

Short communication

**SULPHAMOYLATED ESTRADIOL ANALOGUE
INDUCES ANTIPROLIFERATIVE ACTIVITY AND APOPTOSIS
IN BREAST CELL LINES**

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Abstract: Research into potential anticancer agents has shown that 2-methoxyestradiol exerts antiproliferative activity *in vitro* and *in vivo* in an estrogen receptor-independent manner. Due to its limited biological accessibility and rapid metabolic degradation, several new analogues have been developed in recent years. This study investigated the *in vitro* effects of a novel *in silico*-designed compound (C16) in an estrogen receptor-positive breast adenocarcinoma epithelial cell line (MCF-7), an estrogen receptor-negative breast adenocarcinoma epithelial cell line (MDA-MB-231) and a non-tumorigenic breast cell line (MCF-12A). Light microscopy revealed decreased cell density, cells blocked in metaphase and the presence of apoptotic characteristics in all three cell lines after exposure to C16 for 24 h. Polarization-optical transmitted light differential interference contrast revealed the presence of several rounded cells and decreased cell density. The xCELLigence real-time label-independent approach revealed that C16 exerted antiproliferative activity. Significant inhibition of cell growth was demonstrated after 24 h of exposure to 0.2 μ M C16 in all three cell lines. However, the non-tumorigenic MCF-12A cell line recovered extremely well after 48 h when compared to the tumorigenic cell lines. This indicates that C16 acts as an antiproliferative agent, possesses antimetabolic activity and induces apoptosis *in vitro*. These features warrant further investigation.

Key words: C16, Cancer, Proliferation, MCF-7, MCF-12A, MDA-MB-231, Metaphase, Apoptosis, Xcelligence, Tumorigenic

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Abbreviations used: 2ME – 2-methoxyestradiol; C16 – compound 16

INTRODUCTION

2-methoxyestradiol (2ME) is a 17- β estradiol derivative that exerts antiproliferative activity and destroys the tubulin structure in an estrogen receptor-independent manner. 2ME failed to advance to United States Food and Drug Administration approval because of its low efficacy [1-4]. It also shows rapid metabolic degradation and has limited bioavailability. However, several promising analogues have been developed in recent years [2].

2-methoxyestradiol-bis-sulphamate is a bis-sulphamoylated analogue of 2ME with antiproliferative activity. It has been found to induce apoptosis in several cell lines, including MCF-7 estrogen receptor-positive breast adenocarcinoma cells, PC-3 prostate cancer cells, human umbilical vein endothelial cells (HUVEC) and CAL51 human breast adenocarcinoma cells [2, 5-8]. 2-methoxyestradiol-bis-sulphamate exposure resulted in a G₂/M block in cell lines, including the estrogen receptor-positive human breast adenocarcinoma cell line MCF-7, the drug-resistant human adenocarcinoma cell line MCF-7 DOX40, and the highly tumorigenic breast carcinoma cell line MDA-MB-231 [9]. Another analogue, 2-methoxyestra-1,3,5(10)16-tetraene-3-carboxamide (also known as ENMD-1198, C24, 883, or ENMD-0998) exerts antiproliferative and antiangiogenic activity in the MCF-7 cell line and the HCT 116 colon cancer cell line [10-12]. Sulphamoylated analogues, including methylcoumarin-sulphamate and 2-methoxyestradiol-sulphamate, exert steroid sulphatase inhibitory activity, with methylcoumarin-sulphamate also possessing additional weak aromatase activity. They are currently being evaluated in Phase I clinical trials for post-menopausal women with metastatic breast cancer [13-15].

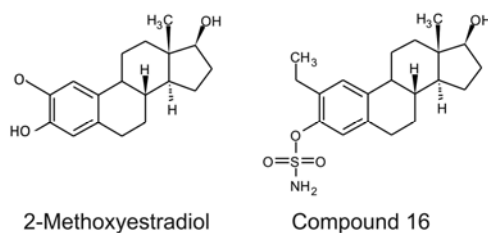


Fig. 1. Summary of the structural differences between 2ME and its sulphamoylated analogue C16.

This preliminary study focuses on the differential effects of a novel sulphamoylated 2ME compound called compound 16 (C16). It was *in silico*-designed in our laboratory based on the anticancer activity of other above-mentioned sulphamoylated compounds (Fig. 1). Docking studies revealed that 2-ethyl-3-O-sulphamoyl-estra-1,3,5(10)16-tetraene (compound 12), 2-ethyl-3-O-sulphamoyl-estra-1,3,5(10),15-tetraen-3-ol-17-one (compound 9) and 2-ethyl-17-(1'-methylene)estra-1,3,5(10)-trien-3-O-sulphamate (compound 10) have a superior carbonic anhydrase IX to carbonic anhydrase II ratio compared to

other synthesized molecules and their parental molecule (2ME). Furthermore, hydrophobic interactions between C12 and ala250.B, leu242.B, leu248.B, leu252.B, leu255.B, lys352.B, and val318.B were demonstrated [16]. These studies showed that the *in silico*-designed compounds are more potent than 2ME and they have a 50% growth inhibitory (GI₅₀) ability at nanomolar concentrations [16]. We investigated the *in vitro* effects of C16 on cell proliferation, morphology and possible cell death induction in an estrogen receptor-positive breast adenocarcinoma cell line, an estrogen receptor-negative metastatic cell line and a non-tumorigenic breast epithelial cell line.

MATERIALS AND METHODS

Cell lines

MCF-7 is an estrogen receptor-positive tumorigenic adherent breast epithelial cell line derived from metastatic sites in adenocarcinoma. MCF-7 cells were supplied by Highveld Biological (Pty) Ltd. (Sandringham, South Africa). MDA-MB-231 is an estrogen receptor-negative breast adenocarcinoma cell line supplied by Microsep (Pty) Ltd, Johannesburg (South Africa). MCF-12A is a non-tumorigenic transformed adherent human breast epithelial cell line. These cells are produced by long-term cultures and form domes in confluent cultures. The MCF-12A cells were a gift from Professor Parker of the Department of Medical Biochemistry, University of Cape Town, South Africa.

Reagents

All the required reagents were of cell culture analytical grade and were purchased from Sigma (St. Louis, United States of America) unless otherwise specified. Heat-inactivated fetal calf serum (FCS), sterile cell culture flasks and plates were purchased from Sterilab Services (Kempton Park, Johannesburg, South Africa). Penicillin, streptomycin and fungizone were obtained from Highveld Biological (Pty) Ltd. (Sandringham, South Africa).

The sulphamoylated analogue (C16) of 2-methoxyestradiol was synthesized by iThemba Pharmaceuticals (Pty) Ltd. (Modderfontein, Gauteng, South Africa), since this compound was *in silico*-designed in our laboratory and is therefore commercially unavailable [13]. A stock solution of C16 dissolved in dimethyl sulphoxide (DMSO) was prepared with a concentration of 10 mM and was stored at 4°C. The vehicle control sample was composed of DMSO and growth medium where the DMSO content of the final dilutions never exceeded 0.05% (v/v).

Cell culture

Cells were grown and maintained in 25-cm² tissue culture flasks in a humidified atmosphere at 37°C and 5% CO₂. MCF-7 and MDA-MB-231 cells were cultured in Dulbecco's minimum essential medium eagle (DMEM) and supplemented with 10% heat-inactivated FCS (56°C, 30 min), 100 U/ml penicillin G, 100 µg/ml streptomycin and 250 µg/l fungizone [9]. MCF-12A maintenance medium consisted of a 1:1 mixture of DMEM and Ham's-F12 medium, 20 ng/ml

epidermal growth factor, 100 ng/ml cholera toxin, 10 µg/ml insulin and 500 ng/ml hydrocortisone, supplemented with 10% heat-inactivated FCS (56°C, 30 min), 100 U/ml penicillin G, 100 µg/ml streptomycin and 250 µg/l fungizone [2].

Light microscopy (haematoxylin and eosin staining)

The investigation of the *in vitro* influence of C16 on cell morphology was conducted using the haematoxylin and eosin staining method. It yielded both quantitative and qualitative information.

Cells were seeded on sterile coverslips in 6-well plates at a density of 500,000 cells per well and incubated overnight. Then, cells were exposed to 0.2 µM C16 for 24 h, since previous studies have shown that sulphamoylated compounds exert antiproliferative and apoptotic activity at 0.2 µM for 24 h [16].

Haematoxylin and eosin staining was conducted according to Visagie *et al.* [2]. Coverslips were mounted on microscope slides with resin and left to dry. Photos were taken using a Zeiss Axiovert MRc microscope (Zeiss, Oberkochen, Germany). The haematoxylin- and eosin-stained cells were also used to determine mitotic indices. Quantitative data for the mitotic indices was acquired by counting 1,000 cells on each slide of the biological replicates and expressing the data as the percentages of cells in each phase of mitosis (prophase, metaphase, anaphase and telophase), cells in interphase, and cells demonstrating hallmarks of apoptosis.

Polarization-optical transmitted light differential interference contrast

Polarization-optical transmitted light differential interference contrast (PlasDIC) is a method to view cell morphology. It was conducted according to Visagie *et al.* [2, 9]. Images were obtained before and after exposure using the Axiovert 40 CFL microscope (Carl Zeiss, Goettingen, Germany).

xCELLigence monitoring

The xCELLigence system is a novel approach developed by Roche Applied Science (Penzberg, Germany) to investigate cell growth, adhesion and morphology in real time in a label-independent manner. It was employed to confirm whether the dosage exerts optimal antiproliferative activity [17]. This system measures electrical impedance across the micro-electrodes integrated on the bottom of tissue culture 96-well plates therefore allowing real-time and continuous cellular analysis as cells attach and proliferate. The change in impedance is expressed as the cell index. The cell index is an indication of cell number, cellular attachment and morphology. Cells were seeded at a density of 5000 cells per well, placed for 30 min on a rotator plate and subsequently placed in the xCELLigence system, which was linked to the incubator in a humidified atmosphere at 37°C and 5% CO₂ [17].

Statistical analysis

Qualitative data were obtained by PlasDIC, light microscopy with haematoxylin and eosin staining, and fluorescent microscopy. Quantitative information was gained by means of time- and dose-dependent studies using the real time

xCELLigence system. Data were obtained from three independent experiments with 6 technical repeats each and statistically analyzed for significance using the analysis of variance (ANOVA) single factor model followed by a two-tailed Student's *t*-test. *P*-values < 0.05 were regarded as statistically significant.

RESULTS

Light microscopy

Light microscopy was used to investigate the *in vitro* effects of C16 exposure on the morphology of MCF-7, MCF-12A and MDA-MB-231 cells (Fig. 2). C16 exposure resulted in compromised cell density in all three cell lines when compared to vehicle-treated cells. Several apoptotic hallmarks (apoptotic bodies, shrunken cells and cell debris) were observed in treated MCF-7 cells (Fig. 2D), treated MDA-MB-231 cells (Fig. 2E) and treated MCF-12A cells (Fig. 2F) and in all three cases, cells were found that were blocked in metaphase. MDA-MB-231 cells were more strongly affected by C16 than the other cell lines. Mitotic indices (Table 1) revealed that a significant percentage of cells were in metaphase and demonstrated features of apoptosis after exposure to C16 in all three cell lines when compared to vehicle-treated cells.

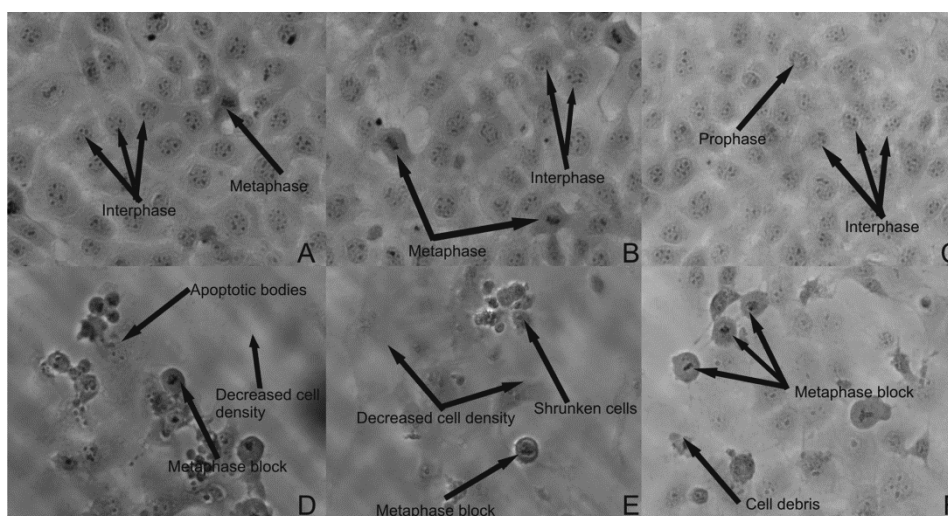


Fig. 2. Light microscopy of haematoxylin and eosin staining of vehicle-treated MCF-7 (A), MDA-MB-231 (B) and MCF-12A cells (C), and C16-treated MCF-7 (D), MDA-MB-231 (E) and MCF-12A cells (F). All of the cell lines treated with C16 revealed decreased cell density and an increased number of cells present in metaphase when compared to vehicle-treated cells. In addition, apoptotic bodies, shrunken cells and cell debris were observed. All micrographs were taken at 40x magnification.

Table 1. Percentages of cells in the various phases of mitosis, in interphase, or featuring characteristics of apoptosis.

	Interphase	Prophase	Metaphase	Anaphase	Telophase	Apoptotic cells
MCF-7						
Vehicle-treated cells	93.5%	3.6%	1.2%	0.9%	0.8%	0.0%
C16-treated cells	59.4%	1.1%	28.8 %	0.1%	0.2%	10.4%
MDA-MB-231						
Vehicle-treated cells	93.3%	3.3%	1.6%	1.0%	0.8%	0.0%
C16-treated cells	53.9%	0.9%	32.5%	0.2%	0.1%	12.4%
MCF-12A						
Vehicle-treated cells	94.5%	3.2%	1.2%	0.6%	0.5%	0.0%
C16-treated cells	56.7%	1.9%	30.4%	0.5%	0.2%	10.3%

Polarization-optical transmitted light differential interference contrast

Polarization-optical transmitted light differential interference contrast (PlasDIC) revealed the induction of apoptosis in all C16-treated MCF-7, MDA-MB-231 and non-tumorigenic MCF-12A cells (Fig. 3). Cells were exposed to C16 for 24 h. The C16-treated cells displayed decreased cell density and a rounded appearance compared to the vehicle-treated cells. In addition, the C16-treated cells showed shrunken size, cell debris and apoptotic bodies, which are all characteristics of apoptosis.

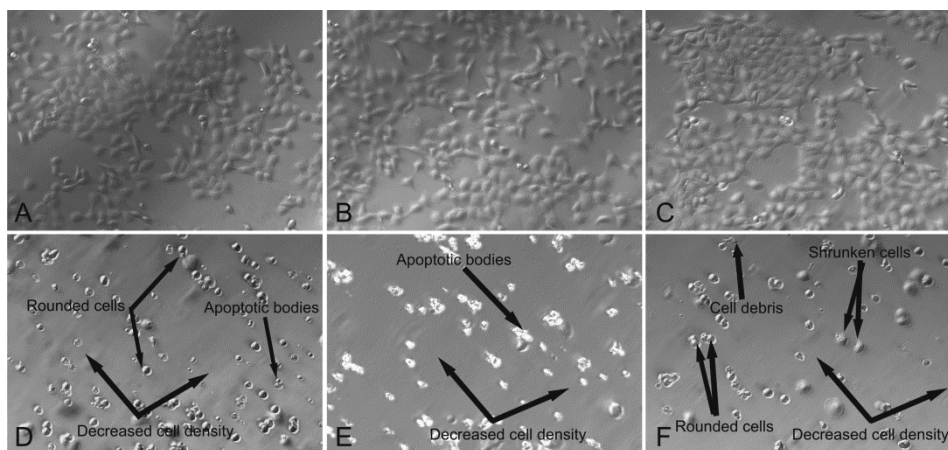


Fig. 3. PlasDIC micrographs of vehicle-treated MCF-7 (A), MDA-MB-231 (B) and MCF-12A cells (C), and C16-treated MCF-7 (D), MDA-MB-231 (E) and MCF-12A cells (F). Cells were exposed to 0.2 μ M of C16 for 24 h. Vehicle-treated cells showed no abnormal morphology. C16-treated cells revealed decreased cell density, rounded appearance, apoptotic bodies and cell debris.

xCELLigence system

This novel real-time label-independent approach was used to measure cell adhesion and cell proliferation of C16-treated MCF-7, MDA-MB-231 and MCF-12A cells (Fig.4A, B and C). Cell proliferation was significantly inhibited in all of the cell lines (0.2-1.0 μM), decreasing the cell index in the MCF-7 and MDA-MB-231 cell lines. The MCF-12A cell line recovered after 48 h of exposure.

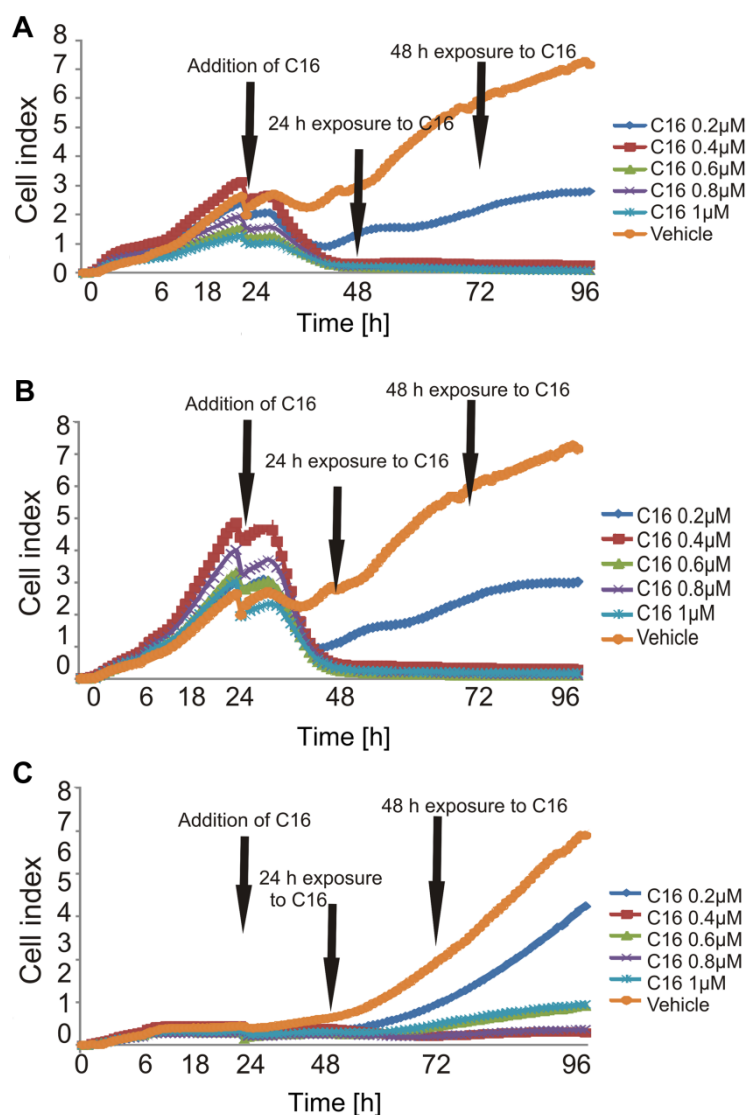


Fig. 4. The xCELLigence method demonstrated the *in vitro* effects of C16 on proliferation in MCF-7 cells (A), MDA-MB-231 cells (B) and MCF-12A cells (C). C16 had a statistically significant inhibitory effect on all of the treated cell lines. The MCF-7 and MDA-MB-231 cells recovered after 48 h of exposure to 0.2 μM C16. The MCF-12A cell line recovered more effectively than the other cell lines.

DISCUSSION

This *in vitro* pilot study investigated the effects of a novel *in silico*-designed sulphamoylated 2ME compound in an estrogen receptor-negative breast adenocarcinoma cell line (MDA-MB-231), an estrogen receptor-positive breast adenocarcinoma cell line (MCF-7) and an estrogen receptor-negative non-tumorigenic breast cell line (MCF-12A). To date, no research regarding the *in vitro* or *in vivo* activity of C16 has been published due to the novelty of this compound. Studies performed in our laboratory revealed that other *in silico*-designed compounds in a concentration range of 0.11-0.22 μM inhibited the growth of various cancer cell lines [16]. In this study, C16 exerted effective antiproliferative activity at a concentration of 0.2 μM . Light microscopy, mitotic indices and PlasDIC demonstrated that cells were blocked in metaphase and had several characteristics associated with apoptosis induction and decreased cell density.

We find that C16 exerts antiproliferative and antimitotic activity in MCF-7 cells, MDA-MB-231 cells and MCF-12A cells and that C16 exposure leads to states characteristic for apoptosis. The tumorigenic MCF-7 and MDA-MB-231 cells were more susceptible to C16 treatment than the non-tumorigenic MCF-12A cells. Further research will focus on the molecular signal transduction that C16 utilizes in its induction of cell death and an in-depth analysis of specific targets *in vitro* and subsequently *in vivo*. The latter will contribute to the discovery of targets for cancer therapies that will aid in the design of antiproliferative and microtubule-disrupting agents.

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