In vitro changes in mitochondrial potential, aggresome formation and caspase activity by a novel 17-β-estradiol analogue in breast adenocarcinoma cells

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2-Methoxyestradiol, a natural metabolite of estradiol, exerts antiproliferative and antitumour properties in vitro and in vivo. Because of its low oral bioavailability, several promising analogues of 2-methoxyestradiol have been developed. In this study, the in vitro influence of the compound, 2-ethyl-3-O-sulphamoyl-estra-1,3,5(10)16-tetraene (C19), a non-commercially available 17-β-estradiol analogue, was tested on the breast adenocarcinoma MCF-7 cell line. The in vitro influence of 24 h exposure to 0.18 μM of C19 on MCF-7 cells was evaluated on cell morphology, cell cycle progression and possible induction of apoptosis and autophagy. Polarization-optical transmitted light differential interference contrast and fluorescence microscopy revealed the presence of cells blocked in metaphase, occurrence of apoptotic bodies and compromised cell density in C19-treated cells. Hallmarks of autophagy, namely an increase in the number of acidic vacuoles and lysosomes, were also observed in C19-treated samples. An increase in the number of cells present in the sub-G1 fraction, as well as a reduction in mitochondrial membrane potential was observed. No significant alterations in caspase 8 activity were observed. A twofold increase in aggresome formation was observed in C19-treated cells. C19 induced both apoptosis and autophagy in MCF-7 cells. Copyright © 2013 John Wiley & Sons, Ltd.

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INTRODUCTION

Worldwide, there are approximately 7.4 million cancer deaths per year.1 As the incidence of common cancer types increases at the age of 40, the prevalence of breast cancer per se augments in the late twenties.2 Risk factors for breast cancer involve genetic makeup, mammographic density, atypical hyperplasia, radiation and the effect of oestrogen, which can be divided into endogenous and exogenous oestrogen.2 Lifestyle factors such as diet, alcohol intake and cigarette smoking also influence the incidence of breast cancer development.2

2-Methoxyestradiol (2ME2) is an analogue of 17-β-estradiol, which is synthesized by the human body. 2ME2 has a strong anticancer activity and induces apoptosis in cancer cells.3 2ME2 activates c-jun N-terminal kinase (by phosphorylation) as a proapoptotic signal; it also inactivates (by phosphorylation) the anti-apoptotic (B cell lymphoma) Bcl-2 family proteins, namely Bcl-2 and Bcl-XL, culminating in apoptosis through the mitochondrial pathway. Furthermore, 2ME2 activates extracellular signal–regulated kinase and p38 mitogen-activated protein kinase as anti-apoptotic signals. Phosphorylated extracellular signal–regulated kinase and p38 mitogen-activated protein kinase contribute to the degradation of the proapoptotic Bcl-2 family proteins.3,9 2ME2 apoptotic actions include the inhibition of cell cycle progression, changes in the mitochondrial membrane potential and the disruption of DNA.

Despite all the benefits of the drug, its bioavailability is low because of rapid degradation due to metabolism. To overcome the disadvantage of its low bioavailability, many estradiol analogues have been synthesized. Stander et al.4 designed novel 2ME2 derivatives in silico by modification of position 2 and the D-ring of 2ME2 to create novel estradiol derivatives (Figure 1). Position 3 was replaced by a sulphamate group in the newly designed drug and changes were made at position 2 to increase the antimitotic properties of the new compound. The 2-methoxyestradiol-bis-sulphamate (2-MeOE2bisMATE) analogue of 2ME2 is more resistant to metabolism and its increased bioavailability is due to its sulphamoyl moieties.12 Improved oral bioavailability is argued to be a result of the potential of aryl sulphamoyl-containing compounds to reversibly bind to carbonic anhydrase II present in blood cells and in turn circumvent first-pass liver metabolism.13

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ENMD-1198, another analogue of 2ME2, is undergoing clinical trials and the D-ring modification seems to improve bioavailability when compared with 2ME2.10–15

Figure 1. Chemical structures of 2ME2 (A) and compound C19 (B). (Drawings by B. A. Stander using MarvinSketch software available from ChemAxon at http://www. Chemaxon.com/product/marvin_land.html.)

Materials and Methods

Materials

Cell line. The MCF-7 (oestrogen receptor positive) cell line is a tumourigenic immortalized adherent breast adenocarcinoma cell line that has the ability to chemically transform estradiol via oestrogen receptors located in the cytoplasm and can form domes in culture. Cells were purchased from Highveld Biological, Pty Ltd (Sandringham, South Africa). MCF-7 cells were propagated in Dulbecco’s modified Eagle’s minimal essential medium (DMEM) enriched with 10% heat-inactivated foetal calf serum, 100 U/ml of penicillin G, 100 μg/ml of streptomycin and 250 μg/l of fungizone. C19 was dissolved in dimethyl sulfoxide (DMSO) and the final concentration did not exceed 0.05% in cell culture. Cells were exposed to 0.18 μM of C19 for 24 h at 37°C. This concentration was selected because it was previously established that 0.18 μM of C19 inhibited cell growth by 50% (GI50) after 24 h at 37°C.4

Reagents

All the reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise indicated. Foetal calf serum as well as sterile cell culture flasks and plates were supplied by Sterilab Services (Kempton Park, Johannesburg, South Africa). The antibiotics, namely penicillin, streptomycin, fungizone and gentamycin were supplied by Highveld Biological. Trypsin-EDTA, DMEM and buffers were purchased from Highveld Biological. Actinomycin D served as a positive control for apoptosis, and DMSO served as vehicle control. The caspase 8 colorimetric assay, caspase 7 antibody, the aggresome detection kit, MitoCapture™ mitochondrial apoptosis detection kit and the LC3 antibody were purchased from BIOCOM Biotech Pty Ltd (Clubview, South Africa).

Drug synthesis

The compound C19 is not commercially available and was in silico-designed at the Bioinformatics and Computational Biology Unit, Department of Biochemistry, University of Pretoria, South Africa and synthesized by Ithemba Pharmaceuticals Pty Ltd (Modderfontein, Midrand, South Africa).

Cell culture

MCF-7 cells were propagated as monolayers in growth medium at 37°C in a humidified environment containing 5% carbon dioxide. Cells were seeded and allowed for attachment overnight. Appropriate controls were included in all experiments. Cells were propagated in growth medium only; vehicle-treated controls were exposed to 0.6 μl of DMSO in 3 ml of DMEM. Cells were exposed to 1.2 μl of tamoxifen in 3 ml of DMEM for autophagy detection, as well as cells exposed to 0.3 μl of actinomycin D in 3 ml of DMEM served as the controls for apoptosis. Experiments were conducted in 25 cm² culture flasks and six-well plates as stipulated for a specific technique. For 25 cm² culture flasks, 10⁶ or 500 000 cells were seeded in 3 ml of growth medium. For six-well plates, 250 000 cells were seeded per well in a 3-ml growth medium on heat-sterilized cover slips. For each experiment, growth medium was renewed prior to cell exposure.

Methods

Morphology

Triple staining with acridine orange, Hoechst 33342 and propidium iodide. Fluorescence microscopy was conducted using a triple fluorescent staining technique to identify possible intracellular markers of cell death. Acridine orange detects and accumulates in autophagic vacuoles to emit a green fluorescent light. Hoechst 33342 stains the nuclei of apoptotic cells blue, whereas propidium iodide red fluorescence aggregates in the nuclei of necrotic cells. Cells were seeded at a density of 250 000 cells per well in six-well plates and allowed to attach overnight. Cells were then exposed to 0.18 μM of C19 and appropriate controls in fresh medium for 24 h. Subsequently, 0.5 ml of 0.9 μM Hoechst 33342 and 0.5 ml of 50 μM acridine orange in phosphate saline buffer (PBS) were added to all six wells and samples were incubated for 25 min at 37°C. Next, 0.5 ml of 12 μM propidium iodide was added for an additional 5 min. Cells were washed twice with PBS. The experiment was performed in the dark to
prevent bleaching. Samples were examined under a Zeiss inverted Axiovert CFL40 microscope and photomicrographs were taken with a Zeiss Axiovert MRm monochrome camera with different fluorescence filters to distinguish between the different stains. Zeiss filter 2 was used for Hoechst 33342 (blue emission), Zeiss filter 9 for acridine orange (green emission) and Zeiss filter 15 for propidium iodide (red emission) stained cells.

_Polarization-optical differential interference contrast._ Polarization-optical transmitted light differential interference contrast (PlasDIC) is an enhanced method in which linearly polarized light is emitted after the objective, generating quality images. This technique uses a beam of polarized light divided into two beams polarized at 90° to each other; each beam of light takes a slightly different path through the sample. The absorbance of the sample causes the two beams to interfere with each other before they recombine. PlasDIC gives a three-dimensional image of individual cells and cell clusters in plastic cell culture flasks. PlasDIC microscopy was used to non-invasively assess the morphology of the cell population. MCF-7 cells were seeded at a density of 250,000 cells per well in six-well plates. After an incubation period of 24 h, cells were exposed to 0.18 M of C19, DMSO and actinomycin D. Cells were trypsinized and centrifuged at 10,000 × g. Cells were resuspended in 50 μl of chilled cell lysis buffer and incubated on ice for 10 min. Cells were centrifuged at 10,000 × g for 1 min. The supernatant was transferred to a new tube and left on ice for 15 min. After the protein concentration was determined using a standard curve, 100 μg of protein/50 μl of cell lysis buffer was mixed with 50 μl of reaction buffer containing 10 mM of DTT. Five microlitres of 4 mM VEID-pNA substrate with a final concentration of 200 μM was added to the mixture and incubated at 37°C for 2 h. Absorbances were read at 405 nm using the ELx800 Universal Microplate Reader purchased from BioTek Instruments Inc. (Winooski, VT, USA).

**Flow cytometry**

_Caspase 7 activation assay._ The activation of caspase 7 (an effector caspase of apoptosis) was determined using an FITC Caspase 7 kit. MCF-7 cells were seeded at a density of 10⁶ cells per 25 cm² flask. After 24 h of attachment, the medium was discarded and cells were exposed to 0.18 μM of C19, DMSO and actinomycin D. Cells were trypsinized and centrifuged at 10,000 × g. Cells were washed with 0.5 ml of PBS, centrifuged and then the supernatant was discarded. Fixation buffer (0.5 ml) was added and the samples were incubated at room temperature for 20 min. After the supernatant was discarded, the cell suspension was resuspended in 0.5 ml of PBS. After the supernatant was centrifuged and discarded, the cell suspension was resuspended in 0.5 ml of ice-cold cell lysis buffer containing 0.5% Tween 20 and incubated at 4°C for 20 min. After another centrifugation step, 0.5 ml of 1× assay buffer was added to the cell suspension. The supernatant was discarded and 100 μl of 1× assay buffer was added followed by 100 μl of primary antibody cocktail. The cell samples were incubated at 4°C for 90 min. Nine hundred microlitres of 1× assay buffer was added and then centrifuged, and the supernatant was discarded. Then, 100 μl of 1× assay buffer was added along with 100 μl of secondary antibody cocktail. The samples were protected from light to avoid bleaching. Samples were incubated at 4°C for 60 min. The samples were then centrifuged and the supernatant was discarded. Samples were washed twice using 0.5 ml of 1× assay buffer and were analysed using flow cytometry. Data from at least 10,000 cells were analyzed with a Cytofluor version 1.2.1 software (Perttu Therho, Turko, Finland).

**Flow cytometry**

_Cell cycle progression._ Influences on cell cycle progression and mitochondrial membrane potential were investigated using flow cytometry. Cells were seeded at a density of 10⁶ cells per 25 cm² flask and were incubated overnight to allow for attachment. Cells were subsequently exposed to 0.18 μM of C19 for 24 h and appropriate controls were included as previously described. Cells were trypsinized...
this challenge, the supernatant was collected. The cells were pelleted and washed in 1 mL of PBS. After centrifugation, the cells were resuspended in 200 μL of ice-cold PBS containing 0.1% foetal bovine serum. Cells were fixed in 4 mL of ice-cold 70% ethanol and the samples were kept overnight at 4°C. Cells were washed and resuspended in 1 mL of PBS containing propidium iodide (40 μg/mL), RNase A (100 μg/mL) and Triton X-100 (0.1%) for 40 min at 37°C. Analyses were performed using a Beckman Coulter Cytomics FC500 instrument (Beckman Coulter Inc., Fullerton, CA). Data from at least 30,000 cells per sample were analyzed with a CXP software (Beckman Coulter Pty Ltd, South Africa).

Data obtained from at least three biological repeats for mitochondrial membrane potential, cell cycle analysis, caspase 7 and 8, LC3 and aggresome quantification were obtained. Only one set of representative data is shown. Quantitative data were statistically analyzed for significance using the analysis of variance—single factor model followed by a Student t-test. Means are represented in bar charts with T-bars referring to standard deviations. P < 0.05 values were regarded as statistically significant. Qualitative experiments were repeated at least twice where data were obtained from PlasDIC, haematoxylin and eosin staining and fluorescent microscopy.

RESULTS

Morphology

Triple staining. Fluorescent triple staining was conducted to examine the cells for possible hallmarks of apoptosis and autophagy after a 24-h exposure to 0.18 μM of C19. Acridine orange stains autophagic vacuoles and lysosomes with an increase in green fluorescence. Hoechst 33342 penetrates cell membranes of viable cells and cells undergoing apoptosis, and the stained nuclei will emit a blue fluorescence.
Propidium iodide stains the nuclei of cells that have lost their membrane’s integrity due to oncotic or necrotic processes with a red fluorescence. An increase in green fluorescence was observed in the C19-treated sample (Figure 2D) when compared with the controls (Figures 2A and 2B). Hypercondensed chromatin was observed in the actinomycin D-treated cells (Figure 2C) and cell density was compromised in the C19-treated sample (Figure 2D). No red fluorescence was observed, indicating that cell membranes were not disrupted.

**Polarization-optical differential interference contrast.** PlasDIC was used to visualize the morphological effects of 0.18 μM of C19 on MCF-7. MCF-7-treated cells revealed an increase in the number of cells blocked in metaphase as well as an increase in the number of shrunken cells (Figure 3D). The density of the treated sample was compromised when compared with the density of controls (Figures 3A–D). Apoptotic bodies were observed in both C19-treated and actinomycin D-treated cells (Figures 3C and 3D).

**Haematoxylin and eosin staining.** Haematoxylin and eosin staining revealed hypercondensed chromatin, apoptotic bodies, metaphase block and compromised density in the C19-treated MCF-7 cells when compared with the vehicle-treated cells (Figure 4).

**Spectrophotometry**

**Caspase 7 and 8 activation assay.** The purpose of this study was to determine whether 0.18 μM of C19 increases caspase 7 and 8 activity in MCF-7 cells after 24 h of exposure. The ratio of caspase 7 and 8 activity was measured with reference to cells propagated in medium only. Positive controls for both caspase 7 and 8 were included in the experimental design (data not shown). The C19-treated sample showed an increase in caspase 8 activity after a 24 h exposure time; however, this increase was not statistically significant (Figure 5). A statistically significant increase in caspase 7 activity in C19-treated cells compared with vehicle-treated cells was observed.

**Flow cytometry**

**Cell cycle analysis.** Flow cytometric analysis revealed the increased amount of cells in sub-G1 phase in the C19-treated sample in comparison with the vehicle control sample (Figure 6). Table 1 indicates the percentage of the C19-treated and vehicle control samples in different phases.

**Mitochondrial membrane potential.** Analysis of the membrane potential showed an increase in the number of cells with a reduced membrane potential in the C19-treated sample when compared with the vehicle control sample (Figure 7). This increase in reduced membrane potential was represented by a shift to the right on the histograms. There was a twofold increase in the number of cells with a reduced membrane potential in the C19-treated sample when compared with the vehicle control (Table 2). The results are expressed as FL1 log histograms.

**Aggresome detection assay.** The assessment of aggresome formation by flow cytometry showed an increase in the number of aggresomes formed in the C19-treated sample when compared with the vehicle control sample (Figure 8). This was represented by a shift to the right. The ratio of aggresomes formed was obtained by calculation of the...
AAF using the MFI of treated and control samples, as indicated in Table 3. Results are expressed as FL3 log histograms.

$$\text{AAF : } 100 \times \frac{(15.0 - 7.28)}{15.0} = 51\%$$

If the AAF value is greater than 25%, this indicates the formation of aggresomes.

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**Figure 4.** Light microscopy displayed MCF-7 cells propagated in medium only (A), vehicle control cells (B) and 0.18 μM C19-treated cells (C) after a 24 h exposure time. MCF-7 exposed to C19 showed mitotic block whereas cells in metaphase, anaphase and interphase were observed in cells propagated in medium only and in the vehicle control sample (magnification, ×400). Quantitative analysis provided in (D) showed an increase in the number of cells in metaphase in the C19-treated cells when compared with the vehicle control sample.

**Figure 5.** Caspase 7 and 8 activity ratios of C19-treated cells compared with cells in medium only after 24 h of exposure. Caspase 8 activity was not statistically significantly increased in the C19-treated cells when compared with cells propagated in medium only. A statistically significant increase in caspase 7 activity in C19-treated cells compared with vehicle-treated cells was observed. An asterisk (*) indicates $P < 0.05$ when compared with the vehicle control.

**Figure 6.** Flow cytometric analysis of cell cycle progression showed an increased sub-G1 in C19-exposed cells when compared with the vehicle control sample. The vehicle control sample is indicated as the red solid filled area, with the C19-treated sample represented as the green solid line.

*Autophagy detection: rabbit polyclonal anti-LC3B conjugated to DyLight 488*

A conjugated rabbit polyclonal anti-LC3 antibody using flow cytometry was used for the detection of autophagy. The latter demonstrated the accumulation of LC3 in 38% of the C19-treated cells when compared with 31% of the vehicle-treated cells (Figure 9).
DISCUSSION

Previous research conducted in our laboratory revealed that 0.18 μM of C19 inhibited MCF-7 cell growth by 50% after 24 h of exposure. Similar results were obtained by Vorster et al. in MCF-12A cells after treatment with 2ME2 and 2-MeOE2bisMATE. Subsequently, and to provide more insight into C19’s antiproliferative effects, the influence of 0.18 μM of C19 was determined on cell morphology, caspase activity, cell cycle progression, mitochondrial membrane potential and aggresome formation after an exposure time of 24 h in MCF-7 cells. Another preliminary study showed that treatment of MDA-MB-435 cells with 2ME2 inhibited growth in a time- and dose-dependent manner. Hallmarks of apoptosis, namely cells blocked in metaphase, hypercondensed chromatin, apoptotic bodies, as well as a compromised cell density were observed within the MCF-7 cell line exposed to 0.18 μM of C19. An intense green fluorescence was also observed in the C19-treated sample, indicating increased lysosomal activity and possible induction of autophagy.

Caspase 8 activity was not statistically increased in the C19-treated samples. Caspase 8 is an initiator in the extrinsic pathway of apoptosis, culminating in the activation of executioner caspases 3, 6 and 7. Caspase 7 activity was increased significantly in the C19-treated cells. Caspase 7 is one of the downstream executioner caspases along with caspases 3 and 6. Effector caspases are responsible for the morphological changes associated with apoptotic cell death, namely shrunken cells, apoptotic body formation and subsequent reduced cell density. Another study revealed an increase in the activation of caspases 3, 7 and 9 in MDA-MB-435 cells after treatment with 2 μM of 2ME2 for 12, 24 and 36 h in a time-dependent manner. Azab et al. showed that the combination of doxorubicin, a prominent chemotherapeutic agent for the treatment of breast cancer, and 2ME2 resulted in an increase in caspase 3 activity by up to 27-fold in a dose-dependent fashion, suggesting that the activation of effector caspases may be involved in the 2ME2 chemosensitizing effect to doxorubicin.

DNA content was measured as an indication of cells in various stages of the cell cycle to evaluate C19’s effect on cell cycle progression. MCF-7 cells displayed a sub-G₁ fraction indicative of apoptosis. Apoptosis was confirmed by PlasDIC by means of decreased cell density, cell debris and the presence of apoptotic bodies. Flow cytometry demonstrated a reduction of mitochondrial membrane potential in the treated cells. Stander et al. also showed an increase in the number of cells in the G₂/M phase after 24 h of exposure and an increase in the number of cell in the sub-G₁.
fraction after 48 h in the MDA-MB-231 cells treated with newly synthesized estrone analogues, namely compounds 2-ethyl-3-O-sulphamoyl-estra-1,3,5(10),15-tetraen-3-ol-17-one and 2-ethyl-estra-17 methylbenzenesulphenohydrazide at different concentrations. Concurrently, Van Zijl et al. confirmed that 2ME2-treated MCF-7 cells caused a metaphase block, membrane blebbing, apoptotic body formation and disruption of spindle formation. These researchers revealed that 10^{-6} M of 2ME2 reduced cell growth by 84% in MCF-7 cells and by only 44% in MCF-12A cells, implying that the tumourigenic cell line is more sensitive to 2ME2 treatment than the normal cell line.

The mitochondrial membrane potential of cells was evaluated to suggest the induction of apoptosis via the intrinsic pathway. The disruption of the mitochondrial membrane is one of the earliest events that occurs after the induction of apoptosis. The induction of apoptosis is accompanied by the mitochondrial permeability transition, which is characterized by a decrease in the electrochemical gradient across the mitochondrial membrane due to the formation of pores in the membrane. The formation of pores is initiated by the activation of proapoptotic members of the Bcl-2 family (Bax, Bid, Bak and Bad proteins). After disruption of the mitochondrial membrane, cytochrome c, normally found in the inner mitochondrial membrane and involved in the electron transport chain, is released in the cytosol and the activity of effector caspase increases.

The intrinsic pathway of apoptosis is mediated via the mitochondria. An increase in Bcl-2 expression and a decrease in Bax expression may foretell the response to chemotherapy in breast cancer cells and the Bcl-2:Bax ratio predicts the cell fate. An increase in the number of cells with reduced membrane potential was observed in the C19-treated MCF-7 cells. Fukui et al. observed similar results after the exposure of MDA-MB-435 cells to 2ME2 for 42 h. A reduction in the mitochondrial membrane potential was observed. In addition, it was demonstrated that pretreatment of cells with an inhibitor of extracellular signal–regulated kinase or p38 enhanced 2ME2-induced reduction in mitochondrial membrane potential. The assessment of aggresome formation suggests cell death by autophagy. C19- and tamoxifen-treated cells showed a shift to the right. This shift suggests that there is an increase in the number of aggresomes formed. A twofold increase in the AAF value was observed in the C19-treated MCF-7 cells.

As previously mentioned, fluorescence microscopy revealed lysosomal staining, thus suggesting the novel discovery of autophagy induction by C19. Conjugated rabbit polyclonal anti-LC3B antibody using flow cytometry verified the induction of autophagy by C19 in the tumourigenic MCF-7 cell line. Autophagy is a process by which the cell’s own components are broken down to maintain a balance of synthesis and degradation in the metabolism of all eukaryotic cells when the cells are undergoing stress. Azab et al. demonstrated that 2ME2 induced autophagy in human glioblastoma–astrocytoma epithelial-like cell line (U87), human cervical adenocarcinoma cells (HeLa) and transformed human embryonic kidney cells (HEK293).

In conclusion, C19 treatment of MCF-7 cells resulted in apoptosis, which was observed by the presence of apoptotic bodies, shrunken cells, disruption of the mitochondrial membrane potential and caspase 7 activation. An increase in aggresome formation and an increase in lysosomal activity in the C19-treated sample suggests the occurrence of autophagy. LC3 accumulation in the C19-treated cells confirmed cell death by autophagy. This is the first time that an increase in aggresome formation was observed in C19-treated MCF-7 cells, and the exact role of the increased aggresome formation and cell death remains to be determined. This novel compound induced cell death by both apoptosis and autophagy in the breast adenocarcinoma MCF-7 cell line. Cross talk between autophagy and apoptosis occurs at many levels because both pathways share mediators. Future studies are under way to further investigate the signal transduction pathway of C19 in MCF-7 cells and to identify cross talk targets affected by C19 in both apoptosis and autophagy in vitro.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.
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