***In vitro* effects of an *in silico*-modelled 17 β -estradiol derivative in combination with dichloroacetic acid on MCF-7 and MCF-12A cells**

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Abstract

Objectives: To investigate anti-proliferative properties of a novel *in silico*-modelled 17 β -oestradiol derivative (C9), in combination with dichloroacetic acid (DCA), on MCF-7 and MCF-12A cells.

Materials and methods: xCELLigence system was employed to determine optimal seeding number for cells, and crystal violet assay was used to assess cell number and to determine IC₅₀ value (24 h) for combination treatment. Light and fluorescent microscopy techniques were used to morphologically detect types of cell death. Flow cytometry was used to analyse cell cycle and apoptosis.

Results: Optimal seeding number for 96-well plates was determined to be 5000–10 000 cells/well for both MCF-7 and MCF-12A cells. IC₅₀ for MCF-7 cells of the combination treatment after 24 h was 130 nM of C9 in conjunction with 7.5 mM of DCA ($P < 0.05$). In contrast, the same concentration inhibited cell population growth by only 29.3% for MCF-12As after 24-h treatment ($P < 0.05$). Morphological studies revealed lower cell density of both types of combination-treated cells. Flow cytometric analyses demonstrated increase in sub-G₁ phase in combination-treated MCF-7 cells.

Conclusions: These results demonstrate that the novel 17 β -oestradiol derivative C9, in combination with DCA is a potent anti-proliferation treatment, with properties of selectivity towards tumourigenic cells. Thus, this warrants further studies as a potential combination chemotherapeutic agent for further cancer cell lines.

Introduction

The mitotic spindle, being composed of long, hollow microtubules, is intimately involved in mitosis and cell division (1,2). Naturally occurring chemical compounds such as paclitaxel, vinca alkaloids, colchicine alkaloids, as well as 2-methoxyestradiol (2ME), that target microtubules and inhibit the normal function of the mitotic spindle, comprise one of the most researched classes of cancer chemotherapeutic agent (1–5). 2ME (Fig. 1a) is produced in trace amounts in the human body; it is an endogenous metabolite derivative of oestrogenic hormone 17 β -oestradiol, with well-documented anti-mitotic, anti-angiogenic, and pro-apoptotic properties *in vitro* (6–9). 2ME abrogates microtubule dynamics by binding to the colchicine binding site of β -tubulin (2,6,10,11). It is currently in phases I and II clinical trials for treatment of multiple myeloma, glioblastoma multiforme and other solid malignancies, such as carcinoid, prostate and breast tumours (6,12,13). However, this compound has been shown to have characteristics of low oral bioavailability, probably caused by an additional degradation step of oxidation at C3/C17 hydroxyl groups (14,15). Previous studies by Liu *et al.* (16) and Newman *et al.* (15) have emphasized the limitations of 2ME due to its potential for inactivation at the D-ring, C17 position (15,16). Panzem[®] NCD (NanoCrystal Dispersion), patented by Entremed, Inc. (Rockville, MD, USA), is a nanotechnologically enhanced particle of 2ME that has improved dissolution (13,14,17). Zhou *et al.* (18) mentioned that Panzem[®] NCD proved to be well tolerated by cancer patients, but its anti-tumourigenic activity is modest even with heavily pre-treated (1000 mg orally four times daily) cancer patients (18,19). Thus, 2ME analogues are under extensive research amongst interested scientists. Sulphamoylated analogues of 2ME are oestrogen sulphamates that are much more potent than their parent oestrogens (20–22). Newman *et al.* (23) have demonstrated for the first time that the cyanomethyl group at C17 position significantly increased efficacy of compounds STX640 and STX641 *in*

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Figure 1. Chemical structures of 2-methoxyestradiol (a) and its 17 β -estradiol derivative (b). (a) 2-methoxyestradiol (2ME); (b) 2-ethyl-3-O-sulphamoyl-estra-1,3,5(10),15-tetraen-3-ol-17-one (C9) (ACD/Chem-Sketch freeware version 12.0).

vitro and *in vivo* (23). Subsequently, Foster *et al.* (24) demonstrated that 2-MEOe2bisMATE (STX140) and 2-EtE2bisMATE (STX 243), which are compounds exhibiting A-ring (C3 and/or C2) and D-ring (C17) modifications of 2ME, have potent anti-proliferative and anti-angiogenic activity *in vitro*, *in vivo* and *ex vivo* (24–26). The same group also showed efficacy of STX243 against expansion of both (oestrogen receptor) ER+ and ER– breast tumours *in vitro* and *in vivo*, with promising pharmacokinetic properties (25). These oestrogen sulphamates have been proven to provide higher bioavailability as they are able to overcome biotransformation encountered by liver metabolism, as they are capable of reversibly conjugating to cytosolic erythrocyte carbonic anhydrase II (CAII) (20,21,27,28).

Carbonic anhydrases are a family of ubiquitously expressed, zinc containing isozymes that catalyse inter-conversion between CO₂ and HCO₃[–] (28,29). It is known that overexpression of carbonic anhydrases IX (CAIX) and XII (CAXII) is strongly associated with hypoxia, a condition favoured by cells of most solid tumours. For example, the promoter of CAIX gene, which has a hypoxia response element (HRE), binds to hypoxia-inducible factor alpha (HIF- α) in response to increased cell density and a hypoxic microenvironment, and activates transcription of CAIX (21,30). Increased formation of carbonic acid as a result of CAIX expression facilitates formation of an acidic environment surrounding solid tumour cells and this acidosis is known to enhance pathological processes favoured by tumourigenicity (22,28,29). Prospective inhibitors capable of selective inhibition of hypoxia-activated CAIX therefore provide therapeutic promise for treatment of metastatic tumours.

Several promising sulphamoylated analogues of 2ME have been designed in our laboratory and 2-ethyl-3-O-sulphamoyl-estra-1,3,5(10),15-tetraen-3-ol-17-one (C9) (Fig. 1b) is one of those *in silico* modelled, commercially unavailable oestradiol derivatives (21). *In silico* docking performed by Stander *et al.* (21) revealed that 2-ethyl derivatives displayed higher binding affinity to the

colchicine-binding site when compared to other C2-modified analogues (21). This finding is in agreement with results from a further group Leese *et al.* in whose earlier study it was discovered that ethyl substitution at the C2 position of oestrone provided the optimal substitution for high anti-proliferative activity (27,31). Therefore, based on docking results of the analogues, to the colchicine site and CAIX/CAII ratio of the compounds, it was decided to synthesize C9. Furthermore, pilot *in vitro* cell proliferation experiments demonstrated that mode of action of C9, similar to its mother molecule 2ME, can be attributed to its ability to disrupt mitotic spindle dynamics, especially the microtubule structure, which in turn leads to G₂/M arrest, in actively dividing cells, regardless of their oestrogen receptor status.

Not only the carbonic anhydrase pathway contributes to acidosis, but also cytosolic glycolysis contributes to the acidic environment as a result of production and accumulation of lactic acid (30,32). Glucose oxidation in mitochondria is controlled by a gate-keeping mitochondrial enzyme, pyruvate dehydrogenase (PDH). Pyruvate dehydrogenase kinase (PDK) is able to phosphorylate PDH and inhibit its function. Dichloroacetic acid (DCA) is a well-characterized inhibitor of PDK (33) and recent studies suggest that inhibitors of PDKs attenuate inhibition of PDH activity, thus forcing cells into oxidative phosphorylation and suppress cancer cell proliferation (34–36). Xiao *et al.* (37) used up to 40 mM DCA for treatment of A549, HeLa, HCT116 and PLC cell lines *in vitro* and significant decrease in cell number expansion was demonstrated in all.

Liu *et al.* (38) have proposed that combination treatment of a glycolysis inhibitor, in conjunction with a chemotherapeutic agent would open new windows for anti-cancer therapy. A recent study by Tagg *et al.* (39) described that glycolytic inhibitor 2-deoxy-D-glucose in combination with STX140, significantly reduced tumour volume by 76% ($P < 0.001$) *in vivo* compared to 46% ($P < 0.05$) in STX140-only treated xenograft models. However, there is currently no literature available concerning combination of both C9 and DCA for treatment of cancer cells *in vitro* and effects of C9 and DCA on tumourigenic and non-tumourigenic cell lines remains to be elucidated. Both C9 and DCA are regarded as potential anti-tumourigenic therapeutics, with different targets and modes of action. It is of vital importance to examine and compare effects of these compounds by investigating their *in vitro* effects on carcinogenic cells and non-carcinogenic ones. Thus, the aim of this study was to evaluate *in vitro* influence of C9 in combination with DCA on cell proliferation, cell cycle progression, cell morphology and cell death, in tumourigenic adenocarcinoma MCF-7 and non-tumourigenic MCF-12A breast epithelial cell lines.

Materials and methods

Cell lines

The MCF-7 cell line (oestrogen receptor positive) is derived from a pleural effusion of human breast adenocarcinoma provided by Highveld Biological Pty (Ltd) (Sandringham, Johannesburg, South Africa) while MCF-12A cell line (oestrogen receptor negative) is a non-tumorigenic spontaneously immortalized adherent human breast epithelial cell line, established from tissue taken at reduction mammoplasty from a nulliparous patient with fibrocystic breast disease, that contained focal areas of intraductal hyperplasia. The original cell line was produced by long-term culture, and now it forms a monolayer without domes when cell growth reach confluency. These MCF-12A cells were obtained as a gift from Prof MI Parker (Department of Cancer Biology of the University of Cape Town, Cape Town, South Africa).

Reagents

Dulbecco's minimum essential Eagle's medium (DMEM), Ham's F12 medium, trypsin-EDTA, crystal violet and Bouin's fixative were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Heat-inactivated foetal bovine serum (FBS) (PAA Laboratories (Pty) Ltd., Morningside, Qld, Australia), sterile cell culture flasks and plates (96-well plates and 6-well plates) were obtained through Separations (Pty) Ltd. (Randburg, Johannesburg, South Africa). Phosphate-buffered saline (PBS) was purchased from Gibco® BRL (Invitrogen, Carlsbad, CA, USA) and penicillin, streptomycin and fungizone were purchased from Highveld Biological (Pty) Ltd. (Sandringham, Johannesburg, South Africa). Glutaraldehyde, triton X-100, and ethanol were purchased from Merck (Munich, Germany). The demo xCELLigence system, which includes RTCA SP Station, RTCA Analyzer and RTCA Control Unit developed by ACEA Biosciences, Inc. (San Diego, CA, USA), was kindly provided by Roche Products (Pty) Ltd. (Randburg, Johannesburg, South Africa) and E-Plate 96 microtitre plates were purchased from Roche Products (Pty) Ltd. Annexin V-FITC Kit was purchased from BIOCOT biotech (Pty) Ltd. (Clubview, Pretoria, South Africa) and manufactured by MACS Miltenyi Biotech, GmbH, Bergisch Gladbach, Germany. All other chemicals were of analytical grade and were purchased from Sigma Chemical Co., Southern Cross Biotechnology (Pty) Ltd. (Cape Town, South Africa) and Amersham Biosciences (Pittsburgh, PA, USA).

Chemical compounds and composition of appropriate controls

The compound 2-ethyl-3-O-sulphamoyl-estra-1,3,5(10), 15-tetraen-3-ol-17-one (C9) was synthesized by iThemba

Pharmaceuticals (Pty) Ltd (Modderfontein, Midrand, South Africa). DCA (powder) was purchased from Sigma Chemical Co. A stock solution of 0.75 μM of C9 in DMSO was prepared for all subsequent experiments which was diluted with culture medium to required final concentrations prior to exposure. Several controls were incorporated. First, cells grown under normal conditions propagated in culture medium only. Secondly, to determine whether the vehicle dimethyl sulphoxide (DMSO) had any effect on proliferating cells, vehicle-treated controls were included and final DMSO concentration used to expose cells never exceeded 0.1% (v/v). Thirdly, to observe differences between combination/dual treatment of 130 nM of C9 in conjunction with 7.5 mM of DCA and individual treatment of C9 (130 nM) or DCA (7.5 mM), cells exposed with either C9 (130 nM) or DCA (7.5 mM) were included as single treatment controls. Lastly, cells were treated with actinomycin D (0.2 $\mu\text{g}/\text{ml}$) to induce apoptosis and the latter served as a positive control for studying induction of apoptosis.

Cell culture

The MCF-7 breast adenocarcinoma tumorigenic cells were cultured in DMEM and supplemented with 10% heat-inactivated FBS, 1% penicillin G (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) and fungizone (250 $\mu\text{g}/\text{l}$). MCF-12A non-tumorigenic breast epithelial cells were cultured in 1:1 mixture of DMEM and Ham's F12 medium made up with epidermal growth factor (20 ng/ml), cholera toxin (100 ng/ml), insulin (0.01 mg/ml), hydrocortisone (500 ng/ml), 10% heat-inactivated FCS, 1% penicillin G (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) and fungizone (250 $\mu\text{g}/\text{l}$). All cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂, in air.

Experiments were conducted either in 25 cm² culture flasks, 96-well plates or six-well plates. For 25 cm² culture flasks, exponentially growing cells were seeded at 500 000 cells/3 ml maintenance medium per flask. For six-well plates, exponentially growing cells were seeded at 375 000 cells per well in 3 ml maintenance medium on heat-sterilized cover slips. For 96-well plates, exponentially growing cells were seeded at 5000 cells/well, 200 μl volume of maintenance medium. For E-Plate 96 microtitre plates, exponentially growing cells were seeded at densities of 20 000, 10 000, 5000, 2500/well/200 μl medium for proliferation assays and subsequently seeded at 10 000/well/200 μl medium for cytotoxicity assays, as suggested by supplier's manual. Cells used in all experiments were allowed 24-h incubation for cell adherence, and medium were renewed prior to cell exposure.

Cell quality assessment

Real-time cell proliferation assays. The xCELLigence System (Real-Time Cell Analyzer Single Plate (RTCA SP[®]) system) was developed by ACEA Biosciences, Inc. in conjunction with Roche Diagnostics GmbH (Roche Applied Science, Mannheim, Germany) to monitor cell events in real time, without incorporation of dyes, by measuring electrical impedance created by cells (40,41). The RTCA SP Station was connected to the RTCA Analyzer and subsequently joined the RTCA Control Unit. The xCELLigence System was connected and tested by Resistor Plate Verification before the RTCA SP Station was placed inside the incubator at 37 °C and 5% CO₂. Cells were seeded at four different densities: 20 000, 10 000, 5000 and 2500 cells/well in E-Plate 96 microtitre plate devices (E-Plate[™], as suggested by supplier's manual). Briefly, cells were trypsinized and counted using the trypan blue exclusion method and haemocytometer; they were then resuspended in culture medium. Background measurements were taken by adding 100 µl of appropriate medium to the wells of the E-Plate 96. Subsequently, RTCA Software Package 1.2 was used to calibrate the plates. Cells were trypsinized and prepared for a 400 000 cell/ml stock solution prior to exposure. A volume of 100 µl of cell suspension was added to wells of the E-Plate 96, which contained 100 µl of proliferation medium, which composed final volume equal to 200 µl. MCF-7 and MCF-12A at densities of 20 000, 10 000, 5000 and 2500 cells/well were seeded and allowed to settle at the bottom of wells, at room temperature for 30 min, before placing the E-Plate 96 on the RTCA SP Station. Cell attachment and proliferation were continuously monitored for a period of 48 h as indicated using the RTCA Control Unit. While seeded cells were propagated in the incubator, impedance values were converted into cell index (CI) values corresponding to each well. CI value is defined as relative change in measured electrical impedance to represent cell status, and is directly proportional to quantity, size, and attachment forces of the cell.

Cell number

Crystal violet assay. To determine growth inhibitory effect of dual treatment on both cell lines, as well as optimal exposure conditions, various time- (24, 48 and 72 h) and dose- (C9: 100–200 nM; DCA: 2.5–40 µM) dependent cell proliferation studies, were conducted prior to final combination of dose selection. Doses chosen for C9 were based on previous *in vitro* cell proliferation assays conducted in our laboratory (data not shown) and concentrations of DCA chosen were supported in the literature (37,42,43). Quantification of fixed monolayer cells was

spectrophotometrically determined by employing crystal violet as the DNA stain. Staining nuclei of fixed cells with crystal violet allows for rapid, accurate and reproducible quantification of cell number in cultures grown in 96-well plates (44,45). Growth inhibitory effect (IC₅₀) was calculated as described by the National Cancer Institute (NCI, USA) to compare growth inhibition induced by the compounds of the two cell lines (46).

Morphological studies

Optical transmitted light differential interference contrast. Polarization-optical differential interference contrast (PlasDIC) is a polarization-optical transmitted light differential interference method from Zeiss (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Unlike the traditional DIC method (Smith/Nomarski DIC method), the improved PlasDIC equipment positions polarizer and Wollaston prism after light has already passed through the object and objective to create image quality of high superiority (47,48). The PlasDIC contrast system allows use of plastic dishes for microscopic examinations and delivers quality optical imaging, in particular, assessment of cells of high dimension that lie in close proximity to each other, or form groups. Images of the living cells were captured before and after appropriate exposure, to gain insight into potential effects of the newly synthesized compounds, on cell morphological changes.

Fluorescence microscopy. Fluorescence microscopy was employed to differentiate between viable, apoptotic, autophagic and oncotic cells. A triple fluorescent dye staining method was developed utilizing acridine orange (green), Hoechst 33342 (blue) and propidium iodide (PI; red) fluorescent dyes. Acridine orange is a lysosomotropic fluorescent compound, a tracer for acidic vesicular organelles including autophagic vacuoles and lysosomes (49). Hoechst 33342 is a fluorescent dye that penetrates intact cell membranes of viable cells and cells that are undergoing apoptosis and stain the nucleus. PI is a fluorescent dye that is unable to penetrate an intact membrane and therefore stains nuclei of cells that have lost membrane integrity due to oncotic and/or necrotic processes.

Exponentially growing MCF-7 and MCF-12A cells were seeded at 375 000 cells/well in six-well plates. After 24-h recovery and attachment, cells were exposed to treatments as described. After 24-h exposure, 500 µl of Hoechst 33342 solution (3.5 µg/ml) was added to the medium at final concentration 0.9 µM, and the plates were incubated for 25 min more. Subsequently, 500 µl acridine orange solution (4 µg/ml) and 500 µl PI solution (40 µg/ml) were added simultaneously to provide final concentration of 1 µg/ml and 12 µM respectively. Plates

were incubated for five further minutes. To prevent fluorescent dye quenching, all procedures were performed in a dark room. Cells were examined using a Zeiss inverted Axiovert CFL40 microscope and Zeiss Axiovert MRm monochrome camera (Carl Zeiss MicroImaging GmbH, Göttingen, Germany), Zeiss filter 2, 9 and 15 for Hoechst 33342- (blue), acridine orange- (green) and PI-stained cells respectively.

Cell cycle analysis

Flow cytometry was utilized to analyse effects of C9 and DCA on cell cycle progression of MCF-7 and MCF-12A cells. PI was used to stain the nucleus to determine the amount of DNA present and the amount of PI fluorescence correlates with stages of the cell cycle during cell division. PI fluorescence (relative DNA content per cell) was measured using fluorescence activated cell sorting (FACS) FC500 System flow cytometer (Beckman Coulter SA (Pty) Ltd., Randburg, Gauteng, South Africa) equipped with an air-cooled argon laser excited at 488 nm. Data from at least 10 000 cells/sample were analysed with CXP software (Beckman Coulter SA (Pty) Ltd). Data from cell debris (particles smaller than apoptotic bodies) and clumps of two or more cells were removed to display more accurate data. Cell cycle distributions were calculated with non-commercially available Cyflogic software 1.2.1 (Pertu Therho, Turku, Finland) by assigning relative DNA content per cell to sub-G₁, G₁, S and G₂/M fractions. PI molecules emit light at 617 nm; therefore, data obtained from the log forward scatter detector nr 3 (F13 log, detects 600 nm emissions) were represented as histograms on the x-axis.

Exponentially growing MCF-7 and MCF-12A cells were seeded at 500 000 cells/25 cm² flask. After 24-h attachment, medium was discarded and cells were exposed to the compounds. After a 24-h exposure, cells were trypsinized and resuspended in 1 ml of culture medium. Cells were centrifuged for five minutes to form a pellet and supernatant was discarded. Thereafter, cells were resuspended in 200 μ l of ice-cold PBS containing 0.1% FBS. Subsequently, 4 ml ice-cold 70% ethanol were added dropwise and cells were stored for at least 24 h at 4 °C. Prior to the analysis, cells were pelleted by centrifugation at 300 g for 5 min and resuspended in 1 ml of PBS containing PI (40 μ g/ml) and incubated at 37 °C for 45 min.

Cell death quantification

Annexin V-FITC. One of the earliest indications of apoptosis is translocation of membrane phospholipid phosphatidylserine (PS) from inner to outer leaflet of the plasma-

membrane (50). Once exposed to the extracellular environment, binding sites on PS become available for Annexin V, which is a phospholipid-binding protein with high affinity for PS. Annexin V is conjugated to a fluorochrome, fluorescein isothiocyanate (FITC) and used in flow cytometry for identification for early stages of apoptosis. PS translocation also occurs during necrosis; therefore, PI is used to distinguish between necrotic and early apoptotic cells.

Exponentially growing MCF-7 and MCF-12A cells were seeded (500 000 cells/25 cm²), attached and exposed. After 24-h exposure, cells were trypsinized and resuspended in 1 ml of 1 $\times$ binding buffer and were centrifuged at 300 g for 10 min. Supernatant was removed and cells were washed and resuspended in 100 μ l of 1 $\times$ binding buffer. Annexin V-FITC (10 μ l) was added and incubated with cells for 15 min in the dark. Thereafter, cells were washed in 1 ml of 1 $\times$ binding buffer and were centrifuged at 300 g for 10 min. Supernatant was discarded and cells were resuspended in 500 μ l of 1 $\times$ binding buffer solution. Immediately prior to analysis, 5 μ l of PI (100 μ g/ml) was added and mixed gently. PI fluorescence (oncototic cells) and annexin V fluorescence (apoptotic cells) were measured using FACS FC500 System flow cytometer (Beckman Coulter SA (Pty) Ltd.) equipped with an air-cooled argon laser excited at 488 nm. Data from at least 10 000 cells were analysed with non-commercially available Cyflogic software 1.2.1 (Pertu Therho).

Statistical analysis

Cell index calculations for real-time dynamic cell proliferation assay ($n = 3$) were performed automatically by the RTCA Software Package 1.2 of the xCELLigence system. Experimental data from at least three biological repeats for crystal violet DNA staining ($n = 6$), cell cycle analysis and Annexin V-FITC cell death quantification were statically analysed. Data obtained from independent experiments are shown as mean, standard deviation and (where appropriate) 95% confidence intervals. Each of the quantitative variables was statistically analysed for significance using two-way analysis of variance (ANOVA) model with main factor treatment and cell type along with a term for interaction between treatment and cell type. Sample sizes for quantitative experiments were at least three and data presented are representative of one of the three such experiments. Student's *t*-test was applied where two groups of data were compared. Means are presented in bar charts, with T-bars referring to standard deviations. *P*-values of <0.05 were regarded as statistically significant and are indicated by an asterisk (*). Qualitative experiments were repeated at least twice where data were obtained from PlasDIC and fluorescent microscopy.

Results

Cell quality assessment

Real-time cell proliferation assays. The xCELLigence System allows for real-time dynamic monitoring of cell attachment, adhesion and proliferation. Dynamic cell proliferation of MCF-7 and MCF-12A cells plated on the E-Plates 96 was monitored at 30-min intervals from time of plating until end of experiment. E-Plates 96 mimic ordinary 96-well microtitre plates except for addition of ultra thin sheath of gold beneath the plastic plate, which serves as microelectrode to conduct current across the plate. Presence of cells atop the thin sheath of gold will disturb the local current and thus introduce electrode impedance to the local ionic environment. Thus, any type of cell morphological alteration will cause changes in impedance value and ultimately convert into a CI value. The latter is directly proportional to impedance value.

MCF-7 and MCF-12A at densities of 20 000, 10 000, 5000 and 2500 cells/well in E-Plates 96 were seeded and observed for a period of 48 h (Fig. 2). Growth curves generated *via* xCELLigence System revealed that MCF-7 and MCF-12A each had distinctive trends of cell proliferation profile. MCF-7s displayed rapid attachment over the first 2 h followed by a relatively long lag phase depending on number of cells seeded per well, and finally entered an exponential growth phase (Fig. 2a). MCF-12A cells had increase in cell index after one hour seeding when compared to MCF-7 cells (Fig. 2b). The negative control (medium only without any cells) revealed that the medium was free from any contamination (Fig. 2). Therefore, increases in impedance value of the curves, other than negative control, were purely from cell proliferation of MCF-7 (Fig. 2a) and MCF-12A (Fig. 2b) cells. Impedance cell index of 20 000, 10 000, 5000 and 2500 cells/well increased proportionally to cell number of both MCF-7 and MCF-12A cells (Fig. 3) with the exception of 20 000 cells/well MCF-12A cells. Cell index value of MCF-7 and MCF-12A at 20 000 cells/well was statistically significant with $P < 0.05$ (Fig. 3).

It is of vital importance to ensure cell quality and correct number of cells per specific surface area for any *in vitro* cell experiments to avoid false positive or false negative results. Proliferation curves of MCF-12A cells revealed that higher densities, 20 000 cells/well (Fig. 2b), cell numbers plateaued rapidly and CI declined. This possibly was caused by contact inhibition. At density of 20 000 cells/well, MCF-7 and MCF-12A cells stopped proliferation at 40 and at 24 h respectively post-seeding, due to contact inhibition, thus, making it unsuitable to conduct 24-h cell proliferation studies. In contrast, at

2500 cells/well, neither cell showed typical characteristics of proliferation. Furthermore, at densities of 5000 and 10 000 cells/well, cells were still actively proliferating 24 h after seeding (Fig. 3). It was demonstrated that best quantity of cells per 0.32 cm^2 (surface area of a single well of 96-well plate), for these comparative studies was either 10 000 or 5000 cells. Subsequently, 5000 cells/ 0.32 cm^2 were chosen for crystal violet experiments as the cells reached confluence later than that of 10 000 cells/well.

Cell population growth

Crystal violet assay. Dose- and time-dependent cell population growth studies (data not shown) were conducted for selection of optimal cell exposure conditions. A previous study conducted in our laboratory by Stander *et al.* (21) has revealed GI_{50} value for C9-exposed MCF-7 cells to be 130 nM after 48 h of exposure. This concentration served as our benchmark for concentration selection for dual drug treatment. Selection of concentration for combination therapy was conducted by setting up one non-variable constant (C9 with concentration of 130 nM) and combining this with a range of different concentrations of dichloroacetic acid (2.5, 5.0, 7.5, 10.0, 15.0 and 40.0 mM). Range of concentrations for DCA was based on other studies using similar concentrations (37). Neither 2.5 nor 5.0 mM DCA in combination with 130 nM of C9 inhibited MCF-7 cell population growth by more than 30%; we found 40 mM DCA to be too highly concentrated and therefore not suitable for further *in vivo* use. Amongst concentrations of 7.5, 10.0 and 15 mM DCA, 7.5 mM DCA combined with C9 (130 nM) achieved similar inhibition effects compared to the other two concentrations combined with C9, with no statistically significant differences (data not shown). Finally, 130 nM of C9 in combination with 7.5 mM of DCA inhibited MCF-7 cell population growth to about 50% after 24 h; thus, this concentration and time point were chosen for all subsequent experiments.

Cell proliferation analysis by means of crystal violet DNA staining revealed that compound C9-exposed MCF-7 cells demonstrated approximately 20% inhibition compared to vehicle-exposed MCF-7 cells ($P < 0.05$) after 24-h of exposure (Fig. 4); DCA-exposed (24 h) MCF-7 cells did not show significant cell inhibition compared to controls (Fig. 4). However, MCF-7 cell numbers after 24-h exposure to 130 nM C9 in combination with DCA (7.5 mM) revealed a statistically significant decrease of 50.84% ($P < 0.05$) (Fig. 4). Therefore, a synergistic effect of DCA in combination with C9 was observed, in which DCA served as adjuvant to enhance pharmacokinetics of the microtubule disruptor, C9, in rapidly proliferating tumourigenic cells.

Figure 2. Real-time dynamic monitoring of cell adhesion and proliferation *via* the xCELLigence system. MCF-7 (a) and MCF-12A (b) seeded at densities of 20 000, 10 000, 5000 and 2500 cells per well in E-Plates 96 were observed for a period of 48 h.

Figure 3. Cell number titration expressed as impedance value, namely cell index for both MCF-7 and MCF-12A cells with cell densities of 20 000, 10 000, 5000 and 2500 cells/well in the E-Plate 96 after being monitored for 48 h. **P*-value < 0.05 when MCF-7 and MCF-12A cells were compared.

For MCF-12A cells, C9 also induced cell inhibition in these non-tumourigenic cells and revealed approximately 28% inhibition compared to vehicle-treated controls ($P < 0.05$) (Fig. 4). Growth inhibition for combination-exposed MCF-12A cells was approximately 29% and was not statistically significantly different from C9-treated MCF-12A cells (Fig. 4). Thus, no synergistic effect for combination-exposed MCF-12A cells was observed (Fig. 4).

MCF-12A cells responded differently in a statistically significant way, to combination treatment when compared to MCF-7 cells, with MCF-7 cell growth inhibited to a greater extent (50.84%) when compared to MCF-12A-treated cells (29%) ($P < 0.05$) (Fig. 4). The vehicle (DMSO) used to dissolve C9 was found to be biologically and molecularly inert with no toxic effect observed on the cells with (v/v) not exceeding 0.01% (data not shown).

Figure 4. MCF-7 and MCF-12A cell population growth expressed as percentage of control (cells propagated in medium and the vehicle, DMSO < 0.01%) after 24-h exposure to different conditions. *, †*P*-value < 0.05 after comparison of cells and controls within the same cell line. **P*-value < 0.05 when MCF-7 and MCF-12A cells were compared for the same treatment.

Cell morphology

Optical transmitted light differential interference contrast. PlasDIC images of both MCF-7 and MCF-12A C9-treated cells (Fig. 5b,g) showed increase in metaphases and formation of apoptotic bodies. DCA-treated cells (Fig. 5c,h) revealed shrunken cells compared to cells grown to confluence in vehicle-treated control (Fig. 5a,f). Cells exposed to C9 in combination with DCA (Fig. 5d,i) displayed characteristics of cells in metaphase, formation of apoptotic bodies, shrunken cells, compromised cell density and appearance of cell 'ghosts'. Upon dual treatment of C9 with DCA, MCF-7 cells (Fig. 5d) displayed

Figure 5. PlasDIC images of MCF-7 cells (left column, images a–e) compared to MCF-12A cells (right column, images f–j) after 24-h exposure to different conditions. Vehicle-treated (a and f) cells were confluent and showed no sign of distress. C9 (130 nM)-exposed (b and g) cells had decreased cell density and increase in metaphases. Cells exposed to 7.5 mM of DCA (c and h) indicated no significant decrease in cell number. Cells exposed to C9 (130 nM) in combination with DCA (7.5 mM) (d and i) showed significant inhibition in cell population growth. Actinomycin D (0.2 µg/ml)-treated cells (e and j) exhibited hallmarks of late stages of apoptosis.

not only a severe degree of compromised cell density but also vast numbers of existing cells either in metaphase, shrunken or displaying characteristics of cell death (apoptosis) compared to MCF-12A cells. Thus, MCF-7 cells (Fig. 5d) appeared morphologically to be more susceptible to treatment, compared to MCF-12A cells (Fig. 5i).

Fluorescence microscopy. The fluorescence microscopy study revealed increases in MCF-7 cells in metaphase after 24-h exposure to C9 (130 nM) (Fig. 6b). DCA-exposed (7.5 mM) MCF-7 cells had slightly increased acridine orange staining (Fig. 6c) compared to controls (Fig. 6a). However, combination treatment of C9+DCA-exposed MCF-7 displayed severely compromised cell density, increased acridine orange staining and high number of existing cells in metaphase (Fig. 6d). Furthermore, the microscopy study revealed slight increases in acridine orange staining in C9-, DCA- and C9+DCA-exposed MCF-12A cells (Fig. 6g–i). The effect of C9 or combination treatment on MCF-12A cells (Fig. 6g,i) was moderate compared to MCF-7 cells (Fig. 6b,d) that were treated under the same exposure conditions. This observation is in agreement with findings from previous cell number studies in which addition of DCA enhanced efficacy of compound C9 on tumourigenic cells.

Cell cycle analysis

Previous studies have demonstrated that sulphamoylated derivatives of 2ME are able to induce cell cycle arrest in a number of tumourigenic cell lines *in vitro*, as well as with xenograft models (21,24). In this study, the effect of C-17 modified sulphamoylated derivatives in conjunction with a glycolysis inhibitor on cell cycle arrest were examined in the hormone-dependent MCF-7 and hormone-independent MCF-12A cell lines. In vehicle-exposed MCF-7 cells, <1% of the cells were in sub-G₁ and 27% were in G₂/M (Figs 7a and 8a) phases. The anti-mitotic agent C9-exposed (130 nM) MCF-7 cells showed slightly elevated numbers of cells present in sub-G₁ (5%) ($P = 0.13$) (Figs 7a and 8a). However, C9 induced tumourigenic cell cycle arrest with 36% of cells present in G₂/M ($P < 0.05$) compared to controls (Figs 7a and 8a). After treatment with 7.5 mM of DCA on MCF-7 cells, no significant change in cell cycle events was observed. In contrast, we observed that when DCA was added together with the anti-mitotic agent C9, efficacy of combination therapy was superior to that of C9 alone exposed MCF-7 cells, with increased sub-G₁ (19%, $P < 0.05$) and G₂/M (35%, $P < 0.05$) phases compared to controls (Figs 7a and 8a). Alternatively, agent C9 at 130 nM after 24-h exposure did not induce non-tumourigenic MCF-12A G₂/M (22%) cell cycle arrest compared to control values (23%) (Figs 7b

and 8b). Combination of C9 and DCA-exposed MCF-12A had statistically significant increase in sub-G₁ (10%) compared to controls (5%) ($P < 0.05$). No sign of elevated G₂/M with combination treatment was observed on MCF-12A cells (Figs 7b and 8b). Combination treatment of anti-mitotic agent with glycolysis inhibitor resulted in 19% tumourigenic cells to be in sub-G₁. However, only 10% of non-tumourigenic cells were in sub-G₁ under the same conditions. These observations were statistically significant ($P < 0.05$) (Fig. 8).

Cell death quantification

Analysis of apoptosis is one of the methods to be able to understand possible mechanisms for C9+DCA-mediated cell death. Externalization of phosphatidylserine was detected with Annexin V-FITC and measured using a flow cytometer. In terms of apoptosis induction, in both types of cells, neither anti-mitotic agent C9 (130 nM) nor glycolysis inhibitor DCA (7.5 mM) alone had any significant effect compared to controls (Fig. 9). However, when both cell types had treatment with combination therapy (C9+DCA) for 24 h, there were 15.3% MCF-7 and 5.7% MCF-12A cells present in the early apoptotic phase – the increase being statistically significant when compared to relative controls ($P < 0.05$) (Fig. 9). This finding is in agreement with previous cell cycle analysis sub-G₁ results (Fig. 8). When we evaluated apoptosis induction results between the two cell lines, it was observed that differences between early apoptosis for MCF-7 (15.3%) and MCF-12A (5.7%) cells were statistically significant ($P < 0.05$) (Fig. 9).

Discussion and conclusion

It was hypothesized a decade ago by Liu *et al.* that a glycolysis inhibitor in combination with a conventional chemotherapeutic agent may exhibit synergistic effects. This team further proposed that chemotherapeutics would target fast dividing tumour cells on edges of tissues, while the glycolysis inhibitor would focus on cells at hypoxic centres (38). Furthermore, Tagg *et al.* (39) recently demonstrated that combination of sulphamoylated 2ME analogue with 2-deoxy-D-glucose was a potent therapeutic agent in breast and prostate cancers *in vivo* (39). In our study, combination of an anti-mitotic agent C9 with a glycolytic inhibitor DCA served as a powerful inhibitor for breast tumourigenic cell growth *in vitro*. This study has not only demonstrated that the results are in accord with previous studies by Liu *et al.* (38) and Tagg *et al.* (39), but it also contributes towards understanding combination therapy.

In the present comparative study between non-tumourigenic MCF-12A epithelial cells and breast adenocarcinoma MCF-7 cells, the synergistic effects of

Figure 6. Fluorescence microscopy utilizing triple fluorescent stains of Hoechst 33342 (stains DNA blue), acridine orange (stains acidic vacuoles green) and propidium iodide (penetrate cell membrane). Fluorescence images of MCF-7 cells (left column, images a–e) compared to MCF-12A cells (right column, images f–j) after 24 h of exposure to different conditions. Fluorescence images of MCF-7 cells (left column, images a–e) compared to MCF-12A cells (right column, images f–j) after 24-h exposure to different conditions. Vehicle-treated (a and f) cells were confluent and showed no sign of distress. C9 (130 nM)-exposed (b and g) cells showed decreased cell density and increase in metaphases. Cells exposed to 7.5 mM of DCA (c and h) indicated no significant decrease in cell number. Cells exposed to C9 (130 nM) in combination with DCA (7.5 mM) (d and i) showed significant inhibition of cell population growth. Actinomycin D (0.2 µg/ml)-treated cells (e and j) exhibited hallmarks of late stages of apoptosis.

Figure 7. Cell cycle histograms of vehicle-, C9- and C9+ DCA-exposed cells after 24-h treatment for (a) MCF-7 and (b) MCF-12A cells.

Figure 8. Distribution of DNA content relative to phase of cell cycle of both (a) MCF-7 and (b) MCF-12A cells. Data are sub-ordered to vehicle-, C9-, DCA-, C9 plus DCA- and actinomycin D (positive control for apoptosis)-exposed cells. Both cell lines indicated statistically significant increase in sub-G₁ phase in C9+DCA-exposed samples compared to vehicle-treated cells. MCF-7 cells were more susceptible to combination compounds treatment. **P*-value <0.05 when exposed cells were compared to vehicle controls of the same cell line.

dichloroacetic acid (7.5 mM) combined with the new anti-mitotic compound, C9 (130 nM), on cell proliferation, cell cycle progression and apoptosis were demonstrated. Real-time dynamic monitoring of cell adhesion and proliferation *via* the xCELLigence system revealed that optimal seeding number of cells for this comparative studies in a 96-well plate was between 5000 and 10 000 cells/well. Cell population growth studies employing crystal violet as DNA stain revealed that combination treatment of DCA at concentration of 7.5 mM and C9 at concentration of 130 nM inhibited cell proliferation in tumourigenic

MCF-7 cells to 50.84% after 24-h exposure (IC₅₀). The same concentration inhibited cell population growth by only 29.29% in non-tumourigenic MCF-12A cells, indicating that non-tumourigenic MCF-12A cells to be less susceptible to expansion inhibition compared to tumourigenic MCF-7 cell line, for this specific combination of DCA and C9.

Previous research has demonstrated similar results for DCA when combined with anti-mitotic compounds whereby normal cells experienced lower cytotoxicity compared to tumourigenic cells (51–53). Olszewski *et al.* (51)

Figure 9. Apoptosis detection by means of flow cytometry and annexin V-FITC of MCF-7 (a) and MCF-12A (b) cells. Propidium iodide (FL3 Log) versus annexin V-FITC (FL1 Log) dot-plots of cells propagated in culture medium, vehicle (DMSO)-, C9-, DCA-, C9+DCA- and actinomycin D-exposed MCF-7 (a) and MCF-12A (b) cells. Cells treated with vehicle control (DMSO v/v <0.01%) revealed no toxic effect in both cell lines. Neither C9-treated nor DCA-treated MCF-7 (a) and MCF-12A (b) cells had severe degree of apoptosis compared to C9+DCA-exposed cells. **P*-value <0.05 compared to vehicle control of the same cell line. MCF-7 cells exposed to dual treatment-induced increased early apoptosis compared to MCF-12A cells. [†]*P*-value <0.05 when MCF-7 cells were compared to MCF-12A cells.

demonstrated that HEK293 normal epithelial kidney cells had less cytotoxicity at 10 mM DCA in combination with selected platinum drugs. Fiebiger *et al.* (52) demonstrated that DCA potentiates cytotoxicity of selected platinum drugs, including satraplatin. Dhar and Lippard (53) showed that mitaplatin, another platinum-based anti-cancer compound, in combination with DCA, selectively killed cancer cells. The present study is the first to demonstrate *in vitro* effects of an anti-mitotic compound in combination with DCA on tumourigenic and non-tumourigenic cells.

Morphological investigation *via* plasDIC light microscopy indicated that DCA-treated MCF-12A and MCF-7 cells showed no significant qualitative morphological differences compared to vehicle-treated controls. Compromised cell density was, however, observed in both cell lines treated with C9 when compared to vehicle-treated controls. Formation of apoptotic bodies and compromised cell density in MCF-7 and MCF-12A cells treated with C9+DCA were observed. However, MCF-12A cells appeared to be less affected when compared to MCF-7 cells. Increase in acridine orange suggests increase in lysosomal and/or autolysosomal activity and thus suggests an increase in lysosomal and/or autolysosomal activity.

Cell cycle analyses revealed increase in G₂/M phase in C9-exposed and C9+DCA-exposed MCF-7 cells, decrease in cells present in S-phase, as well as increase in number of cells in sub-G₁ in MCF-7-treated C9+DCA cells. This observation on MCF-7 agrees with previous studies, which showed similar findings with MCF-7 cells, by Tagg *et al.* (39). There was also an increase in sub-G₁ phase in MCF-12A-treated C9+DCA cells. In comparison, difference between sub-G₁ status on tumourigenic and non-tumourigenic cell lines is statistically significant ($P < 0.05$). Apoptosis analyses showed that C9-treated MCF-7 and MCF-12A cells have comparatively similar numbers of cells in early apoptosis. DCA-treated cells had no statistically significant difference in early and late apoptotic cells when compared to vehicle-treated controls. However, when cells were treated with C9+DCA, a synergistic effect was observed whereby MCF-7 cells in early apoptosis increased approximately 3-fold when compared to C9-treated cells. This effect was not as pronounced in MCF-12A cells (2-fold increase), suggesting that the synergistic effect was selective towards tumourigenic cells. Statistical analysis between the two cell lines confirmed this finding that C9+DCA-exposed MCF-7 cells exhibited significantly higher percentages of cells in early apoptosis ($P < 0.05$).

IC₅₀ suggests that MCF-7 cell population growth was half as much compared to vehicle-treated control. This does not necessarily imply that 50% of the tumourigenic

cells in fact underwent cell death. The Annexin V-FITC apoptosis study, however, suggested that approximately 16% of cells were apoptotic after 24-h exposure with C9+DCA. Thus, from the data, it can be concluded that apoptosis is but one mechanism of population growth inhibition. Our morphological studies qualitatively confirm that apoptotic processes were present; however, they also suggest that autophagic processes (increase in acridine orange staining) might also, in part, be responsible for cell population growth inhibition. Previous studies in which 2ME has been used for anti-cancer treatment *in vitro* have demonstrated similar findings. For example, Fukui *et al.* (54) demonstrated that 1.5–2.0 μ M of 2ME inhibited 50% cell population growth in MDA-MB-435 cells after 48 h compared to vehicle-treated control and only 18.1% of the cell population went through apoptosis after exposure to 1.5 μ M of 2ME. Also, our group has demonstrated that 2ME inhibits cell growth in MCF-7 cells after 48 h to 50% at 1 μ M and that it induces apoptosis in 15% of cells at the same concentration (7).

The present study demonstrates that combination of a new anti-mitotic compound in conjunction with PDK inhibitor, DCA, has the potential to selectively target tumourigenic MCF-7 cells over non-tumourigenic MCF-12A cells, by inducing apoptosis. MCF-7 cells have a high glycolytic capacity and they also have significantly more hyperpolarized mitochondrial membrane potential compared to normal cells (55,56). Inhibitors of PDKs such as DCA attenuate inhibition of PDH activity. Increased PDH activity shifts metabolism from glycolysis to oxidative phosphorylation, decreasing mitochondrial membrane potential hyperpolarization, which in turn opens mitochondrial transition pores (56). This allows for translocation of ROS from mitochondrial matrix to cytoplasm and increases ROS-signalling, as well as restoring normal metabolism (56). Restoring normal mitochondrial membrane potential in turn results in sensitizing cells to apoptotic signalling (56).

C9 is a potent anti-mitotic analogue of 2ME; however, it is suggested that its mechanism of action with regard to induction of apoptosis is similar to that of 2ME. In 2ME-treated cells, cell cycle arrest leads to apoptosis *via* reactive oxygen species generation and the intrinsic apoptosis induction pathway as a result of an increase in mitochondrial permeabilization and cytochrome *c* leakage (7). Autophagic activity is also regulated by reactive oxygen species (57). It is proposed that selectivity of our combination treatment is associated with restoration of oxidative phosphorylation in MCF-7 cells, which in turn contributes to reactive oxygen species formation, mitochondrial permeabilization and ultimately culminating in apoptosis and/or autophagy induction. In conclusion, the synergistic effect of a specific novel anti-mitotic compound in

conjunction with DCA on tumourigenic and non-tumourigenic cells was demonstrated for the first time. This study suggests that the specific combination DCA and C9 is more harmful to tumourigenic cells than treatment with DCA or C9 alone. Further mechanistic studies are underway to further characterize the exact mechanisms of this action.

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