Research article

THE *in vitro* EFFECTS OF 2-METHOXYESTRADIOL-BIS-SULPHAMATE ON CELL NUMBERS, MEMBRANE INTEGRITY AND CELL MORPHOLOGY, AND THE POSSIBLE INDUCTION OF APOPTOSIS AND AUTOPHAGY IN A NON-TUMORIGENIC BREAST EPITHELIAL CELL LINE

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Abstract: 2-methoxyestradiol (2ME2) exerts estrogen receptor-independent anti-proliferative, anti-angiogenic and anti-tumor activity *in vitro* and *in vivo*. Due to its low bioavailability and rapid metabolic degradation, several analogues have been developed in recent years. 2-methoxyestradiol-bis-sulphamate (2-MeOE2bisMATE) is a bis-sulphamoylated derivative of 2ME2 with anti-proliferative activity. The aim of this study was to investigate cell signaling events induced by 2-MeOE2bisMATE in a non-tumorigenic cell line (MCF-12A) by analysing its influence on cell number, morphology and membrane integrity, and the possible induction of apoptosis and autophagy. Dose- and time-dependent studies revealed that 48 h exposure to 2-MeOE2bisMATE (0.4 μM) resulted in a decrease in cell numbers to 79%. A slight increase in the level of lactate dehydrogenase production was observed in the 2-MeOE2bisMATE-treated cells. Morphological studies revealed an increase in the number of cells in metaphase. Hallmarks of apoptosis were also found, namely nuclear fragmentation and apoptotic bodies. In addition, increased lysosomal staining was observed via fluorescent microscopy, suggesting the induction of another type of cell death, namely autophagy. Since 2-MeOE2bisMATE is regarded as a potential anti-cancer agent, it is also

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imperative to investigate the susceptibility of non-tumorigenic cells to its influence. The data generated from this study contributes to the understanding of the action that 2-MeOE2bisMATE exerts on the non-tumorigenic MCF-12A breast epithelial cell line.

Key words: 2-methoxyestradiol-bis-sulphamate, Apoptosis, Autophagy

INTRODUCTION

The endogenous metabolite of estradiol, 2-methoxyestradiol (2ME2) exerts its anti-proliferative, anti-angiogenic and anti-tumor activity *in vitro* and *in vivo* in an estrogen receptor-independent mode [1-3]. 2ME2 demonstrates biphasic effects on cell lines including the non-small cell lung adenocarcinoma cell line HOP 62, the colorectal carcinoma epithelial cell line HCT-116, the melanoma tumorigenic cell line UACC-62, the human ovarian adenocarcinoma cell line OVCAR-3, the renal carcinoma cell line SN12-C, the estrogen receptor-positive human breast adenocarcinoma cell line MCF-7, the highly tumorigenic estrogen receptor-negative breast carcinoma cell line MDA-MB-435, and the immortalized T-lymphocyte cell line used to study acute T-cell leukemia (Jurkat cells) [2]. Lower 2ME2 concentrations (1 μM) have a proliferating effect on tumor cells due to increases in the expression of vascular endothelial growth factor (VEGF), but higher 2ME2 concentrations (5 μM or 10 μM) inhibit tumor cell proliferation [4]. 2ME2 (Panzem[®]) is currently being evaluated in Phase II clinical trials for multiple myeloma [5], ovarian cancer [6], glioblastoma multiforme [7], and breast and prostate cancer [8]. Patients treated with 2ME2 displayed minimal side effects, which included hot flushes, reversible liver enzyme elevations, fatigue and diarrhoea [9, 10]. A clinical benefit was observed with the administration of 1600 to 3200 mg Panzem[®]/kg/day in patients with breast cancer and myeloma [11].

Because 2ME2 has a low oral bioavailability and rapid metabolic degradation, attempts to manufacture analogues were made, yielding several promising analogues in recent years. 2-methoxyestradiol-bis-sulphamate (2-MeOE2bisMATE) is a bis-sulphamoylated derivative of 2ME2 possessing anti-proliferative and anti-tumor activity [10, 11].

Recent studies demonstrated that the novel 2-MeOE2bisMATE is ten-fold more powerful than 2ME2, with improved bioavailability, slower metabolic degradation and an enhanced pharmacokinetic profile due to its capability of binding to carbonic anhydrase II (CA II) in red blood cells [10, 12, 13]. CA II regulates the acid/base balance by controlling the CO_2/bicarbonate ratio. A high aerobic glycolysis rate is found in several tumorigenic cells, and this results in the production of excess lactic acid and a decreased pH in the environment of the hyperproliferative tumorigenic cells. The above-mentioned acidic environment contributes to the metastatic processes associated with malignant cancers through the breakdown of the basement membrane and breachment into the circulation [12].

The increased potency of 2-MeOE2bisMATE has been attributed to the additional sulphamate group that is added to the precursor 2ME2 molecule [14, 15]. 2-MeOE2bisMATE inhibits steroid sulphatase (STS), which regulates the conversion of oestrone sulphate to oestrone [16]. STS also plays a vital role in the formation of dehydroepiandrostene (DHEA) from DHEA-sulphate. Androstenediol (adiol), produced by the reduction of DHEA, can attach to estrogen receptors and may increase mammary tumorigenic cellular proliferation. Adiol production is therefore dependent on the sulphatase pathway. The inhibition of STS can thus be considered for cancer treatment [16]. Research performed in our laboratory involving *in silico* modeling demonstrated that a common tubulin-binding mode is shared between 2ME2 and 2-MeOE2bisMATE. This suggests that the action mechanism of 2-MeOE2bisMATE is derived from its ability to disrupt microtubule dynamics, which in turn leads to a G_2/M arrest in actively dividing cells including estrogen receptor-positive human breast adenocarcinoma cell line (MCF-7), mitoxantrone-resistant breast adenocarcinoma cell line (MCF-7 MR), drug-resistant human adenocarcinoma cell line (MCF-7 DOX40) and highly tumorigenic estrogen receptor-negative breast carcinoma cell line (MDA-MB-231) [17, 18]. Apoptosis induction by 2-MeOE2bisMATE in a human breast adenocarcinoma cell line (CAL51) was associated with the rapid activation of caspase 3 and 9 through the intrinsic pathway [19].

2-MeOE2bisMATE demonstrates therapeutic promise as a prospective STS inhibitor and anti-angiogenic and anti-proliferative drug [20]. This compound is not currently commercially obtainable and research on the action mechanism and cell signaling events of 2-MeOE2bisMATE is limited, so the effects of 2-MeOE2bisMATE on tumorigenic and non-tumorigenic cell lines remain unclear and warrant further investigation. Since 2-MeOE2bisMATE is regarded as a potential anti-cancer agent, it is of vital importance to investigate its influence on non-tumorigenic cell lines. The data generated from this study contributes to understanding the mechanism of the action exerted by 2-MeOE2bisMATE on the non-tumorigenic MCF-12A breast epithelial cell line.

MATERIALS AND METHODS

Cell line
The MCF-12A cell line is a non-tumorigenic spontaneously immortalized adherent human breast epithelial cell line that forms domes in confluent cultures. The MCF-12A cells were donated by Professor I. Parker (Department of Medical Biochemistry, University of Cape Town, South Africa).

Reagents
All of the required reagents of cell culture analytical grade were purchased from Sigma (St. Louis, USA) unless otherwise specified. Heat-inactivated fetal calf serum (FCS), and 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 Annexin V-FITC Kit and lactate dehydrogenase (LDH) cytotoxicity assay kit were purchased from BIOCOM biotech Pty Ltd. (Clubview, South Africa). The used 2-MeOE2bisMATE was synthesized by Professor R. Vleggaar from the Department of Chemistry (University of Pretoria, Pretoria, South Africa), since the compound is not commercially available.

Cell culture

Cells were grown in sterile 25-cm^2 tissue culture flasks in a humidified atmosphere at 37ºC and 5% CO_2. MCF-12A cells were cultured in medium consisting 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 fetal calf serum (56ºC, 30 min), 100 U/ml penicillin G, 100 µg/ml streptomycin and 250 µg/l fungizone. A stock solution of $2x10^{-3}$ M 2-MeOE2bisMATE dissolved in dimethyl sulphoxide (DMSO) was prepared and diluted with medium to the desired concentrations prior to the exposure of the cells. The media for all the control experiments were supplemented with an equal volume of DMSO (vehicle). The DMSO content of the final dilutions never exceeded 0.05% (v/v). Experiments were conducted in either 96-well tissue plates, 6-well plates or 25-cm^2 cell culture flasks. For the 96-well plates, exponentially growing cells were seeded at 5,000 cells per well in 200 µl maintenance medium. For the 6-well plates, exponentially growing cells were seeded at 350,000 cells per well in 3 ml maintenance medium on heat-sterilized coverslips. For the 25-cm^2 cell culture flasks, exponentially growing MCF-12A cells were seeded at $1x10^6$ cells per 25-cm^2 flask to a final volume of 5 ml of maintenance medium. A 24 h incubation period at 37ºC was allowed for cell adherence, and then the medium was discarded and the cells were exposed to 2-MeOE2bisMATE at concentrations ranging from 0.2 to 1 µM, including vehicle-treated controls, and incubated for 48 h at 37ºC. These conditions were selected as previous studies in our laboratory had demonstrated that they yielded successful anti-proliferative activity in tumorigenic cell lines. Sample controls for apoptosis and autophagy were also included. The controls for apoptosis comprised cells exposed to actinomycin D with a concentration of 0.1 µg/ml in the growth medium for 48 h. The controls for autophagy consisted of cells that had been starved in a 1:3 ratio growth medium:phosphate buffered saline (PBS) mixture.

Optical transmitted light differential interference contrast

Optical transmitted light differential interference contrast (PlasDIC) is an improved polarization-optical transmitted light differential interference contrast method, where, unlike in the conventional Smith-Nomarski's method, linearly polarized light is only generated after the objective, yielding images of outstanding quality [21]. PlasDIC has the required phase profile, which is

relative to the product of the section thickness and the refractive index difference between the environment and the average refractive index of quartz. PlasDIC gives high-quality imaging of individual cells, cell clusters and thick individual cells in plastic cell culture vessels [21]. Cells were photographed before and after the appropriate exposure.

Cell number determination: Crystal violet staining

Crystal violet is a method used to determine cell number by staining DNA. Gillies *et al*. [22] used crystal violet to quantify the cell number in monolayer cultures as a function of the absorbance of the dye taken up by the cells. A time-dependent study was conducted with time intervals of 24 and 48 h. A dose-dependent study was conducted with a concentration range of 0.2 to 1 μM of 2-MeOE2bisMATE, as previous research conducted in our laboratory and elsewhere had revealed anti-proliferative activity within this concentration range in tumorigenic breast epithelial cell lines [16]. Exponentially growing MCF-12A cells were seeded in 96-well tissue culture plates at a cell density of 5,000 cells per well. The cells were incubated at 37°C for 24 h to allow for attachment. After 24 h, the attachment medium was discarded and the cells were exposed to a concentration series of 2-MeOE2bisMATE from 0.2 to 1 μM for 24 and 48 h, including vehicle-treated controls, and incubated for the appropriate exposure time at 37°C. A baseline was also included, seeded in a separate 96-well plate, and the cells were stained before exposure to determine the starting number of cells. The cells were fixed with 100 μl of 1% gluteraldehyde (incubation for 15 min at room temperature). Subsequently, the glutaraldehyde was discarded and the cells were stained using 100 μl 0.1% crystal violet (incubated at room temperature for 30 min). The crystal violet solution was discarded and the 96-well plate was submersed under running water. The crystal violet dye was solubilized using 200 μl 0.2% Triton X-100 and incubated at room temperature for 30 min. 100 μl of the solution was transferred to a new microtitre plate. Afterwards, the absorbance was read at 570 nm using an EL_x800 Universal Microplate Reader (Bio-Tek Instruments Inc., USA).

This study focused on the effects of 2-MeOE2bisMATE on a non-tumorigenic cell line. However, it was important to select a dose that would cause anti-proliferative activity in tumorigenic cell lines with minimal anti-proliferative effects on non-tumorigenic cell lines. Therefore, a concentration was selected with a high level of activity in tumorigenic cell lines to be compared to its activity in a non-tumorigenic cell line. As described by the National Cancer Institute, the 50% growth inhibitory concentration (IC_{50}) was calculated for an adenocarcinoma cell line (MCF-7) in another study conducted in our laboratory in order to determine the growth inhibition induced by 2-MeOE2bisMATE (data not shown). The IC_{50} that was found in that study was then incorporated in subsequent studies as described below to investigate to differential effects of 2-MeOE2bisMATE on the MCF-12A cell line.

Membrane integrity: LDH assay
Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme that catalyzes the interconversion of lactate and pyruvate. Cells release LDH during injury or cell damage, following the loss of membrane integrity resulting from either apoptosis or necrosis [23]. LDH activity can thus be used as an indicator of cell membrane integrity, and serves as a general means to assess cytotoxicity resulting from exposure to chemical compounds. Cells were seeded in 96-well plates at a cell density of 5,000 cells per well with an overnight attachment policy (incubated at 37ºC at 5% CO_2). After 24 h, the cells were exposed to 2-MeOE2bisMATE or the appropriate control media. Subsequently, 200 μl of medium was transferred and centrifuged at 5,000 rpm for 10 min. Afterwards, 10 μl was transferred to a clear 96-well plate. Subsequently, 100 μl of the LDH reaction mix (mixed according to the kit pamphlet instructions) was added to the sample. After 90 min incubation at room temperature, the absorbance was read at 460 nm, with a reference wavelength of 630 nm using an EL_x800 Universal Microplate Reader (Bio-Tek Instruments Inc., USA).

Morphology: Light microscopy (Haematoxylin and eosin staining)
The haematoxylin and eosin staining method was used to determine the influence of 2-MeOE2bisMATE on the cytoplasm and nucleus. Cells were seeded at a volume of 500,000 per well on sterile coverslips in 6-well plates and incubated overnight. The cells were exposed to 0.4 μM 2-MeOE2bisMATE for 48 h or the appropriate control media. The coverslips were transferred to staining dishes, and the cells were fixed with Bouin's fixative for 30 min. Subsequently, Bouin's fixative was discarded and 70% ethanol was added to the coverslips for 20 min at room temperature before they were rinsed with tap water. Mayer's haematoxylin was added to the coverslips for 20 min. The coverslips were rinsed with tap water for 2 min. Afterwards, 70% ethanol was added to the coverslips followed by 1% eosin for 5 min. The eosin was discarded and the coverslips were consecutively rinsed twice for 5 min with 70, 96 or 100% xylol. The coverslips were mounted on microscope slides with resin and left to dry. Photos were taken with a Zeiss Axiovert MRc microscope (Zeiss, Oberkochen, Germany). Mitotic indices were also determined from the haemotoxylin- and eosin-stained cells. Quantitative data for the mitotic indices was obtained by counting 1,000 cells on each slide of the biological replicates and expressing the data as the percentage of cells in each phase of mitosis, cells in interphase and cells presenting abnormal morphology. A distinction was made between the cells in the mitotic phases (prophase, metaphase, anaphase and telophase), the cells in interphase and the cells demonstrating abnormal morphology, which included cells displaying hypercondensed chromatin, membrane blebbing, apoptotic bodies and abnormal chromosome segregation. This H & E staining yielded both qualitative and quantitative information.

Morphology: Transmission electron microscopy

Transmission electron microscopy (TEM) was employed to visualize the morphology of the cells after exposure to 2-MeOE2bisMATE and the morphology of the vehicle-treated cells. TEM was used to view autophagic lysosomes and to identify apoptotic bodies formed during the final stages of apoptosis. Cells were seeded in a 25-cm^2 flask at a density of 500,000 cells per flask with an overnight attachment policy. Subsequently, the medium was discarded and the cells were exposed to 0.4 μM 2-MeOE2bisMATE for 48 h. Appropriate controls were included. After 48 h, cells were trypsinised and resuspended in 1 ml medium. The cells were fixed with 2.5% glutaraldehyde in 0.075 M phosphate buffer for 1 h. The cells were rinsed with 0.075 M phosphate buffer (three times), fixed with osmium tetroxide for 30 min, rinsed three times with distilled water, and dehydrated with increasing ethanol concentrations (30, 50, 70, 90 and 100%). The cells were infiltrated with 50% quetol in ethanol for 1 h and then with 100% quetol for 4 to 6 h. Ultra-thin sections were prepared using a microtome, contrasted using 4% uranyl acetate for 10 min, then rinsed with water. The samples were viewed using TEM (Electron Microscopy Unit, University of Pretoria, South Africa).

Morphology: Fluorescent microscopy

A triple staining method was employed to investigate the effects on the morphology of MCF-12A cells exposed to 2-MeOE2bisMATE. Hoechst 33342 stains the DNA, and propidium iodide was used as a probe to detect if the membrane was compromised. Acridine orange is a lysosomotropic fluorescent compound that served as a tracer for acidic vesicular organelles including autophagic vacuoles and lysosomes [24]. Cells (500,000) per well were seeded in 6-well plates and incubated overnight. Afterwards, the cells were exposed to 0.4 μM 2-MeOE2bisMATE or to the appropriate control media. 0.5 ml of Hoechst 33342 solution (3.5 μg/ml in PBS) was added to the medium to give a final concentration of 0.9 μM, and was then incubated for 25 min at 37ºC. Subsequently, 0.5 ml of acridine orange solution (4 μg/ml in PBS) was added to the medium to give a final concentration of 1 μg/ml, and incubated for 5 min at 37ºC. Then 0.5 ml of propidium iodide solution (40 μg/ml in PBS) was added to the medium to provide a final concentration of 12 μM. The cells were washed three times with PBS. Photos were taken with the appropriate filters in a dark room to prevent quenching. A Zeiss Axiovert CFL40 microscope and Zeiss Axiovert MRm monochrome camera (Zeiss, Oberkochen, Germany) were used, employing a Zeiss Filter 2 for the Hoechst 33342-stained (blue) cells and a Zeiss Filter 9 for the acridine orange-stained (green) cells, and a Zeiss filter 15 for the propidium iodide-stained (red) cells.

Apoptosis detection: Annexin V-FITC

The occurrence of apoptosis as a possible induction of cell death was evaluated and quantified using flow cytometry in combination with Annexin V-FITC. In apoptosis, the calcium-dependent phospholipid scramblase activity is

activated, resulting in the externalization of the phosphatidylserine layer of the cell membrane. The externalization of the phosphatidylserine layer during apoptosis provides binding sites for Annexin V (a Ca^{2+}-dependent, phospholipid binding protein), which is conjugated to a fluorochrome (fluorescein isothiocyanate). This allowed for the identification of different stages of apoptosis (early and late) and necrosis. After 48 h of exposure to 0.4 µM 2-MeOE2bisMATE, the cells were trypsinized, and 10^6 cells were resuspended in 1 ml of 1x Binding Buffer and centrifuged at 300 x g for 10 min. The supernatant was removed and the cells resupended in 100 µl of 1x Binding Buffer. 10 µl of Annexin V-FITC was added and the whole was incubated for 15 min in the dark at room temperature. After 15 min, the cells were washed by adding 1 ml of 1x Binding Buffer and centrifuged at 300 x g for 10 min. The supernatant was carefully pipetted off and the cells were resuspended in 500 µl of 1x Binding Buffer solution. Immediately prior to analysis, 12.5 µl of propidium iodide (40 µg/ml) was added and gently mixed. The propidium iodide fluorescence (oncotic cells) and Annexin V fluorescence (apoptotic cells) were measured with a FACS FC500 System flow cytometer (Beckman Coulter South Africa Pty Ltd) equipped with an air-cooled argon laser excited at 488 nm. Data from at least 30,000 cells was analyzed with cyflogic version 1.2.1 software (Pertu Therho, Turku, Finland).

Statistics
Qualitative data was obtained from PlasDIC, TEM, light microscopy and fluorescent microscopy. Quantitative data was supplied by means of cell number determination (crystal violet staining) and mitotic indices. The data obtained from three independent experiments (each conducted in six replicates) is shown as the mean ± SD and the data for mitotic indices was obtained by counting 1,000 cells (repeated three times) on each slide of the biological replicates. The data was statistically analysed for significance using the analysis of variance-single factor model, followed by a two-tailed Student's *t*-test. Means are presented in bar charts, with T-bars referring to standard deviations. *P*-values < 0.05 were regarded as statistically significant and are indicated by an asterisk (*).

RESULTS

Optical transmitted light differential interference contrast
The effects of 2-MeOE2bisMATE on the morphology of MCF-12A cells were investigated by means of PlasDIC. The slight rounding MCF-12A 2-MeOE2bisMATE-treated cells indicated an increase in the number of cells in metaphase when compared to the vehicle-treated controls and the cells propagated in growth medium (Fig. 1A, B and C). However, the effects were not prominent and the cell density was not compromised when compared to the vehicle-treated controls and the cells propagated in growth medium.
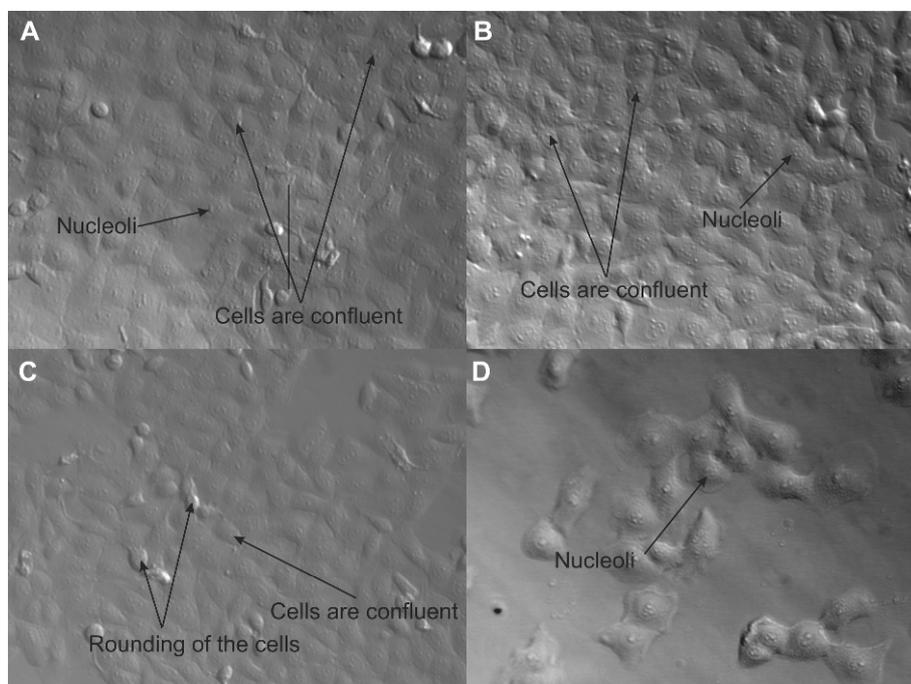
Fig. 1. PlasDIC micrographs of MCF-12A cells propagated in growth medium (A) and of vehicle-treated cells (B) after 48 h of exposure. They show confluent cells displaying no signs of distress where nucleoli were clearly visible. PlasDIC of MCF-12A 2-MeOE2bisMATE–treated cells are shown in (C) and (D). C reveals a slight increase of the number of cells in metaphase with unaffected cell density when compared to the vehicle-treated control and cells propagated in the growth medium (magnification: 100x).

Cell number determination: Crystal violet

Dose- and time-dependent studies were performed using a DNA stain known as crystal violet. Spectrophotometrical studies indicated that 2-MeOE2bisMATE decreased cell numbers to 92% after 24 h of exposure (Fig. 2A). Furthermore, the 48 h exposure of 2-MeOE2bisMATE (0.4 μM) resulted in a decrease in cell growth to 79% (Fig. 2B). Neither the 0.02% nor the 0.05% vehicle-treated cells revealed significant influences on the cell numbers.

The previous data obtained in our laboratory revealed that the IC_{50} of 2-MeOE2bisMATE for the tumorigenic MCF-7 cell line was at 48 h of exposure and a concentration a 0.4 μM (data not shown). All of the subsequent experiments on the MCF-12A cell line were performed with a 48-h exposure period and a 0.4 μM 2-MeOE2bisMATE concentration. The vehicle control did not reveal significant effects on cell numbers.
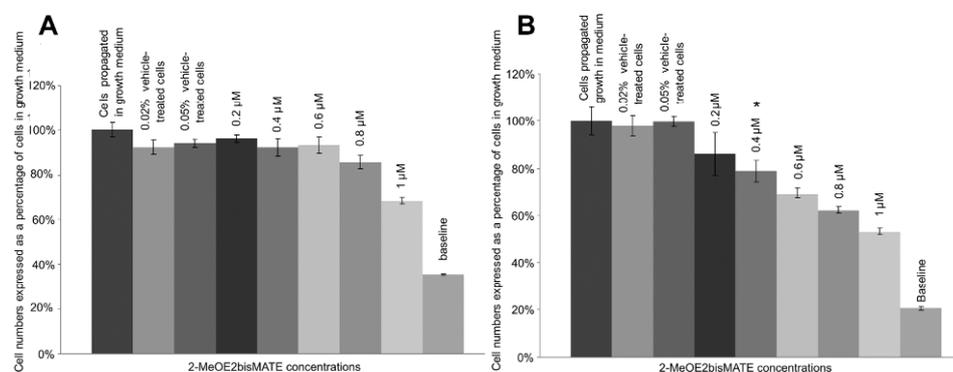
Fig. 2. Cell number determination. Crystal violet staining during the 24 h exposure of the MCF-12A cell line to 2-MeOE2bisMATE resulted in a slight inhibition of cell growth (A). 0.4 μM 2-MeOE2bisMATE treatment decreased cell numbers to 92% in the MCF-12A cell line. However, the decrease in cell numbers was not statistically significant. The 48 h exposure of the 0.4 μM 2-MeOE2bisMATE-treated cells had an inhibitory growth effect of 21% when compared to cells in growth medium (B). * indicates a statistically significant *(P*-value < 0.05) difference for growth inhibition between the 2-MeOE2bisMATE-treated cells and the cells in growth medium.

Membrane integrity: Lactate dehydrogenase assay

LDH leakage was measured in the medium after exposure by conducting spectrophotometry at a wavelength of 450 nm (reference wavelength of 630 nm). A slight increase in the LDH (Fig. 3) leakage was found (not statistically significant) in the 2-MeOE2bisMATE-treated cells after exposure when compared to the vehicle-treated cells. However, these results were found not to be statistically significant. The background control consists of growth medium only. The low control refers to cells resuspended in growth medium and the high control to cells resuspended in growth medium with cell lysis solution added to the cells shortly before the experiment was terminated (according to the manufacturer's instructions).
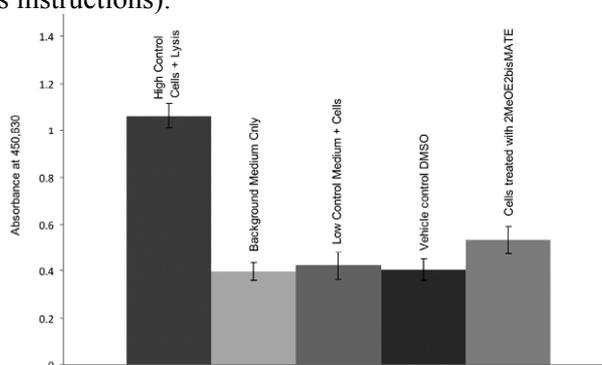

Fig. 3. LDH production measured by means of spectrophotometry revealed a slight increase in 2-MeOE2bisMATE-treated cells after 48 h of exposure when compared to the vehicle-treated control cells. The increase found in the exposed cells was not statistically significant (P > 0.05).

Morphology: Light microscopy
Haematoxylin and eosin staining revealed no indications of apoptosis such as hypercondensed chromatin or apoptotic bodies in the 2-MeOE2bisMATE-treated MCF-12A cells when compared to the vehicle-treated cells (Fig. 4A and B). In addition, there was no difference in cell density between the 2 -MeOE2bisMATE-treated MCF-12A cells and the vehicle-treated cells.
Mitotic indices were obtained by counting 1,000 cells on each H & E stained slide and by expressing it as the percentage of cells in each phase of mitosis and interphase (Fig. 5). An increase of 5% was observed in cells occupying metaphase when compared to the vehicle control cells. Cells presenting characteristics of apoptosis and cell death had a slight increase of 0.4%.
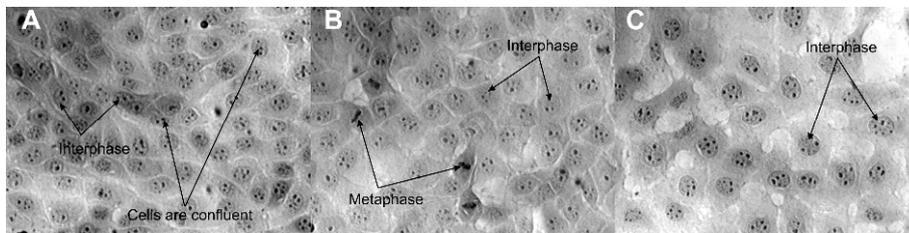

Fig. 4. Light microscopy revealed that confluent vehicle-treated MCF-12A cells (A) showed no signs of cellular distress. In addition, 48 h of exposure to 0.4 μM 2-MeOE2bisMATE revealed no hypercondensed chromatin (B and C). Several cells were observed in interphase, and cells in metaphase were also present (magnification: 100x).
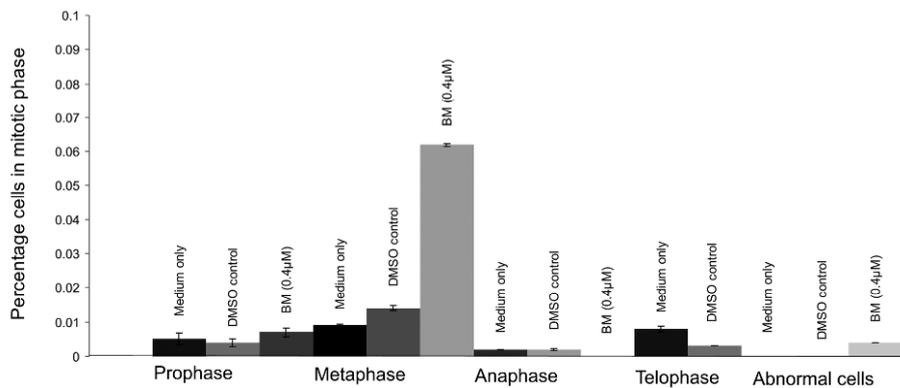

Fig. 5. Mitotic indices of MCF-12A cells propagated in growth medium, DMSO vehicle-treated cells and 2-MeOE2bisMATE-treated cells, the last indicated in the figure as BM (0.4 μM). The 2-MeOE2bisMATE-treated cells revealed an increase in the number metaphase cells when compared to cells propagated in growth medium and vehicle-treated cells. Mitotic indices were determined by counting 1,000 cells on each slide (3 repeats) of the biological replicates and calculating which percentage of the cells were in mitosis and interphase. A distinction was made between mitotic cells (included prophase, metaphase, anaphase and telophase), cells in interphase and abnormal cells which included those displaying hypercondensed chromatin, membrane blebbing, apoptotic bodies and abnormal chromosome segregation.

Morphology: Transmission electron microscopy

TEM provides an illustration of the interior of the cell at a much larger magnification than light microscopy. Thus, effects on the cell morphology not visible in light microscopy photos were revealed by TEM. Although not prominent, nuclear fragmentation and apoptotic bodies were observed in the 2-MeOE2bisMATE-treated cells when compared to the vehicle-treated control (Fig. 6A and B).
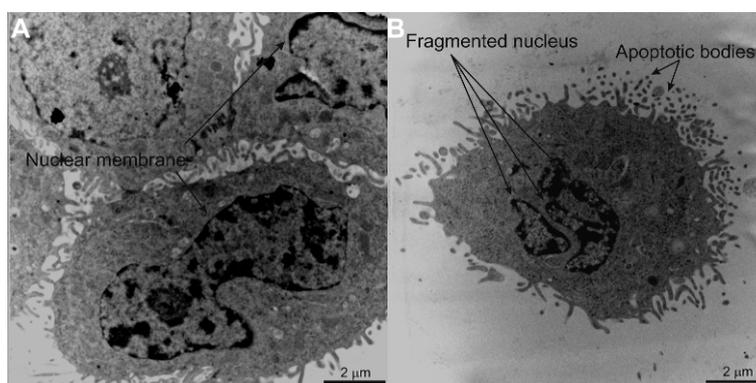

Fig. 6. Transmission electron microscopy (TEM; images 6000x magnification) revealed that the vehicle-treated cells showed no signs of distress (A), while the 0.4 μM 2-MeOE2bisMATE-treated cells (48 h exposure) displayed nuclear fragmentation, extensions of the cell membrane and apoptotic bodies (B). TEM allows for the illustration of the cell at a higher magnification. Morphological effects and changes not detectable using light microscopy were observed by means of TEM.

Morphology: Fluorescent microscopy

Hoechst 33342, acridine orange and propidium iodide staining were used to visualize possible apoptotic and autophagic characteristics. Hoechst stains the DNA of viable and non-viable cells, while acridine orange acts as a lysotropic tracer and indicates autophagy. Propidium iodide can only penetrate cells where the cell membrane has been compromised. Slightly increased lysosomal staining was observed in the 2-MeOE2bisMATE-exposed cells when compared to the vehicle-treated cells (Fig. 7A and B). No propidium iodide staining was found in either the treated cells or the control cells, revealing the absence of necrosis. Necrosis is an energy-independent process that takes place after the cell is dead, and is characterized by the swelling of the cell (oncosis) and uncontrollable release of the cellular contents into the surrounding area due to the damaged cell membrane, which causes damage to the neighbouring cells [25]. The absence of propidium iodide staining indicated that the cell membranes were intact, and necrotic and oncotic processes were absent.
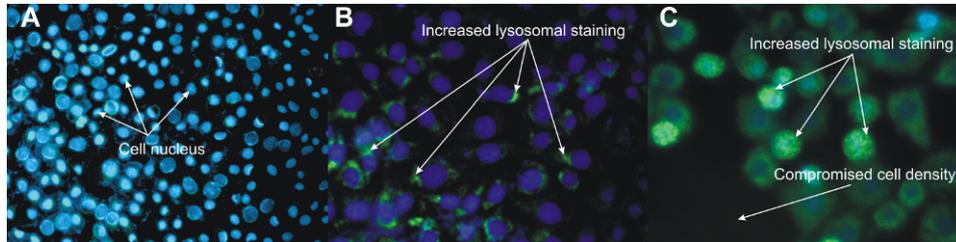
Fig. 7. Fluorescent microscopy. A – Vehicle-treated control cells were confluent. Hoechst 33342 stains the DNA displaying the nucleus (blue). Minimal lysosomal staining (green) was observed indicating the presence of negligible autophagic activity (100x magnification). B – A representative sample of MCF-12A cells treated with 2-MeOE2bisMATE for 48 h. Lysosomal staining was observed and cell density was affected. C – Another representative image of cells treated with 2-MeOE2bisMATE for 48 h revealed a decrease in cell density when compared to the vehicle-treated cells. Increased lysosomal staining revealed the presence of increased acidic vesicles suggesting the presence of autophagy. However, no propidium iodide staining was observed suggesting the absence of necrosis (100x magnification). The cell density of the 2-MeOE2bisMATE-treated cells was thus affected and also confirmed by TEM.

Apoptosis detection: Annexin V-FITC

The presence of apoptosis was investigated by means of flow cytometry employing Annexin V-FITC. After the exposure of the cells to 0.4 μM 2-MeOE2bisMATE for 48 h, 76% of the cells were viable when compared to the 98% of the vehicle-treated control (Fig. 8A and B). In addition, 10.9% of the 0.4 μM 2-MeOE2bisMATE-treated cells were found in early apoptosis, 9% in late apoptosis and 4% in necrosis.
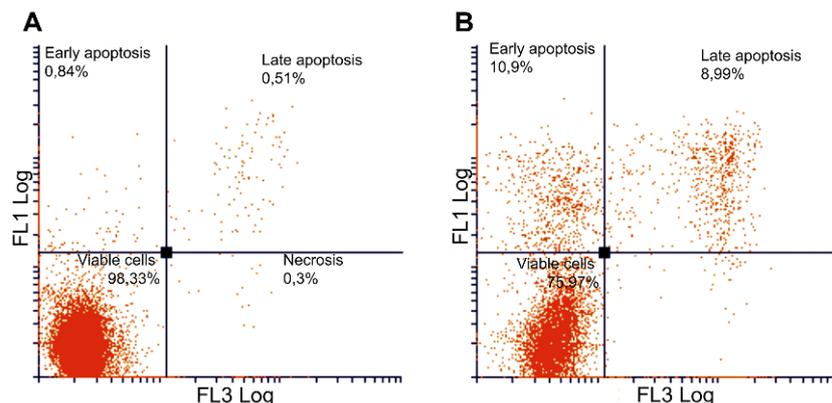

Fig. 8. An investigation of apoptosis by means of flow cytometry revealed that vehicle-treated cells (A) had a cell viability of 98% when compared to 2-MeOE2bisMATE-treated MCF-12A cells (B), which showed a decreased cell viability of 76%. Of the cells treated with 2-MeOE2bisMATE, 11% were in early apoptosis, 9% in late apoptosis and 4% in necrosis.

DISCUSSION

Previous data collected in our own laboratory and reported by other researchers illustrated that 2-MeOE2bisMATE possesses anti-proliferative activity in tumorigenic cell lines [1-3, 8, 9, 11-20]. However, no research has been published to date reporting the *in vitro* effects of 2-MeOE2bisMATE on non-tumorigenic cells. Tumorigenic cell lines are more susceptible to the influence of 2-MeOE2bisMATE when compared to non-tumorigenic cells (data not shown). Furthermore, it is imperative to investigate the effects of 2-MeOE2bisMATE on non-tumorigenic cell lines if 2-MeOE2bisMATE is going to be considered for treatment. In this study, we demonstrated the dose-dependent (0.2 to 1.0 µM) effects of 2-MeOE2bisMATE on MCF-12A cells after 48 h. During another study performed in our laboratory, it was found that the IC_{50} of 2-MeOE2bisMATE was 0.4 µM at 48 h in the MCF-7 cell line (data not shown). Thus, all subsequent studies were conducted using 0.4 µM 2-MeOE2bisMATE at an exposure period of 48 h to determine the effect that 2-MeOE2bisMATE has on LDH production (injury or stress results in a comprised cell membrane and leakage of LDH into the growth medium) and the possible induction of cell death (apoptosis and autophagy).

This study revealed that 2-MeOE2bisMATE did not increase LDH production in a statistically significant manner in 2-MeOE2bisMATE-treated cells when compared to the vehicle-treated cells. This suggests that the cell membrane was not compromised severely enough to allow for acute LDH leakage. Previous reports indicated that the precursor molecule, 2ME2, increased LDH production in a cell line derived from the bone marrow of an individual with myelodysplastic syndrome (the MDS-RAEB MUTZ-1 cell line) and in the human promyelocytic leukaemia cell line (HL-60) [26, 27]. Nonetheless, the relationship between lactate dehydrogenase and apoptosis remains elusive, and literature on 2-methoxyestradiol and 2-MeOE2bisMATE influence on LDH is rare.

Membrane blebbing, membrane budding, minor vacuoles and nuclear fragmentation were revealed by conducting qualitative morphological studies on the 2-MeOE2bisMATE-treated cells when compared to the vehicle-treated cells. Previous studies demonstrated that 2-MeOE2bisMATE induced apoptosis in MCF-7 cells and human umbilical vein endothelial cells (HUVEC) [19, 20]. In this study, fluorescence microscopy revealed minor lysosomal staining. No decrease in cell density was observed in PlasDIC and light microscopy studies. However, fluorescent staining and TEM indicated compromised density in 2-MeOE2bisMATE-treated cells, and mitotic indices demonstrated the presence of a mitotic block. However, these results revealed that the influence of 2-MeOE2bisMATE is not as pronounced when compared to the effects on tumorigenic cell lines [1-3, 8, 9, 11-17, 19, 20]. The latter indicates that 2-MeOE2bisMATE possesses the specificity to effect tumorigenic cell lines more severely than non-tumorigenic cell lines.

STS inhibition can be considered for a possible treatment of cancer. STS is one of twelve sulphatases characterized in human cells. It regulates the formation of oestrone from oestrone sulphate [12]. STS mRNA levels are detected across the non-tumorigenic breast cancer MCF-10A cell line. However, to date, no studies referring to the expression levels of STS in MCF-12A cells have been reported. STS mRNA expression is higher in malignant than in normal breast tissue, and is found in 74% of all breast cancer biopsies [28]. A recently published study revealed that mRNA STS, oestradiol sulfotransferase (EST) and 17-β-hydroxysteriod dehydrogenase II are elevated in breast carcinomas [29]. High levels of STS mRNA expression in breast tumors are associated with a poor prognosis [30]. This suggests regulation by a common metabolite, possibly oestradiol. Furthermore, oestradiol has recently been associated with apoptosis in tumorigenic breast cancer cells [29].

The aims of this study were to determine the effects of 2-MeOE2bisMATE on cell growth, membrane integrity, morphology and possible induction of cell death in the non-tumorigenic MCF-12A cell line. We demonstrated the dose-dependent effects of 2-MeOE2bisMATE on MCF-12A cell growth over 48 h. Minor apoptotic and autophagic characteristics were observed. There is convincing evidence that 2-MeOE2bisMATE exerts differential effects on tumorigenic and non-tumorigenic cell lines. Since 2-MeOE2bisMATE has potential as an anti-cancer agent, it is vital to investigate the susceptibly of non-tumorigenic lines also. However, research on this novel compound and especially its effects on non-tumorigenic cell lines is limited. For this reason, the signaling events and effects exerted by 2-MeOE2bisMATE warrant further investigation.

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