Scoresby, Vic, Australia). RNA samples were treated with RQ1 DNase (Promega Corp; Alexandria, NSW, Australia) prior to RT-PCR for comparison between MDA-468 and MDA-2J2 mRNA expression. Primer sequences and PCR cycling conditions are shown in Table 1. RT-PCR was conducted in a Rotor-Gene 6000 thermal cycler (Corbett Life Science, Mortlake, NSW, Australia) using the express one-step SYBR® GreenER™ Universal qPCR supermix (Life Technologies). PCR conditions were validated by melting curve or agarose gel analysis. Relative gene expression was calculated by the ΔΔCt method.

2.5. RNA interference

Cells were seeded and transfections were carried out using lipofectamine® RNAiMAX transfection reagent (Life Technologies). siRNA that targeted ALDH1A1 or CYP2J2 (siGENOME human ALDH1A1 or CYP2J2 siRNA, SMARTpool) or green fluorescent protein (control) (eGFP siRNA) were obtained from Dharmacon (Millennium
Science Pty Ltd, Mulgrave, VIC, Australia) and were used at a final concentration of 8 pM. Cells were incubated with siRNA for 6 h before replacement of media with DMEM (10% FBS). After 24 h, cells were either treated with serum-free DMEM for 18 h or collected for analysis.

2.6. Cell proliferation, apoptosis and mitochondrial membrane potential

For the estimation of proliferation, cells were seeded (1 x 10^4 cells/96-well), allowed to adhere for 24 h and then cultured in serum-free DMEM for 18 h; in some experiments cells were transfected with siRNA as described above. After culture in serum-free DMEM, cells were treated for 24 h with either paclitaxel (0.1, 10 µM), doxorubicin (0.5, 1.0 µM) or sorafenib (2.0, 5.0 µM), or for 48 h with staurosporine (0.05, 0.1 µM). Effects of drug treatments on cellular MTT reduction were determined spectrophotometrically and IC_{50} values in the case of paclitaxel were estimated using the sigmoidal, non-linear fit function in Prism (GraphPad Software Inc., La Jolla, CA, USA). Colony formation assays in paclitaxel-treated MDA-MB-231 cells (0.5 µM, 24 h) in soft agar were conducted essentially as described by Franken et al. (2006).

For the assessment of apoptosis, cells were seeded (1 x 10^4 cells/96-well), allowed to adhere for 24 h and then cultured in serum-free DMEM for 18 h; in some experiments cells were transfected with siRNA as described above. After culture in serum-free DMEM for 18 h, cells were treated with paclitaxel (1 µM) or DMSO (0.05%) for 24 h. Caspase activity was measured using the caspase-Glo 3/7 assay (Promega).

Mitochondrial membrane potential was assessed with the dye JC-1 according to the manufacturer’s protocol. Briefly, cells were seeded into black-walled 96-well plates (1.5 x 10^4 cells/well) and allowed to adhere overnight. Serum was removed and cells were treated 18 h later with paclitaxel (0.1, 1.0 or 10 µM, 4 h). Cells were incubated with JC-1 for 20 min
(37°C), centrifuged and then media was aspirated. PBS (200 µL) was added and fluorometric measurements were conducted in a SpectraMax Gemini XS microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.7. Assays of ALDH activity, ROS production and lipid peroxidation

ALDH activity was measured using an Aldehyde Dehydrogenase activity assay kit. Briefly, MDA-MB-468, MDA-CTL and MDA-2J2 cells were harvested and washed twice in PBS before being re-suspended in HEPES buffer (50 mM, pH 8) to a final concentration of ~10⁷ cells/mL for use in assays. All buffers contained protease inhibitor cocktail (Sigma).

The capacity of recombinant human ALDH1A1 to oxidize reactive aldehydes produced from lipid peroxidation was assessed (n=3 experiments). Briefly, incubations contained aldehyde substrate (4-HNE, malondialdehyde tetrabutylammonium or trans-2-hexen-1-al) and ALDH1A1 (10 µg/mL) in 0.1 M Tris.HCl buffer (pH 8.5) containing 0.1 M KCl and 2 mM DTT; reactions were initiated with 1 mM β-NAD and monitored at 339 nm.

For the assay of lipid peroxidation, cells (1 x 10⁷/mL) were suspended in PBS and sonicated on ice at 40 V (3x5 sec intervals; sonifier 250). Lipid peroxidation was measured using a TBARS kit; the standard curve was prepared with 1,1,3,3-tetramethoxypropane. Briefly, each reaction (1 mL final) contained 100 µl cell lysate, 1% trichloroacetic acid, 29 mM 2-thiobarbituric acid, 0.28 M NaOH and 1% acetic acid. Samples were incubated for 1 h at 90°C, placed on ice for 10 min and then centrifuged (1.6 x 10³ g, 4°C). After transfer to 96-well plates the fluorescence intensity was measured.

For the estimation of intracellular ROS content, cells were seeded (1.2 x 10⁵ cells/well, 12-well plate) and, after 24 h, were either transfected with siRNA or cultured in serum-free DMEM for 18 h. Cells were then treated with paclitaxel (0.01-1 µM), doxorubicin
(0.01µM), sorafenib (2.0 µM), staurosporine (0.01 µM) or DMSO. Experiments were conducted in the presence or absence of the potential ROS inhibitors antimycin (0.1 µM), DPI (0.1 µM) or allopurinol (0.25 µM). After 48 h, cells were washed once with PBS, treated with HyQ®Tase™ cell detachment solution (GE Healthcare Life Sciences, Logan, UT, USA), pelleted and resuspended in PBS (2% FBS, 500 µM EDTA). DCFDA was added to a final concentration of 20 µM. After incubation in the dark for 45 min (37°C, 5% CO₂) cells were placed on ice, propidium iodide was added (final concentration 30 µg/mL) and flow cytometry was conducted (Gallios, Beckman Coulter Australia, Lane Cove, NSW) using Gallios software.

2.8. Statistical analysis

All data are presented as means ± SEM throughout and were derived from three independent experiments unless otherwise stated. Data from multiple treatments were analysed by one-way ANOVA followed by Tukey’s multiple comparison’s test.
3. Results

3.1. Over-expression of CYP2J2 protects MDA-MB-468 breast cancer cells against paclitaxel-mediated cell death

MDA-2J2, MDA-CTL and parental MDA-468 breast cancer cells were treated with paclitaxel (0.1 or 10 µM, 24 h) and cell proliferation, as reflected by MTT reduction, was assessed. As shown in Fig 1A, proliferation of both MDA-CTL and MDA-468 was significantly decreased by paclitaxel (0.1 µM, 24 h) to 63±7% and 67±4% of DMSO-treated control (P<0.05); similar decreases were produced by 10 µM paclitaxel. In contrast, MDA-2J2 cells were much less susceptible to paclitaxel. Proliferation was unchanged at the lower concentration of paclitaxel and only a trend toward decreased proliferation was noted at the 10 µM concentration (to 79±9% of DMSO-treated control; Fig 1A). The capacity of paclitaxel to activate apoptotic cell death was also assessed in these cell lines. Paclitaxel (1 µM, 24 h) increased caspase-3/7 activity in each cell line (P<0.001), but the extent of the increase was less pronounced in MDA-2J2 cells (to 2.05±0.5-fold of DMSO control, compared with increases to 2.50±0.13 and 2.94±0.09-fold of corresponding control in MDA-CTL and MDA-468 cells, respectively; Fig 1B). The apparent protective effects of CYP2J2 over-expression were confirmed in colony formation assays. MDA-2J2 cells maintained 67±10% of colony formation after paclitaxel (0.05 µM; 24 h), compared to the much lower survival of MDA-CTL and MDA-468 cells (21±6% and 24±6%, respectively; P<0.05 relative to MDA-2J2; Fig 1C).

3.2. Effect of CYP2J2 overexpression on cellular lipid peroxidation

Because ROS have been implicated in paclitaxel-mediated cell death we assessed lipid peroxidation in MDA-MB-468-derived breast cancer cells. Basal lipid peroxidation was markedly lower in MDA-2J2 cells (4.8±0.9 nM) than in MDA-CTL and MDA-468 cells...
(12.6±0.9 nM, P<0.01, and 10.7±1.0 nM, P<0.05, respectively; Fig 2A). Following paclitaxel treatment (1 µM, 24 h), lipid peroxidation was increased in all cell types, although the increase was less pronounced in MDA-2J2 cells (8.6±1.5 nM) than in MDA-CTL or MDA-468 cells (20.2±0.6 nM and 17.4±1.3 nM, respectively, Fig 2A) In further studies, MDA-2J2 and MDA-468 cells were treated with paclitaxel (0.01–1.0 µM) and the proportion of cells containing high intracellular concentrations of ROS (ROS$_{\text{high}}$, left shift in histograms; Fig 2A) was estimated by flow cytometry using DCFDA. Treatment of MDA-468 cells with paclitaxel (0.1 or 1 µM) increased the proportion of ROS$_{\text{high}}$ cells from 9.4±1.6% of total, to 20.6±1.0% and 36.6±2.4% of total (P<0.001), respectively (Fig 2B). By contrast, in MDA-2J2 cells the ROS$_{\text{high}}$ cell population was unchanged by low concentrations of paclitaxel (0.01 or 0.1 µM) and was only increased at the highest concentration (1.0 µM: 24.9±2.1% compared to 5.9±4% in DMSO-control; P<0.001, Fig 2B).

Potential sources of ROS production were assessed in paclitaxel-treated MDA-CTL cells relative to DMSO-treated control (Fig 2C). The ROS$_{\text{high}}$ cell population in DMSO-treated cells was decreased by antimycin, but not allopurinol or DPI. In contrast, the ROS$_{\text{high}}$ cell population in paclitaxel-treated cells was decreased by antimycin and DPI, but not allopurinol (Fig 2C). These findings suggest that mitochondrial complex activity mediates basal ROS production and that paclitaxel-mediated ROS production involves both mitochondrial complexes and NADPH oxidases. From studies with JC-1, paclitaxel-dependent ROS production occurred without any alteration to the mitochondrial membrane potential (not shown).

3.3. ALDH1A1 is selectively upregulated in MDA-2J2 cells

To obtain further mechanistic information we examined the expression of genes with potential roles in the modulation of ROS and in cellular protection against paclitaxel. In
agreement with our previous microarray findings (Allison et al., 2016), ALDH1A1 mRNA was strongly upregulated in MDA-2J2 cells (to 227±31 fold of MDA-468; \( P<0.001 \); Fig 3A). Increased ALDH1A1 function in MDA-2J2 cells relative to both MDA-CTL and MDA-468 cells was confirmed at the levels of protein expression and enzyme activity (Fig 3A). In contrast, the expression of eight other ALDH genes (ALDHs 1A2, 1A3, 2, 3A1, 3B1, 3B2, 4A1 and 5A1), five UGTs (UGTs 1A1, 1A3, 1A5, 1A6 and 1A8), the ABC transporters MDR1 and MRP1, and the anti-oxidant enzymes catalase and glutathione peroxidase was minimally changed from control (range: 0.61-2.32-fold of MDA-CTL; not shown).

Treatment of MDA-2J2 cells with siRNA directed against CYP2J2 and the CYP2J2 inhibitor clotrimazole both decreased ALDH1A1 immunoreactive protein (Fig 3B), consistent with a role for CYP2J2-derived EETs in activation of ALDH1A1 expression. We found previously that endogenous EET production was increased in MDA-2J2 cells to ~3.5-fold of that in MDA-CTL (Allison et al., 2016). In the present study we assessed the role of individual EET regioisomers in the activation of ALDH1A1 expression in MDA-2J2 cells. As shown in Fig 3C, treatment with 14,15-EET (5 µM, 24 h) increased the expression of ALDH1A1 mRNA to 1.5±0.2-fold of control (\( P<0.05 \)); 8,9-EET and 11,12-EET were inactive. We also treated MDA-2J2 cells with inhibitors of EET-activated signal transduction pathways. As shown in Fig 3D the PI3-kinase/Akt inhibitor LY294002 (50 µM) decreased ALDH1A1 immunoreactive protein to 43±9% of MDA-2J2 control (48 h treatment; \( P<0.01 \)) whereas the less pronounced decreases produced by the p38 and ERK mitogen-activated protein kinase inhibitors SB203580 (10 µM) and PD98059 (25 µM) did not attain statistical significance.

In comparative studies we evaluated the expression of CYP2J2 and ALDH1A1 in the breast-derived MDA-MB-231, SK-BR-3 and MCF10A lines, lung-derived A549 cells and in prostate-derived PC3 cells. CYP2J2 was detected across the five cell lines, while ALDH1A1
was highly expressed in A549 cells, and at lower level in MDA-MB-231 and SK-BR-3 cells, but was essentially absent from MCF10A and PC3 cells (Fig 3E). We also evaluated the expression of an alternate ALDH1A isoform – ALDH1A3. Unlike ALDH1A1, ALDH1A3 mRNA was found to be most highly expressed in PC3 cells. There was no apparent relationship between the expression of the two ALDH1A isoforms across the different cell types. Considered together, while not uniform across all of the lines that were tested, co-expression of CYP2J2 and ALDH1A1 was found in cells other than those derived from MDA-MB-468 lines following the stable over-expression of CYP2J2.

3.4. ALDH1A1 gene silencing restored the sensitivity of MDA-2J2 cells to paclitaxel

The potential protective role of ALDH1A1 in attenuating the toxicity of paclitaxel in MDA-2J2 cells was evaluated using gene silencing. In initial studies ALDH1A1 knockdown was confirmed at the mRNA (not shown) and protein levels (Fig 4A). On silencing of ALDH1A1 in MDA-2J2 cells, the anti-proliferative activity of paclitaxel (10 nM – 10 µM, 24 h) was restored in MDA-2J2 cells to that in MDA-468 cells (Fig 4B). The IC\textsubscript{50} for paclitaxel in MDA-2J2 cells after transfection with control siRNA was 0.09 µM (95% CI 0.03–0.26 µM) and 0.04 µM (0.01–0.11 µM) with ALDH1A1 siRNA (P<0.05). In MDA-468 cells after transfection with control siRNA the IC\textsubscript{50} was 0.02 µM (0.01–0.04 µM) and 0.02 µM (0.01–0.03 µM) with ALDH1A1 siRNA. Consistent with these findings the paclitaxel-mediated increase in caspase-3/7 activity in MDA-MB-231 cells was also restored after ALDH1A1 gene silencing to levels comparable with those produced in MDA-468 control cells (2.51±0.07–fold versus 2.50±0.33–fold; Fig. 4C). ALDH1A1 siRNA gene silencing also increased ROS formation in MDA-2J2 cells in response to paclitaxel. Thus, after treatment with 0.1 µM paclitaxel, ROS\textsuperscript{high} cells increased from 12±1% of total after control siRNA transfection to 35±11% of total after ALDH1A1 siRNA transfection (P<0.05; Fig. 4D).
comparison, transfection of MDA-468 cells with control or ALDH1A1 siRNAs produced similar ROS\textsuperscript{high} cell proportions after paclitaxel treatment (0.1 µM; Fig 4D).

The paclitaxel-dependent increase in ROS formation and lipid peroxidation was explored further. We found that paclitaxel increased the formation of 4-HNE-protein adducts in MDA-468 and MDA-CTL cells (to ~1.36±0.04-fold of media alone; P<0.05) and that this was attenuated in MDA-2J2 cells (1.04±0.09-fold of media alone; Fig 4E). It has been reported that 4-HNE and other reactive aldehydes that are generated following lipid peroxidation are substrates for ALDH1A1 (Xiao et al., 2009). In the present study we confirmed that recombinant human ALDH1A1 was able to oxidise 4-HNE, malondialdehyde and \textit{trans}-2-hexenal (not shown). Together, these findings strongly suggest that ALDH1A1 protects MDA-2J2 cells against paclitaxel-mediated ROS production and lipid peroxidation because they detoxify reactive aldehydes that generate protein adducts.

We tested the capacity of additional cytotoxic agents to decrease the viability of MDA-MB-468-derived cells. Again, the MDA-2J2 cells showed resilience against the decrease in proliferation mediated by doxorubicin (0.5 µM, 24 h; \(P=0.05\)) and staurosporine (0.1 µM, 24 h; \(P=0.06\); Fig 5A). Although proliferation in MDA-2J2 and MDA-468 cells was comparable after sorafenib treatment, the ability of the MDA-2J2 cells to form colonies after treatment with sorafenib (0.5 µM) was greater than in control cells (102±10% from MDA-2J2 cells versus 57±3% colonies from MDA-468 cells, \(P<0.05\); not shown). Consistent with findings in paclitaxel-treated cells, doxorubicin (0.01 µM), sorafenib (2.0 µM) and staurosporine (0.01 µM) all increased ROS\textsuperscript{high} populations in MDA-468 but not in MDA-2J2 cells (Fig 5B). We also assessed potential sources of ROS production in MDA-468 cells after treatment with these agents. Similar to findings with paclitaxel, the mitochondrial complex inhibitor antimycin attenuated the doxorubicin-, sorafenib- and staurosporine-dependent increase in ROS\textsuperscript{high} cell populations (\(P<0.001\); Fig 5C). In addition, the NADPH oxidase
inhibitor DPI decreased the ROS\textsuperscript{high} populations in cells that were treated with doxorubicin and sorafenib, but not staurosporine. In summary, the present data indicate that CYP2J2 overexpression in triple-negative MDA-MB-468-derived breast cancer cells upregulated ALDH1A1 and conferred protection against paclitaxel and other agents that activated ROS production from mitochondrial complex III and NADPH oxidases.
4. **Discussion**

CYP2J2 is over-expressed in breast tumours and is positively correlated with tumour size and histological grade (Wei et al., 2014; Jiang et al., 2005). CYP2J2-derived EETs are known to promote the survival of tumour cells by decreasing apoptosis and increasing proliferation (Chen et al., 2011; Jiang et al., 2007; Wang et al., 2005). However, although important information has been presented on the activation of proliferative and prosurvival signalling pathways by EETs (Chen et al., 2001; Yang et al., 2007), there is less information on the mechanisms of protection; this was addressed in the present study. The stable overexpression of CYP2J2 protected MDA-MB-468-derived triple-negative breast cancer cells against paclitaxel-mediated killing. MDA-2J2 cells were less susceptible to the decrease in proliferation and the increase in apoptosis (caspase-3 activity) produced by paclitaxel. Consistent with increased protection, subsequent colony formation was more extensive in MDA-2J2 cells than in either parental MDA-468 or MDA-CTL cells after paclitaxel treatment.

Paclitaxel is an anti-tubule inhibitor that also increases the production of cytotoxic ROS in target tumour cells (Alexandre et al., 2006; 2007; Panis et al., 2012; Ramanathan et al., 2005). In the present study, rates of basal and paclitaxel-induced lipid peroxidation were lower in MDA-2J2 cells than in MDA-468 or MDA-CTL cells. Basal ROS production was attenuated by the mitochondrial complex III inhibitor antimycin, while antimycin and the NADPH oxidase inhibitor DPI both modulated paclitaxel-mediated ROS production. We also found a pronounced and selective increase in ALDH1A1 expression in MDA-2J2 cells relative to corresponding control cells. In contrast, the expression of eight other ALDH and five UGT enzymes that could also contribute to anti-cancer drug detoxification was minimally altered in MDA-2J2 cells relative to control. In addition, no evidence was obtained for altered expression of the major antioxidant genes catalase and glutathione peroxidase in
MDA-2J2 cells, or for increased expression of the ABC-transporters MDR1 and MRP1 that have been associated with anti-cancer drug resistance (Gottesman, 2002). The present findings support a major role for ALDH1A1 in the protective mechanism of CYP2J2 in MDA-2J2 cells because silencing of ALDH1A1 restored the capacity of paclitaxel to decrease cell viability to levels that were comparable with those in MDA-468 and MDA-CTL cells. In addition, the extent of 4-HNE–mediated protein modification that mediates a decrease in viability was attenuated in MDA-2J2 cells, which is consistent with the capacity of ALDH1A1 to detoxify reactive aldehydes (Xiao et al. 2009). Although ALDH1A1 is an established stem cell marker the present findings do not suggest that CYP2J2 over-expression increases the stem cell population in MDA-2J2 cells. Expression of the established breast cancer stem cell markers CD24 and CD44 (Al-Hajj et al., 2003) and members of the ABC-transporter gene family (Gillet et al., 2007) was not increased in MDA-2J2 cells (Allison et al., 2016).

In addition to paclitaxel, MDA-2J2 breast cancer cells were resistant to other agents that increased cellular ROS production and decreased cell proliferation, namely doxorubicin, sorafenib and staurosporine. Doxorubicin is known to kill cells via ROS-mediated mechanisms (Friesen et al., 1999; Gouaze et al., 2001) and circulating lipid peroxides are elevated in doxorubicin-treated patients (Panis et al., 2012). ROS production also contributes to the death of breast and liver-derived cells by the multikinase inhibitor sorafenib in vitro and in vivo. Indeed, the extent of sorafenib-mediated ROS formation correlates with the effectiveness of patient treatment (Coriat et al., 2012; Fumarola et al., 2013). In agreement with the findings with paclitaxel, the sources of doxorubicin- and sorafenib-mediated ROS production were mitochondrial complex III and NADPH oxidases. However, it should be added that it was suggested recently that CYP2J2 could also contribute to the biotransformation of TKIs (Narjoz et al., 2014); this possibility cannot be ruled out.
completely by the present findings. In addition, a role for ROS-mediated cell death by the protein kinase inhibitor staurosporine has been suggested (Kruman et al., 1998); this agent targets the mitochondrion and activates the intrinsic pathway of apoptosis (Zhang et al., 2004). Staurosporine-mediated ROS production was inhibited by the mitochondrial complex III inhibitor antimycin. High levels of ALDH1A1 have been also associated with resistance to other agents that promote ROS-mediated cytotoxicity, including temozolomide in glioblastoma cells (Schafer et al., 2012), and cyclophosphamide in hematopoietic and pulmonary tumor cells (Magni et al., 1996; Moreb et al., 2005). In the present study, the sensitivity of cells to paclitaxel was restored by ALDH1A1 knockdown.

The functional role of ALDH1 in tumor biology is incompletely understood but it appears to modulate cell proliferation and survival, and the response to certain oncology drugs (Magni et al., 1996; Moreb et al., 2005). Over-expression of ALDH1A1 has been shown to confer resistance to chemotherapeutic agents in breast cancer stem cells (Cojoc et al., 2015; Croker and Allan, 2012; Tanei et al., 2009). In triple-negative and HER2-positive breast cancers, high level expression of ALDH1A1 has been correlated with metastatic activity and poor prognosis, as well as to the outcome of chemotherapy in patients (Khoury et al., 2012; Liu et al., 2014; Wu et al., 2015). The present findings are in accord with reports that ALDH1A1 attenuates anti-cancer drug efficacy but now implicate CYP2J2 over-expression, which is a common feature of many human tumors, to increased expression and function of ALDH1A1 (Wei et al., 2014; Jiang et al., 2005).

The finding that CYP2J2 overexpression in MDA-MB-468 breast cancer cells markedly upregulates ALDH1A1 expression provides new insight into potential treatment strategies in CYP2J2-containing tumors. We found recently that over-expression of CYP2J2 in MDA-MB-468 cells activates EET production (Allison et al., 2016). In the present study the decrease in ALDH1A1 expression in MDA-2J2 cells after silencing of the CYP2J2 gene
or treatment with the CYP2J2 inhibitor clotrimazole implicated CYP2J2-dependent EETs in ALDH1A1 activation. In further studies we found that 14,15-EET activated CYP2J2 expression in MDA-2J2 cells while the regioisomers 8,9-EET and 11,12-EET were inactive. It was also found that the PI3-kinase/Akt inhibitor LY294002 decreased ALDH1A1 protein expression in MDA-2J2 cells. This finding is compatible with reports that EETs act in part through the PI3-kinase/Akt cascade in cells and implicate that pathway in ALDH1A1 activation in MDA-2J2 cells (Wang et al., 2005; Yang et al., 2007). Because knockdown of ALDH1A1 in MDA-2J2 cells restored the efficacy of paclitaxel the targeting of ALDH1A1 could be a viable approach for optimizing the efficacy of anti-cancer agents that act in part by ROS generation. There is some evidence that suggests that this approach could be useful. Agents such as diethylaminobenzaldehyde and disulfiram are established ALDH1 inhibitors that are relatively well tolerated (Koppaka et al., 2012). Disulfiram resensitized glioma cells to gemcitabine and depleted breast cancer stem cells that express ALDH1A1 (Wang et al., 2014; Liu et al., 2012) and also reportedly induced remission in a patient with stage IV metastatic melanoma (Brar et al. 2004).

The present findings support the mechanism proposed in Fig 6. CYP2J2-derived 14,15-EET activates ALDH1A1 expression that protects tumor cells against ROS-dependent lipid peroxidation and reactive aldehyde formation by cytotoxic agents such as paclitaxel. Reactive aldehydes like 4-HNE generate protein adducts that decrease cell viability; this is attenuated in CYP2J2 cells. While inhibitors of CYP2J2 have been found to decrease the growth of tumours (Chen et al., 2009), CYP inhibitors often lack selectivity and could elicit clinically significant pharmacokinetic drug interactions by modulating the activity of multiple CYPs (Farrell and Murray, 1990). In addition, CYP2J2-derived EETs are physiologically important in many extrahepatic tissues (Zeldin, 2001), which may undermine the strategy of targeting the CYP2J2 enzyme. Instead, approaches in which ALDH1A1 is targeted in
adjuvant treatments to be used in combination with cytotoxic agents may be a more refined approach to enhance outcomes from cancer chemotherapy.
Acknowledgements

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Conflict of interest

The authors have no conflicts of interest.
References


mechanism of action, substrate specificity, and clinical application. Pharmacol Rev 64: 520–539


Table 1. Primer sequences and RT-PCR cycling conditions.

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<th>Gene</th>
<th>forward primer</th>
<th>reverse primer</th>
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<td>ATGGATGCTTCCGAGAGG</td>
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<td>ALDH1A2</td>
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UGT1A6  GGCCTACCACATCTGTGATCTCTTTC  ATCCACATCTCTTTGAGGACAGC
UGT1A9  AGCCCCCTTCTCTATGTTG  TGGCATGACTACAACCACCT
MDR1  AATTACAGCAAGCCTGGAACC  TGCTCACAGACGGATGTAGTTG
MRP1  TCACACCAAGCCGGCGTCTTT  CTGACAAGCTAGACCATGAATGT
Catalase  CGAGCACCGTAGGGACA  TGAAGATGCGGCGAGAC
Glutathione peroxidase  GCACCGTTACCCTCGCAGCT  AGTCGGTGATGCGCTTCTCGG
CYP2J2  CGAAGGTGATGGGAGC  AGCTTAGAGGAACGC
Reverse transcription was carried out at 50°C (30 minutes), followed by 95°C (15 minutes), after which PCR cycling was conducted. Cycling conditions used for each primer set: (40 cycles) of 94°C (15 sec), and 60°C (60 sec) (ALDH genes), 94°C (60 sec), 63°C (30 sec), and 72°C (45 sec) (UGT genes except UGT1A1), 94°C (60 sec), 63°C (60 sec), and 72°C (60 sec) (UGT1A1), 94°C (60 sec), 60°C (30 sec), and 72°C (60 sec) (MRP1, catalase), 94°C (30 sec), 55°C (60 sec), and 72°C (30 sec) (Glutathione peroxidase), 94°C (60 sec), and 60°C (60 sec) (MDR1) or 94°C (15 sec), 60°C (10 sec), and 72°C (60 sec) (CYP2J2).
**Figure legends**

**Fig. 1.** (A) MTT reduction in MDA-CTL, MDA-468 and MDA-2J2 cells after treatment with paclitaxel (0.1 or 10 µM, 24 h). (B) Caspase-3 activity in MDA-CTL, MDA-468 and MDA-2J2 cells after treatment with paclitaxel (1.0 µM, 24 h) (C) Colony formation in MDA-CTL, MDA-468 and MDA-2J2 cells after treatment with paclitaxel (0.05 µM, 24 h). *P<0.05, **P<0.01, ***P<0.001 different from corresponding control treatments in each cell type. Other differences between treatments are indicated by P values. Data are presented as mean±SEM (n=3 individual experiments).

**Fig. 2.** (A) Basal and paclitaxel-activated lipid peroxidation (1 µM, 24 h) in MDA-CTL, MDA-MB-468 and MDA-2J2 cells. **P<0.01, different from DMSO-treated MDA-CTL cells; †P<0.05, different from DMSO-treated MDA-468 cells; ‡P<0.05, different from DMSO-treated MDA-2J2 cells. Other differences between treatments are indicated by P values. (B) ROS\textsuperscript{high} live cell populations in MDA-2J2 and MDA-468 cells after treatment with paclitaxel (0.01 or 1.0 µM, 48 h; as % of total cells). Representative images: DCFDA fluorescence profiles in MDA-468 and MDA-2J2 cells. The more pronounced left shift in MDA-468 cells consistent with increased ROS formation after paclitaxel treatment (dotted profile). *P<0.05, ***P<0.001 different from corresponding DMSO-treated cells; other differences between treatments are indicated by P values. (C) ROS\textsuperscript{high} cell populations in MDA-468 cells after cotreatment with paclitaxel (1 µM, 2 h) and either antimycin a (0.1 µM), DPI (0.1 µM) or allopurinol (0.2 µM). *P<0.05, **P<0.01, ***P<0.001 different from DMSO-treated cells; ††P<0.01, †††P<0.001 different from paclitaxel treatment alone. Data are presented as mean±SEM (n=3 individual experiments).
Fig. 3. (A) ALDH1A1 mRNA, ALDH enzyme activity and ALDH1A1 immunoreactive protein in MDA-2J2 compared to control cells. (B) Effect of CYP2J2 siRNA gene silencing and clotrimazole (CTZ, 5 µM, 24 h) on expression of ALDH1A1 protein in MDA-2J2 cells; the control (eGFP) siRNA was transfected in control experiments. (C) Effect of regioisomeric EETs on the expression of ALDH1A1 mRNA in MDA-2J2 cells. (D) Effects of inhibitors of EET-activated signalling cascades on ALDH1A1 immunoreactive protein in MDA-2J2 cells. Inhibitor treatments were: p38 MAP kinase (SB203580, 10 µM), ERK MAP kinase (PD98059, 25 µM) and PI3 kinase/Akt (LY294002, 50 µM). (E) CYP2J2 and ALDH1A1 immunoreactive proteins in multiple cell types. *P<0.05, **P<0.01 with respect to corresponding control. Data are presented as mean±SEM (n=3 individual experiments).

Fig. 4. (A) Effect of ALDH1A1 siRNA gene silencing on expression of ALDH1A1 protein in MDA-2J2 cells; the control (eGFP) siRNA was transfected in control experiments. (B) ALDH1A1, but not control siRNA, restores the anti-proliferative action of paclitaxel in MDA-2J2 cells to that in MDA-468 cells. IC$_{50}$s and 95% confidence intervals are shown. (C) ALDH1A1 siRNA, but not control siRNA, restores the pro-apoptotic action of paclitaxel in MDA-2J2 cells to that in MDA-468 cells. (D) ALDH1A1 siRNA, but not control siRNA, restores the ROS$^{high}$ live cell population in MDA-2J2 cells to that in MDA-468 cells. In panels (A)-(D) *P<0.05, **P<0.01 and ***P<0.001 relative to control siRNA-transfected, DMSO-treated cells; other differences between treatments are indicated by P values. (E) Activation of 4-HNE protein adduct formation by paclitaxel is attenuated in MDA-2J2 cells. *P<0.05, **P<0.01 relative to
corresponding DMSO-treated cells. Data are presented as mean±SEM (n=3 individual experiments).

Fig. 5. (A) MTT reduction in MDA-2J2 and MDA-468 cells after treatment with doxorubicin (0.5, 1.0 μM), sorafenib (2.0, 5.0 μM) and staurosporine (0.05, 0.1 μM). **P<0.01, ***P<0.001 different from DMSO-treated MDA-468 cells; †P<0.05, †††P<0.001 different from DMSO-treated MDA-2J2 cells; other differences between treatments are indicated by P values. (B) ROS$^{\text{high}}$ populations as percentages of total MDA-468 and MDA-2J2 cells after treatment with doxorubicin (0.01 μM), sorafenib (2.0 μM), staurosporine (0.01 μM) or DMSO. **P<0.01, ***P<0.001 different from DMSO-treated MDA-468 cells; other differences between treatments are indicated by P values. (C) Effects of antimycin (0.1 μM), allopurinol (0.2 μM) or DPI (0.1 μM) on the ROS$^{\text{high}}$ population in MDA-468 cells after treatment with doxorubicin, sorafenib or staurosporine. *P<0.05, **P<0.01 and ***P<0.001 different from DMSO-treated MDA-468 cells; †P<0.05, †††P<0.001 different from doxorubicin, sorafenib or staurosporine treatment alone. Data are presented as mean±SEM (n=3 individual experiments).

Fig. 6. Overview of proposed mechanism of paclitaxel-mediated ROS production, lipid peroxidation and reactive aldehyde formation that leads to increased 4-HNE-dependent protein modification and decreased cell proliferation. Over-expression of CYP2J2 increases the formation of 14,15-EET in cells that activates ALDH1A1 expression and attenuates the loss of cell proliferation that is elicited by reactive aldehydes.
**Allison et al. Figure 1**

(A) MTT reduction (% of control)

(B) Caspase-3 activity (fold of control)

(C) Scratch assay images for MDA-CTL, MDA-468, and MDA-2J2.
Allison et al. Figure 2
Allison et al. Figure 5
Paclitaxel → Mitochondrial complex → NADPH oxidases → ROS → Lipid peroxidation → Reactive aldehydes → 4-HNE-protein modification → Cell proliferation

Allison et al., Fig 6
Paclitaxel $\rightarrow$ ROS $\rightarrow$ Cell death

CYP2J2 $\rightarrow$ 14,15-EET $\rightarrow$ ALDH1A1

*Cell survival and anti-cancer drug resistance*