

## **2-Methoxyestradiol-bis-sulphamate induces apoptosis and autophagy in a tumorigenic breast epithelial cell line**

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### **Abstract**

In anticancer research where the focus is on finding agents that induces cell death while leaving non-tumorigenic cells less affected, a novel 2-methoxyestradiol derivative has come forth. 2-Methoxyestradiol-bis-sulphamate (2-MeOE2bisMATE) is a 2-methoxyestradiol derivative produced by bis-sulphamoylation which possesses increased antiproliferative activity and biological availability. Several questions remain regarding the the type of cell death mechanisms and possible induction of autophagy by 2-MeOE2bisMATE. The aim of this *in vitro* study was to investigate the cell death mechanisms exerted by 2-MeOE2bisMATE in an adenocarcinoma cell line (MCF-7) by analyzing its influence on cell growth, morphology and possible induction of cell death. Spectrophotometry (crystal violet staining), transmission electron microscopy (TEM), light microscopy (haematoxylin and eosin staining) and fluorescent microscopy (Hoechst 33342, propidium iodide and acridine orange) were employed. Spectrophotometrical studies indicated that 2-MeOE2bisMATE decreased cell numbers to 75% in MCF-7 cells after 24 h and to 47% after 48 h of exposure. TEM demonstrated membrane blebbing, nuclear fragmentation and chromatin condensation indicating hallmarks of apoptosis. Light microscopy revealed the presence of several cells blocked in metaphase and apoptotic cells were also observed. Fluorescent microscopy demonstrated increased lysosomal staining; suggesting the induction of autophagy. 2-MeOE2bisMATE shows therapeutic potential as an anticancer agent and the investigation of the cell death mechanisms used by 2-MeOE2bisMATE thus warrants further investigation.

Keywords: 2-methoxyestradiol-bis-sulphamate, apoptosis, autophagy, MCF-7

## **Introduction**

2-Methoxyestradiol (2ME2) is an estradiol metabolite, characterized in recent years to possess *in vitro* (dose- and cell line-dependent) and *in vivo* antiproliferative, antiangiogenic and antitumor activity [1, 2, 3, 4, 5]. Antiproliferation activity is mainly contributed to apoptosis induction as it appears that 2ME2 pursues active proliferating cells and quiescent cells are therefore less affected [2]. 2ME2 has a low affinity for estrogen receptors and the antiproliferating effect is independent of the presence of estrogen receptors [2, 5].

Presently, phase II clinical trials for 2ME2 (Panzem<sup>®</sup>) are being conducted for multiple myeloma [6], ovarian cancer [7], glioblastoma multiforme [8], breast- and prostate cancer [9]. Due to the limited biological availability and rapid metabolic breakdown of 2ME2 several promising analogues of 2ME2 have been developed in recent years. 2-Methoxyestradiol-bis-sulphamate (2-MeOE2bisMATE) is a 2ME2 derivative with antiproliferative activity produced by means of bis-sulphamoylation [10, 11].

Additional studies performed in our laboratory imply that 2ME2 and 2-MeOE2bisMATE share a common tubulin binding mode. Microtubule disruption by 2-MeOE2bisMATE resulted in a G<sub>2</sub>/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 MDA-MB-231 cell line [11, 12, 13, 14].

As anticancer research focuses on agents that induces cell death in cancer cells leaving non-tumorigenic cells unaffected, 2-MeOE2bisMATE has beneficial potential as an antiproliferative- and anticancer agent [15]. Due to the novelty of 2-MeOE2bisMATE, this compound is not currently commercially available. Furthermore, the *in vitro* signaling mechanism of 2-MeOE2bisMATE remains elusive. The aim of this study was to investigate the types of cell death events exerted by 2-MeOE2bisMATE in an adenocarcinoma cell line (MCF-7) by exploring its influence on cell growth, morphology and possible induction of apoptosis and autophagy.

## **Materials and Methods**

### **Cell line**

MCF-7 is a estrogen positive tumorigenic adherent breast epithelial cell line derived from metastatic sites in adenocarcinoma and is formed by pleural effusion of human breast adenocarcinoma. MCF-7 cells were acquired from Highveld Biological (Pty) Ltd. (Sandringham, South Africa).

### **Reagents**

All required reagents of cell culture analytical grade 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 Annexin V fluorescein isothiocyanate (FITC) kit and a rabbit polyclonal anti-LC3B conjugated to DyLight 488 were purchased from BIOCOT biotech (Pty) Ltd. (Clubview, South Africa). Since 2-MeOE2bisMATE is not commercially available, it was synthesized by Professor Vlegaar from the Department of Chemistry (University of Pretoria, Pretoria, South Africa).

### **Cell culture**

For maintenance cells were grown in 25cm<sup>2</sup> tissue culture flasks in a humidified atmosphere at 37°C and 5% CO<sub>2</sub>, and routinely cultivated 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 fungizone (250 µg/l).

A stock solution of 2x10<sup>-3</sup> M 2-MeOE2bisMATE dissolved in dimethyl sulphoxide (DMSO) was prepared and diluted with medium to the desired concentrations prior to exposure of the cells. The medium of control cells was 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<sup>2</sup> cell culture flasks. For 96 well plates, exponentially growing cells were seeded at 5 000 cells per well in 200 µl maintenance medium. For 6 well plates, exponentially growing cells were seeded at 350 000 cells per well in 3 ml maintenance medium on heat-sterilized coverslips. For 25 cm<sup>2</sup> cell culture flasks, exponentially growing MCF-7 cells were seeded at 1x10<sup>6</sup> cells per 25 cm<sup>2</sup> flask to a final volume of 5 ml of maintenance medium. After a 24 h incubation period at 37°C to allow for cell adherence, medium was discarded and cells were exposed to 2-MeOE2bisMATE with concentrations ranging from 0.2-1 µM. The latter series was selected since previous studies conducted at our laboratory demonstrated successful antiproliferative activity within this concentration range in the MCF-7 cell line. All experiments included a control consisting of cells grown in growth medium and vehicle-treated cells. Positive controls for apoptosis comprised of cells exposed to actinomycin D with a concentration of 0.1 µg/ml in growth medium for 48 h. A positive controls for autophagy comprised of cells with induced starvation (1:3 ratio relative to growth medium: phosphate buffered saline (PBS)).

### **Three dimensional view: Optical transmitted light differential interference contrast**

Optical transmitted light differential interference contrast (PlasDIC) is an improved method where interference and polarization has been expertly combined so that the complete cell is located outside the polarization-sensitive area [16]. PlasDIC provides high-quality imaging of individual cells, cell clusters and thick individual cells in plastic cell culture vessels and allows for identification of growth interaction, apoptosis and necrosis [16]. Images were attained before and after the exposure by means of the Axiovert 40 CFL microscope (Carl Zeiss, Goettingen, Germany).

### **Cell number determination: Crystal violet**

Crystal violet is a technique used to determine cell number by staining the deoxyribonucleic acid (DNA). Gillies et al. (1986) used crystal violet to quantify cell number in monolayer cultures as a function of the absorbance of the dye taken up by cells [17]. A time-dependent study was conducted with time intervals of 24 h, 48 h and 72 h. A dose-dependent study was conducted with a concentration range of 0.2  $\mu$ M-1  $\mu$ M. Exponentially growing MCF-7 cells were seeded in 96 well tissue culture plates at a cell density of 5000 cells per well. Cells were incubated at 37°C for 24 h to allow for attachment. Subsequently, the medium was discarded and the cells were exposed to the 0.2-1  $\mu$ M 2-MeOE2bisMATE concentration range including vehicle-treated controls and incubated for the appropriate exposure time at 37°C. A baseline determination was also conducted before exposure to establish the starting number of cells. Cells were fixed with 100  $\mu$ l 1% gluteraldehyde (incubation for 15 min at room temperature). Subsequently the gluteraldehyde was discarded and cells were stained using 100  $\mu$ l 0.1% crystal violet (incubated at room temperature for 30 min). Crystal violet was discarded and the 96 well plate was submersed under running water. Cells were solubilized using 200  $\mu$ l 0.2% Triton X-100 and incubated at room temperature for 30 min. 100  $\mu$ l of the solution was transferred to a new microtitre plate. Afterwards, the absorbance was determined at 570 nm using an EL<sub>x</sub>800 Universal Microplate Reader available from Bio-Tek Instruments Inc. (Vermont, United States of America). The 50% maximal growth inhibition concentration (IC<sub>50</sub>) was determined and used in subsequent studies as described below [18].

### **Cytotoxicity: Lactate dehydrogenase 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 consequential from either apoptosis or necrosis. LDH activity can therefore be used as an indicator of cell

membrane integrity and serves as a general means to assess for cytotoxicity resulting from exposure to chemical compounds. Cells were seeded in 96 well plates at a cell density of 5000 cells per well with an overnight attachment policy (incubated at 37°C at 5% CO<sub>2</sub>). After 24 h, cells were exposed to 2-MeOE2bisMATE including appropriate controls. After 48 h of exposure (IC<sub>50</sub> of 0.4 µM was determined by means of crystal violet staining), the 200 µl medium was transferred and centrifuged at 300 x g for 10 min. Afterwards, 10 µl was transferred to an optimally clear 96 well plate. Subsequently, 100 µl of the LDH reaction mix (mixed according to the supplier's manual instructions) was added to the medium. After 90 min incubation at room temperature, the absorbance was read at 460 nm (reference wavelength of 630 nm) with an EL<sub>x</sub>800 Universal Microplate Reader from Bio-Tek Instruments Inc. (Vermont, United States of America).

### **Morphology: Transmission electron microscopy**

Transmission Electron microscopy (TEM) was employed to visualize morphology of MCF-7 cells after exposure to 2-MeOE2bisMATE. TEM was used to view autophagic lysosomes and to identify apoptotic bodies formed during final stages of apoptosis. Cells were seeded in a 25 cm<sup>2</sup> flasks at a density of 1.25 x 10<sup>6</sup> cells per flask with an overnight attachment policy. Subsequently the medium was discarded and cells were exposed to 0.4µM 2-MeOE2bisMATE for 48 h. Cells were trypsinised and resuspended in 1 ml medium. Cells were fixed with 2.5% glutaraldehyde in 0.075 M phosphate buffer for 1 h. Cells were rinsed thrice with 0.075 M phosphate buffer, fixed with osmium tetroxide for 30 min. Cells were rinsed (3x) with distilled water and dehydrated by using increased ethanol concentrations (30%, 50%, 70%, 90%, and 100%). Cells were infiltrated with 50% quetol in ethanol for 1 h and then with 100% quetol (Merck & Co Inc., Munich, Germany) for four to six h. Ultra-thin sections were prepared using a microtome and contrasted using 4% uranyl acetate for 10 min and rinsed with water. Samples were viewed at the Electron Microscopy Unit (University of Pretoria, Pretoria, South Africa).

### **Morphology: Light microscopy (Haematoxylin and eosin staining)**

The haematoxylin and eosin (H & E) staining method was used to determine the influence of 2-MeOE2bisMATE on cell morphology. H & E staining allows for identification of different mitotic phases, interphase, as well as apoptotic and abnormal cells. Cells (500 000) per well were seeded on sterile coverslips in 6 well plates and incubated overnight. Afterwards cells were exposed to 0.4 µM 2-MeOE2bisMATE for 48 h. Coverslips were transferred to staining dishes and 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. Coverslips were rinsed with tap water for 2 min. Afterwards, 70% ethanol was added to the coverslip; followed by 1% eosin for 5 min. Eosin was discarded and coverslips were consecutively rinsed twice for 5 min with 70%, 96%, 100% and xylol. Coverslips were mounted on microscope slides with resin and left to dry. Photos were taken with a Zeiss Axiovert MRc microscope (Zeiss, Oberkochen, Germany).

### **Morphology: Fluorescent microscopy**

A triple staining method was used to investigate the *in vitro* influence of 2-MeOE2bisMATE on morphology of MCF-7 cells. Hoechst 33342 was used to stain DNA and propidium iodide (PI) was used to detect whether the membrane was compromised. Acridine orange is a lysosomotropic fluorescent compound that serves as a tracer for acidic vesicular organelles including autophagic vacuoles and the acidic interior of the lysosome. As autophagy occurrence increases it will stimulate lysosomal proton pumping. Acridine orange is able to cross as uncharged molecules across lysosomal membrane however, the acridine orange becomes trapped within the acidic compartment. The increased lysosomal acidity is accompanied with increased occurrence of autophagy that can be correlated to the fluorescence intensity [19, 20]. Cells (500 000) per well were seeded in 6 well plates and incubated overnight. Afterwards cells were exposed to 0.4 $\mu$ M 2-MeOE2bisMATE for 48 h. 0.5 ml of Hoechst 33342 solution (3.5  $\mu$ g/ml in PBS) was added to the medium to provide a final concentration of 0.9  $\mu$ M and was incubated for 25 min at 37°C. Subsequently, 0.5 ml of acridine orange solution (4  $\mu$ g/ml in PBS) was added to the medium to give a final concentration of 1  $\mu$ g/ml and incubated for 5 min at 37°C and 0.5 ml of propidium iodide solution (40  $\mu$ g/ml in PBS) was added to the medium to provide a final concentration of 12  $\mu$ M. Cells were washed three times with phosphate buffered saline (PBS). Photos were taken with appropriate filters in a dark room to prevent quenching. A Zeiss Axiovert CFL40 microscope and Zeiss Axiovert MRm monochrome camera were used employing a Zeiss Filter 2 for Hoechst 33342-stained (blue) cells, a Zeiss Filter 9 for acridine orange-stained (green) cells and a Zeiss filter 15 for propidium iodide-stained (red) cells.

### **Apoptosis detection: Annexin V-FITC**

The presence of apoptosis as a possible mechanism of cell death induced by 2-MeOE2bisMATE was evaluated and quantified using flow cytometry in combination with Annexin V-FITC. In apoptosis the calcium-dependent phospholipids scramblase activity is activated which results in the externalization of the phosphatidylserine

layer of the cell membrane. Externalization of the phosphatidylserine layer during apoptosis provides binding sites for Annexin V. Annexin V (a  $\text{Ca}^{2+}$ -dependent, phospholipid binding protein) is conjugated to a fluorochrome; this allowed for the identification of early- and late apoptosis and necrosis. After 48 h of exposure to  $0.4\mu\text{M}$  2-MeOE2bisMATE, cells were trypsinised and  $10^6$  cells were resuspended in 1 ml of 1x Binding Buffer and centrifuged at  $300 \times g$  for 10 min. Supernatant was removed and cells were resuspended in  $100 \mu\text{l}$  of 1x Binding Buffer.  $10 \mu\text{l}$  of Annexin V-FITC was added and incubated for 15 min in the dark at room temperature. After 15 min cells were washed by adding 1 ml of 1x Binding Buffer and centrifuged at  $300 \times g$  for 10 min. Supernatant was carefully pipetted off and cells were resuspended in  $500 \mu\text{l}$  of 1x Binding Buffer solution. Immediately prior to analysis  $12.5 \mu\text{l}$  of propidium iodide ( $40 \mu\text{g}/\text{ml}$ ) was added and gently mixed. Propidium iodide fluorescence (oncotic cells) and annexin V fluorescence (apoptotic cells) were measured with a fluorescence activated cell sorting (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 were analyzed with CXP software (Beckman Coulter South Africa (Pty) Ltd). Propidium iodide emits light at 617nm and FITC emit at 530nm therefore, data obtained from the log forward scatter detector nr 1 (Fl Lin, detects 515-545nm emissions) and the log forward scatter detector nr 3 (Fl3 Lin, detects 600nm emissions) were represented as a single dot-plot. FL3 log (propidium iodide) was represented on the *x*-axis and FL1 log (FITC) was represented on the *y*-axis. Distributions of cells within the quadrants were calculated with Cyflogic version 1.2.1 software (Pertu Therho, Turko, Finland).

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

LC3 is the only recognized mammalian protein identified that stably associates with the autophagosome membranes. LC3-II is membrane bound and enriched in the autophagic vacuole fraction. Detection of the conversion of LC3-I to LC3-II is a useful and sensitive marker for identifying autophagy in mammalian cells (21). LC3B antibody will allow for autophagy detection. After 48 h of exposure to  $0.4\mu\text{M}$  2-MeOE2bisMATE cells were trypsinised and centrifuged. Cells were washed with cold PBS, pelleted and fixed with 3 ml 0.01% formaldehyde in PBS for 10 min at  $4^\circ\text{C}$ . Cells were centrifuged and resuspended in  $200 \mu\text{l}$  PBS, followed by 1 ml ice-cold methanol ( $-20^\circ\text{C}$ ) for 15 min at  $4^\circ\text{C}$ . Afterwards cells were washed twice with cold PBS. Cells were stained with 0.5 ml of the antibody cocktail (0.05% Triton X-100, 1% bovine serum albumin (BSA),  $40 \mu\text{g}/\text{ml}$  propidium iodide and  $0.5 \mu\text{g}/\text{ml}$  conjugated rabbit polyclonal anti-LC3B antibody) prepared in PBS for 2 h at  $4^\circ\text{C}$ . Cells were washed trice with PBS/0.05% Triton/1% BSA and analyzed by means of flow cytometry. Data

from at least 10 000 cells were analyzed employing Cyflogic version 1.2.1 software (Pertu Therho, Turko, Finland).

### **Statistical Analyses**

Qualitative data were obtained from PlasDIC, TEM, light microscopy and fluorescent microscopy. Quantitative data were supplied by means of cell number determination (crystal violet staining). Data obtained from three independent experiments (each conducted in six replicates) are shown as the mean  $\pm$ SD. Data were statistically analysed for significance using the analysis of variance (ANOVA)-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**

#### **Three dimensional view: Optical transmitted light differential interference contrast**

The effects of 2-MeOE2bisMATE on morphology of MCF-7 cells were investigated by means of PlasDIC. PlasDIC revealed a decrease in cell density in the MCF-7 2-MeOE2bisMATE-treated cells when compared to the vehicle-treated control and to cells propagated in growth medium (Fig. 1A-1C). 2-MeOE2bisMATE-treated cells were shrunken and the presence of apoptotic bodies was clearly visible in the surrounding area.

#### **Cell number determination: Crystal violet**

Dose- and time-dependent studies were performed using crystal violet staining. Spectrophotometrical studies indicated that 2-MeOE2bisMATE decreased cell numbers to 75% after 24 h of exposure (Fig. 2A). The IC<sub>50</sub> was determined at 0.4  $\mu$ M and a 48 h exposure period (Fig. 2B). All subsequent studies were conducted using the IC<sub>50</sub> (0.4  $\mu$ M) concentration at an exposure period of 48 h. The vehicle control did not have any significant effects on cell numbers.

#### **Cytotoxicity: LDH assay**

LDH production in the medium was measured after exposure to 0.4  $\mu$ M 2-MeOE2bisMATE by conducting spectrophotometry. A slight increase (not statistically significant) in LDH (Fig. 3) production was found after exposure to 0.4  $\mu$ M 2-MeOE2bisMATE after 48 h when compared to the vehicle-treated cells. The insignificant LDH release indicates the absence of necrosis. Various controls were included according to the manufacturer's



instructions namely: background control, low control and high control. The background control consists of growth medium. The low control refers to cells propagated in growth medium and the high control includes cells propagated in growth medium with cell lysis solution added to the cells shortly before the experiment was terminated.

#### **Morphology: Transmission electron microscopy**

TEM revealed hypercondensed chromatin, membrane blebbing, budding and nuclear membrane fragmentation in the 2-MeOE2bisMATE-treated MCF-7 cells when compared to the vehicle control cells (Fig. 4A and 4B). In addition, several intracellular vacuoles were observed in the exposed cells.

#### **Morphology: Light microscopy (Haematoxylin and eosin staining)**

Haematoxylin and eosin staining revealed hypercondensed chromatin, apoptotic bodies, a metaphase block and compromised cell density in the 2-MeOE2bisMATE-treated MCF-7 cells when compared to vehicle-treated cells (Fig. 5A and 5B).

#### **Morphology: Fluorescent microscopy**

Hoechst 33342, acridine orange and propidium iodide staining was used to visualize possible apoptotic- and autophagic characteristics. Hoechst stains the DNA of viable- and non-viable cells, acridine orange acts as a lysotropic tracer and suggests the presence of autophagy. Propidium iodide can only penetrate cells where the cell membrane has been compromised. Increased lysosomal staining and a decrease in cell density were observed in the 2-MeOE2bisMATE-exposed cells when compared to vehicle-treated cells. In addition, several 2-MeOE2bisMATE-exposed cells were present in metaphase (Fig. 6A and 6B). No propidium iodide staining was found in neither the 2-MeOE2bisMATE-treated cells, nor the vehicle control cells. This implicates the absence of necrotic or oncotic processes. Necrosis is the process where the cell releases cellular components uncontrollably into the surrounding area, also characterized by damaged cell membranes and oncosis. This process takes place after the cell has died (23). The absence of propidium iodide staining confirms that the cell membrane was not compromised.

#### **Apoptosis detection: Annexin V-FITC**

Induction of cell death was measured by means of flow cytometry employing Annexin V-FITC. After exposure to 0.4 $\mu$ M 2-MeOE2bisMATE for 48 h only 54% of the cells were viable when compared to the 98% of the vehicle control (Fig. 7A and 7B). Furthermore, 26% of the 0.4 $\mu$ M 2-MeOE2bisMATE-treated cells were found in early apoptosis, 10% in late apoptosis and 9% in necrosis.

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

A conjugated rabbit polyclonal anti-LC3B antibody employing flow cytometry was utilized for the detection of autophagy. The latter demonstrated the accumulation of LC3 in 20% of the 2-MeOE2bisMATE-treated cells when compared to the 1% of the vehicle-treated cells (Fig. 8A and 8B).

#### **Discussion**

As previously mentioned 2-MeOE2bisMATE possesses potential as a future anticancer therapeutic agent, however, the mechanisms of cell death and autophagy must first be unravelled. Low oral bioavailability and rapid metabolic breakdown resulted in the development of several 2ME2 analogues. 2-MeOE2bisMATE exerts antiproliferative activity and exerts anticancer effects [10, 11, 15]. This project focused on the qualitative and quantitative effects of 2-MeOE2bisMATE on the estrogen receptor positive human breast adenocarcinoma MCF-7 cell line.

Dose-dependent (0.2-1.0  $\mu$ M) antiproliferative effects of 2-MeOE2bisMATE on MCF-7 cells were demonstrated after 48 h. The 0.4  $\mu$ M 2-MeOE2bisMATE concentration ( $IC_{50}$ ) was used in subsequent studies to determine the influence on LDH production, as well as the type of cell death (apoptosis and autophagy) induced in MCF-7 cells after 48 h of exposure. Previous research have shown 2-MeOE2bisMATE to be a potent inhibitor of proliferation in a wide range of tumour cell lines with  $IC_{50}$  values approximately 10-fold less than 2ME2 [23]. The  $IC_{50}$  concentration of 0.4  $\mu$ M used in this study is in the same range used by Utsumi et al. (2005) [24]. 2-MeOE2bisMATE inhibited growth in breast cancer mitoxantrone-resistant cells (MCF-7MR) and breast cancer wild type cells (MCF-7wt) xenograft [25, 26]. Furthermore, Wood et al. (2004) showed that 2-MeOE2bisMATE would also cooperate with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to promote apoptosis; however, TRAIL and Fas receptors are not required for 2-MeOE2bisMATE induced apoptosis, since there was a lack of BID (proapoptotic Bcl-2 member) cleavage suggesting that these death receptors were not activated. However, 2-MeOE2bisMATE exposure in combination with TRAIL resulted in

growth inhibition and cell death in the breast tumorigenic CAL51 and MCF-7 cell lines. Therefore, TRAIL and 2-MeOE2bisMATE cooperation enhances cell death in tumorigenic cell lines [27].

LDH production was not statistically significantly increased in 2-MeOE2bisMATE-treated cells when compared to vehicle-treated cells demonstrating the absence of necrosis. The relationship between metabolic lactate and apoptosis has not yet been elucidated [28]. No other studies have been published previously addressing the influence of 2-MeOE2bisMATE on LDH production and cytotoxicity. Nonetheless, 2-methoxyestradiol upregulated LDH production in the MDS-RAEB MUTZ-1 cell line (a cell line derived from the bone marrow of an individual with myelodysplastic syndrome). The study hypothesized the involvement of the G<sub>2</sub>M block and LDH upregulation involvement in subsequent induction of apoptosis [29].

Light microscopy revealed a mitotic block, hypercondensed chromatin, apoptotic bodies and compromised cell density in 2-MeOE2bisMATE-treated cells when compared to vehicle-treated cells. TEM observations confirmed results obtained by light microscopy and included membrane blebbing, budding, apoptotic bodies and nuclear membrane fragmentation in the 2-MeOE2bisMATE-treated cells when compared to vehicle-treated cells. In addition, annexin V-FITC results verified the induction of apoptosis by 2-MeOE2bisMATE in the MCF-7 cell lines. Induction of apoptosis by 2-MeOE2bisMATE was demonstrated in MCF-7, MDA-MB, prostate cancer cells (PC-3), human umbilical vein endothelial cells (HUVEC) and the human breast adenocarcinoma CAL51 cells [16, 24, 27]. Further studies will be required to determine whether 2-MeOE2bisMATE is acting by inhibition of angiogenesis or induction of apoptosis or, as most likely, a combination of both mechanisms [30].

Newman et al. (2007) reported that 2-MeOE2bisMATE caused cell cycle arrest, cyclin B1 induction and subsequent apoptosis in both MCF-7 DOX and MCF-7 WT cells [23]. Furthermore, caspase 3 and caspase 9 were cleaved effectively in CAL51 cells by 2-MeOE2bisMATE supporting the extrinsic pathway of apoptosis activation [27].

As already mentioned, fluorescence microscopy revealed lysosomal staining, thus demonstrating the novel discovery of autophagy induction by 2-MeOE2bisMATE. Conjugated rabbit polyclonal anti-LC3B antibody employing flow cytometry verified the induction of autophagy by 2-MeOE2bisMATE in the tumorigenic 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 cell lacks nutrients. In autophagy parts of the cytoplasm is sequestered into a double-membrane autophagosome and delivered to lysosomes for breakdown and recycling [31]. Azab et al. (2009) revealed that 2ME2 induces

autophagy in human glioblastoma-astrocytoma epithelial-like cell line (U87), human cervical adenocarcinoma cells (HeLa) and transformed human embryonic kidney cells (HEK293) [32].

In conclusion, the present study demonstrated the dose-dependent effects of the novel synthesized compound, 2-MeOE2bisMATE on MCF-7 cell growth and morphology over 48 h. Growth inhibition, the presence of a mitotic block and hallmarks of apoptosis were found in the morphological studies. Results obtained from this study contributes to elucidating the mechanism of action exerted by 2-MeOE2bisMATE. Furthermore, data obtained from this study demonstrates the induction of autophagy in the tumorigenic MCF-7 cell line. In addition, this research supports the notion of crosstalk between different types of cell death including apoptosis and autophagy. Common cellular stressors activate various signaling pathways that regulate both apoptosis and autophagy. ROS induces apoptosis and regulate Atg4 which is essential for autophagy induction. In addition, Atg5 promotes apoptosis-and autophagy induction. proapoptotic stimuli exposure results in calpain activation which cleaves Atg5. The truncated Atg5 moves from the cytosol towards the mitochondria where association with Bcl-XL occurs. this event induces apoptosis. However, calpain depletion inhibits autophagy induction due to exposure to rapamycin and limited presence of amino acids [33]. Beclin 1, a member of the BH3-branch Bcl-2 family plays an essential role in autophagy induction. In addition, microinjection of a peptide encompassing the BH3 domain of Beclin 1 results in BAX-dependent apoptosis [34]. Further studies will focus on the in vitro influence of 2-MeOE2bisMATE on the common signaling molecules involved in the crosstalk.

## **Acknowledgements**

This research was supported by grants from the Medical Research Council of South Africa, the Cancer Association of South Africa, RESCOM (School of Medicine, University of Pretoria, South Africa), National Research Foundation and the Struwig-Germeshuysen Cancer Research Trust of South Africa.

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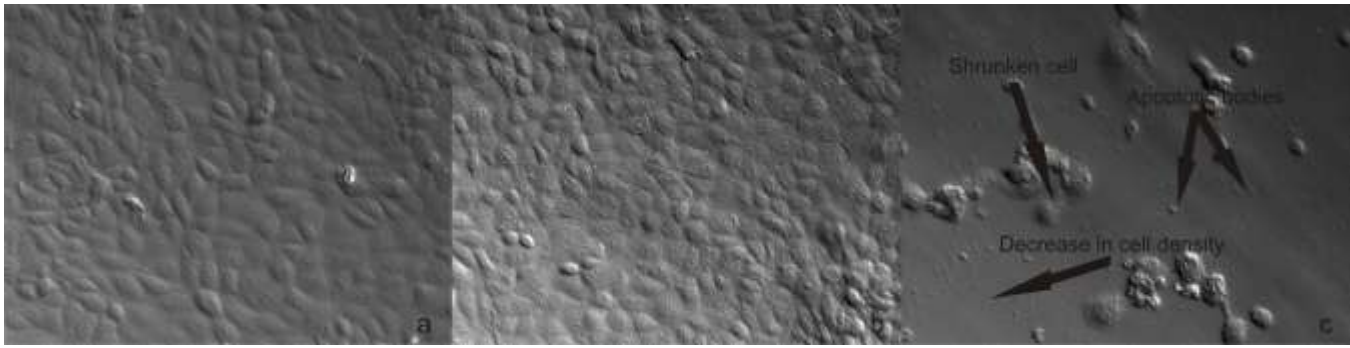
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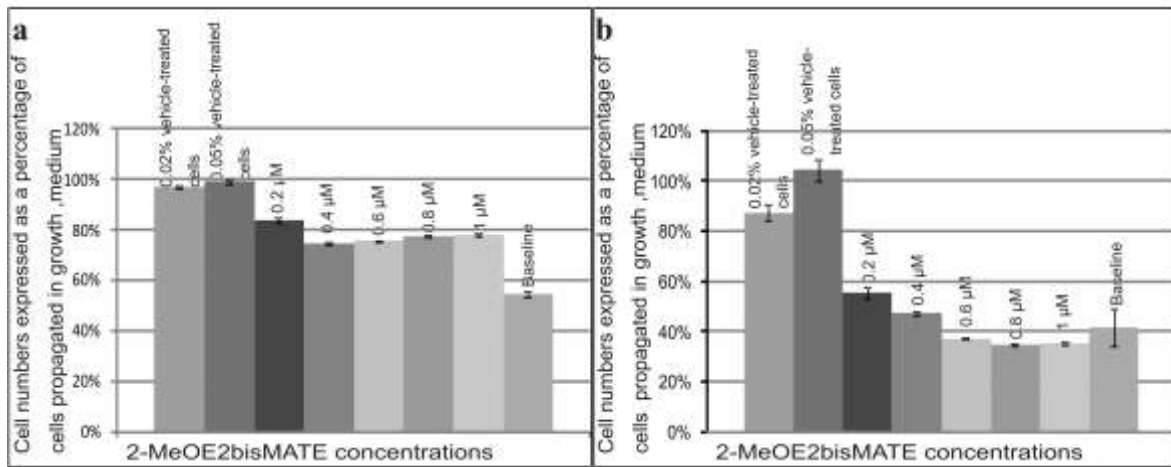
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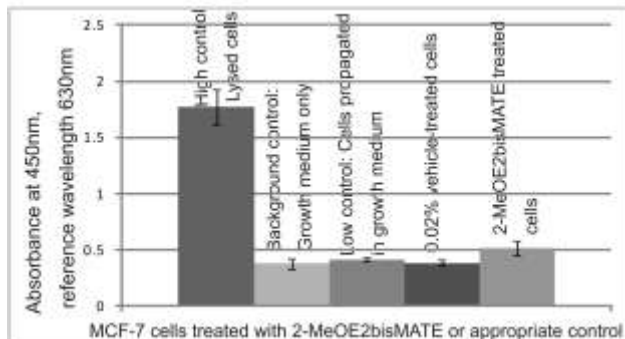
## Figures and Figure Legends



**Fig. 1** PlasDIC micrographs of MCF-7 cells propagated in growth medium and vehicle-treated cells after 48 h revealed confluent cells displaying no characteristics of distress (Fig. 1A and 1B) (20X magnification). PlasDIC showed a decrease in cell density in the MCF-7 cells treated with 2-MeOE2bisMATE (Fig. 1C) when compared to the vehicle-treated control and to cells propagated in growth medium. Information gained from the PlasDIC technique demonstrated that 2-MeOE2bisMATE-treated cells are shrunken accompanied with decreased cell density. Apoptotic bodies were also observed in the 2-MeOE2bisMATE-treated cells (20X magnification).

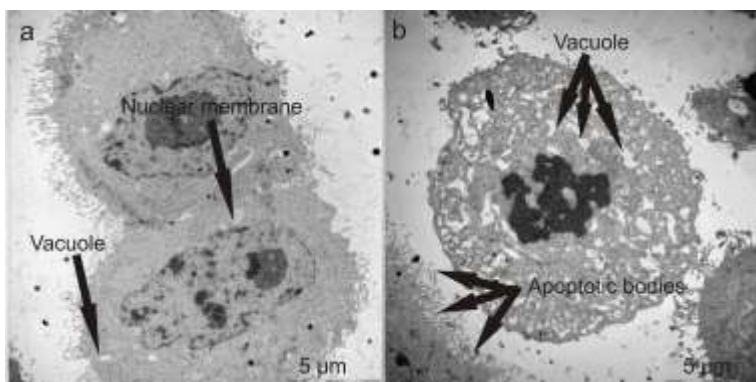


**Fig. 2** Crystal violet staining after 24 h of exposure of the MCF-7 cell line to 2-MeOE2bisMATE (Fig. 2A) resulted in significant growth inhibition. 0.4  $\mu$ M 2-MeOE2bisMATE treatment decreased cell numbers to 75% in MCF-7 cells after 24 h and 48% after 48 h (Fig. 2B). The 50% maximal growth inhibition concentration ( $IC_{50}$ ) was determined at 0.4 $\mu$ M during the 48 h of exposure of the MCF-7 cell line to 2-MeOE2bisMATE.

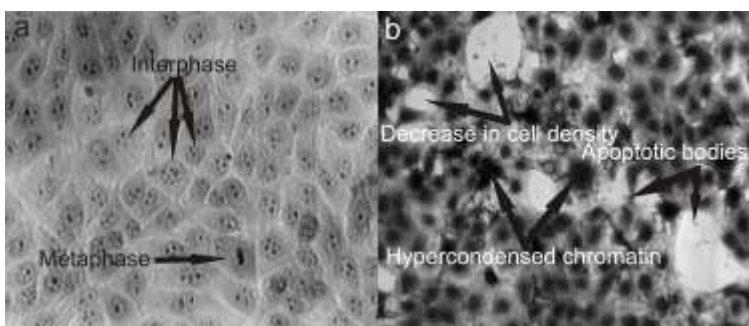




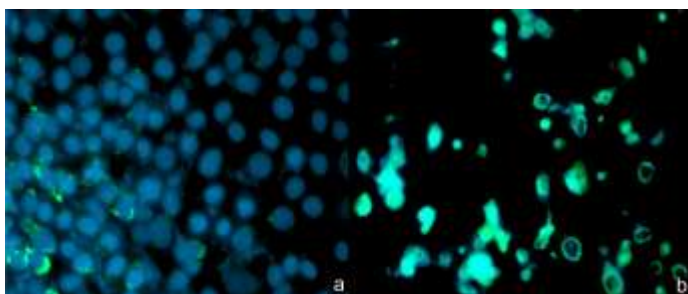
**Fig. 3** LDH production measured by means of spectrophotometry revealed a slight increase in 2-MeOE2bisMATE-treated cells after 48 h of exposure when compared to the vehicle-treated control cells. The increase found in the exposed cells were, however, not statistically significant.



**Fig. 4** TEM illustrated the vehicle-treated sample (Fig. 4A) revealing cells with the presence of minimal vacuoles (6000X magnification). The MCF-7 2-MeOE2bisMATE-treated cells (Fig. 4B) were severely affected. Several vacuoles and apoptotic bodies were present when compared to the vehicle-treated cells.

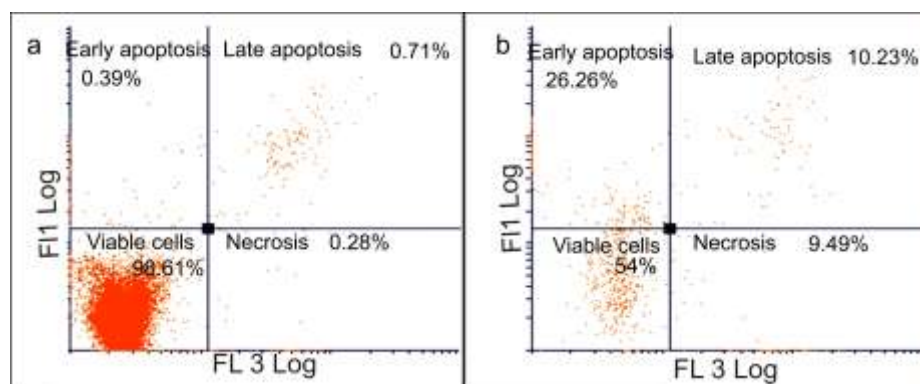


**Fig. 5** Light microscopy displayed the vehicle-treated control MCF-7 cells (Fig. 5A) with no signs of distress (20X magnification). 2-MeOE2bisMATE-treated cells (Fig. 5B) after 48 h of exposure revealed several apoptotic characteristics including hypercondensed chromatin with decreased cell density (20X magnification).

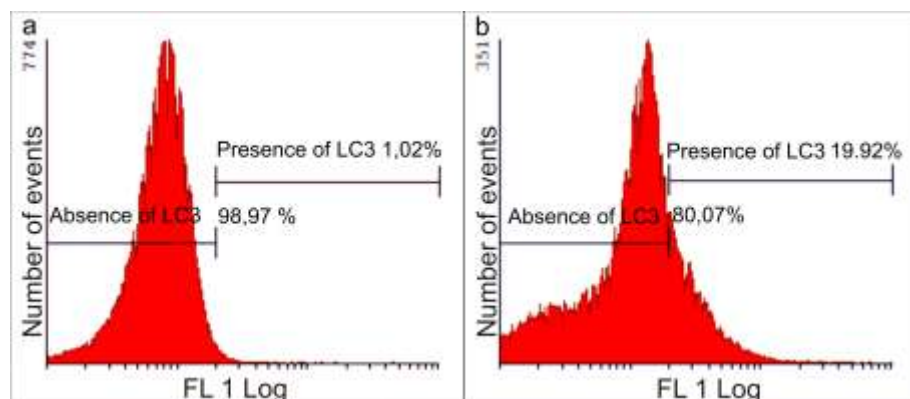


**Fig. 6** Fluorescent microscopy was also used to investigate the effect of 2-MeOE2bisMATE on morphology of MCF-7 cells after 48 h. Vehicle-treated control cells (Fig. 7A) were confluent. The nucleus can be distinguished since Hoechst 33342 stained the DNA. Almost no lysosomal staining was observed (20X magnification). 2-MeOE2bisMATE-treated cells (Fig. 7B) after 48 h of exposure revealed increased lysosomal staining with

decreased cell density when compared to the vehicle-treated cells. In addition, several cells were present in metaphase. However, no propidium iodide staining was observed (20X magnification) indicating the absence of necrotic or oncotic processes.



**Fig. 7** Detection of apoptosis induction in vehicle-treated cells (Fig. 8A) and 2-MeOE2bisMATE-treated cells (Fig. 8B) was performed by means of flow cytometry. Data revealed that 54% of the 2-MeOE2bisMATE-treated MCF-7 cells were viable, 26% were present in early apoptosis, 10% in late apoptosis and 9% in necrosis.



**Fig 8** Flow cytometrical investigation employing the conjugated anti-LC3 antibody to investigate autophagy induction in vehicle-treated cells (Fig. 8A) and 2-MeOE2bisMATE-treated cells (Fig. 8B) revealed that 20% of 2-MeOE2bisMATE-treated MCF-7 cells were present in autophagy when compared to vehicle-treated cells.