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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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Running title: *In vitro* effects of 2-Methoxyestradiol-bis-sulphamate

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ABSTRACT

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

Keywords

2-Methoxyestradiol-Bis-Sulphamate, Apoptosis, Autophagy

INTRODUCTION

The endogenous metabolite of estradiol, namely 2-methoxyestradiol (2ME2) exerts its antiproliferative, antiangiogenic and antitumor *in vitro* and *in vivo* activity in an estrogen receptor independent mode [1, 2, 3]. 2ME2 demonstrate biphasic effects on cell lines including non-small cell lung adenocarcinoma cell line (HOP 62), colorectal carcinoma epithelial cell line (HCT-116), melanoma tumorigenic cell line (UACC-62), human ovarian adenocarcinoma cell line (OVCAR-3), renal carcinoma cell line (SN12-C), estrogen receptor positive human breast adenocarcinoma cell line (MCF-7), highly tumorigenic estrogen receptor negative breast

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Abbreviations: 2-methoxyestradiol (2ME2); 2-Methoxyestradiol-bis-sulphamate (2-MeOE2bisMATE); Androstenediol (adiol); Carbonic anhydrase II (CA II); Dehydroepiandrostene (DHEA); Dimethyl sulphoxide (DMSO); Lactate dehydrogenase (LDH) ; Optical transmitted light differential interference contrast (PlasDIC); Phosphate buffered saline (PBS) ; Steroid sulphatase (STS); Transmission electron microscopy (TEM), carcinoma

cell line (MDA-MB-435), immortalized T lymphocyte cell line used to study acute T cell leukemia (Jurkat cells) (2). Lower 2ME2 concentrations (1 μM) demonstrates a proliferating effect on tumor cells by increasing the expression of the vascular endothelial growth factor (VEGF), however, 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], breast- and prostate cancer [8]. Patients treated with 2ME2 demonstrated minimal side effects, including hot flushes, reversible liver enzyme elevations, fatigue and diarrhoea [9, 10]. Clinical benefit was observed with administration of 1600 to 3200 mg/kg/day Panzem® in both breast- and myeloma patients [11].

Low oral bioavailability and rapid metabolic degradation of 2ME2 resulted in the manufacturing of several promising analogues in recent years. 2-Methoxyestradiol-bis-sulphamate (2-MeOE2bisMATE) is a bis-sulphamoylated derivative of 2ME2 possessing antiproliferative and antitumor activity [10, 11].

Recent studies demonstrated that the novel 2-MeOE2bisMATE is tenfold 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 an excess lactic acid and a decreased pH in the surrounding environment of the hyperproliferative tumorigenic cells. The above-mentioned acidic environment contributes to metastatic processes associated with malignant cancers through the breakdown of the basement membrane and breachment into the circulation [12].

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) that regulates the conversion of oestrone sulphate to oestrone [16]. STS also plays a vital role in the conversion 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. Inhibition of STS can consequently 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. The latter suggests that the action mechanism of 2-MeOE2bisMATE is derived from its ability to disrupt microtubule dynamics. Microtubule dynamic disruption by 2-MeOE2bisMATE led 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 rapid activation of caspase 3 and 9 through the intrinsic pathway [19].

2-MeOE2bisMATE demonstrates therapeutic promise as a prospective STS inhibitor, antiangiogenic-and antiproliferative drug [20]. This compound is at present not commercially obtainable and research on the action mechanism and cell signaling events of 2-MeOE2bisMATE is limited, thus 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. Data generated from this study contribute to understanding the action mechanism of 2-MeOE2bisMATE exerted on the non-tumorigenic MCF-12A breast epithelial cell line.

MATERIALS AND METHODS

Materials

Cell line

The MCF-12A cell line is a non-tumorigenic spontaneously immortalized adherent human breast epithelial cell line and forms domes in confluent cultures. The MCF-12A cells were a gift from Professor Parker (Department of Medical Biochemistry, University of Cape Town, 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-FITC Kit and LDH-cytotoxicity assay kit was purchased from BIOCOM biotech (Pty) Ltd. (Clubview, South Africa). 2-MeOE2bisMATE was synthesized by Professor 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 25cm^2 tissue culture flasks at a humidified atmosphere at 37°C and 5% CO_2. MCF-12A cells were cultured in medium consisting of a 1:1 mixture of DMEM and Ham's-F12 medium, 20ng/ml epidermal growth factor, 100ng/ml cholera toxin, 10µg/ml insulin and 500ng/ml hydrocortisone, supplemented with 10% heat-inactivated fetal calf serum (56°C, 30min), 100U/ml penicillin G, 100µg/ml streptomycin and fungizone (250µg/l).

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 exposure of the cells. Media of all 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 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 in 6 well plates on heat-sterilized coverslips. For 25 cm^2 cell culture flasks, exponentially growing MCF-12A cells were seeded at $1x10^6$ cells per 25 cm^2 flasks to a final volume of 5 ml of maintenance medium. A 24 h incubation period at 37^{o}C was allowed for cell adherence, the medium was discarded and cells were exposed to 2-MeOE2bisMATE with concentrations ranging from 0.2-1 µM including vehicle-treated controls and incubated for 48 h at 37^{o}C. These conditions were selected since previous studies in our laboratory have demonstrated successful antiproliferative activity in tumorigenic cell lines. Sample controls for apoptosis- and autophagy were also included. Controls for apoptosis comprised of cells exposed to actinomycin D with a concentration of 0.1 µg/ml in growth medium for 48 h. Controls for autophagy consisted of cells that were starved (1:3 ratio relative to growth medium: phosphate buffered saline (PBS)).

Methods

Three dimensional visual image: Optical transmitted light differential interference contrast

Optical transmitted light differential interference contrast (PlasDIC) is a polarization-optical transmitted light differential interference contrast method. PlasDIC is an improved method for polarization-optical transmitted light differential interference contrast, where, unlike conventional Smith-Nomarski's method, linearly polarized light is only generated after the objective, giving images of outstanding quality [21]. PlasDIC displays 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 has 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*. (1986) used crystal violet to quantify cell number in monolayer cultures as a function of the absorbance of the dye taken up by the cells [22]. A time-dependent study was conducted with time intervals of 24 h and 48 h. A dose-dependent study was conducted with a concentration range of 0.2 µM - 1 µM of 2-MeOE2bisMATE since previous research conducted in our laboratory and other studies revealed antiproliferative activity within this concentration series 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 5000 cells per well. Cells were incubated at 37°C for 24 h to allow for attachment. After 24 h attachment medium was discarded and cells were exposed to a 0.2-1 µM 2-MeOE2bisMATE concentration series for 24 h and 48 h respectively, including vehicle-treated controls and incubated for the appropriate exposure time at 37^{o}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. Cells were fixed with 100µl of 1% gluteraldehyde (incubation for 15 min at room temperature). Subsequently, glutaraldehyde was discarded and cells were stained using 100µl 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 200µl 0.2% Triton X-100 and incubated at room temperature for 30 min. 100µl of the solution was transferred to a new microtitre plate. Afterwards, the absorbance was read at 570nm using an EL_x800 Universal Microplate Reader (Bio-Tek Instruments Inc., Vermont, United States of America). 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 antiproliferative activity in tumorigenic cell lines with minimal antiproliferative effects on non-tumorigenic cell lines. Therefore, a concentration was selected with increased susceptibility in tumorigenic cell lines to be compared to a non-tumorigenic cell line. Thus, the 50% growth inhibitory concentration (IC_{50}) was calculated in an adenocarcinoma cell line (MCF-7) in another study conducted in our laboratory as described by the National Cancer Institute in order to determine the growth inhibition induced by 2-MeOE2bisMATE (data not shown). The IC_{50} that was found in the latter 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-cytotoxicity assay kit

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 loss of membrane integrity consequential 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 5000 cells per well with an overnight attachment policy (incubated at 37°C at 5% CO_2). After 24 h cells were exposed to 2-MeOE2bisMATE and appropriate controls respectively. Subsequently, 200µl medium was transferred and centrifuged at 5000rpm for 10 min. Afterwards, 10µl was transferred to a clear 96 well plate. Subsequently, 100µl 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 460nm, with a reference wavelength of 630nm by means of EL_x800 Universal Microplate Reader (Bio-Tek Instruments Inc., Vermont, United States of America).

Morphology: Light microscopy (Haematoxylin and eosin staining)

Haematoxylin and eosin staining method was used to determine the influence of 2-MeOE2bisMATE on the cytoplasm and nucleus. Cells (500 000) per well were seeded on sterile coverslips in 6 well plates and incubated overnight. Cells were exposed to 0.4µM 2-MeOE2bisMATE for 48 h and the appropriate controls respectively. 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 for 20 min to the coverslips 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). Mitotic indices were also determined from the haemotoxylin and eosin stained cells. Quantitative data for mitotic indices were obtained by counting 1000 cells on each slide of the biological replicates and expressing data as a percentage of cells in each phase of mitosis, interphase and abnormal cells. Distinction was made between normal mitotic cells (included prophase in which the chromosomes are condensed, metaphase where chromosomes are aligned at the metaphase plate, anaphase in which the chromosomes move to opposite ends of the nuclear spindle and telophase in which chromosomes uncoil the spindle breaks down and during which the chromosomes of daughter cells are grouped in new nuclei), cells in interphase and abnormal cells which included cells displaying hypercondensed chromatin, membrane blebbing, apoptotic bodies and abnormal chromosome segregation. This H & E staining provided both qualitative and quantitative information.

Morphology: Transmission electron microscopy

Transmission electron microscopy (TEM) was employed to visualize the morphology of cells after exposure to 2-MeOE2bisMATE and vehicle-treated cells respectively. TEM was used to view autophagic lysosomes and to identify apoptotic bodies formed during final stages of apoptosis. Cells were seeded in a $25cm^2$ flask at a density of 500 000 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 and appropriate controls were included respectively. After 48 h, cells were trypsinised and resuspended in 1 ml medium. Cells were fixed with 2.5% glutaraldehyde in 0.075M phosphate buffer for 1 h. Cells were rinsed with 0.075M phosphate buffer (three times), fixed with osmium tetroxide for 30 min, rinsed thrice with distilled water and dehydrated with increasing ethanol concentrations (30%, 50%, 70%, 90%, and 100%). Cells were infiltrated with 50% quetol in ethanol for 1h and then with 100% quetol for 4 to 6 h. Ultra-thin sections were prepared using a microtome and contrasted using by means of 4% uranyl acetate for 10 min and rinsed with water. 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 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 cells were exposed to 0.4µM 2-MeOE2bisMATE and appropriate controls were included respectively. 0.5ml of Hoechst 33342 solution (3.5µg/ml in phosphate buffered saline (PBS)) was added to the medium to give a final concentration of 0.9µM and was incubated for 25 min at 37°C. Subsequently, 0.5ml of acridine orange solution **(**4µg/ml in PBS) was added to the medium to give a final concentration of 1µg/ml and incubated for 5min at 37°C and 0.5ml of propidium iodide solution (40µg/ml in PBS)

was added to the medium to provide a final concentration of 12µM. Cells were washed three times with PBS. Photos were taken with the appropriate filters in a dark room to prevent quenching. Zeiss Axiovert CFL40 microscope and Zeiss Axiovert MRm monochrome camera (Zeiss, Oberkochen, Germany) were used employing a Zeiss Filter 2 for Hoechst 33342 stained blue-cells and 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 induction of cell death 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. The externalization of the phosphatidylserine layer during apoptosis will provide binding sites for Annexin V. Annexin V (a Ca^{2+}-dependent, phospholipid binding protein) 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 0.4µM 2-MeOE2bisMATE, cells were trypsinized and 10^6 cells were resuspended in 1ml of 1x Binding Buffer and centrifuged at 300 x g. for 10 min. The supernatant was removed and the cells resupended in 100µl of 1x Binding Buffer. 10µl of Annexin V-FITC was and incubated for 15 min in the dark at room temperature. After 15 min the cells were washed by adding 1ml of 1x Binding Buffer and centrifuged at 300×g for 10 min. The supernatant was carefully pipetted off and the cells were resuspended in 500µl of 1x Binding Buffer solution. Immediately prior to analysis 12,5µl of propidium iodide (40µg/ml) was added and gently mixed. Propidium iodide fluorescence (oncotic cells) and annexin V fluorescence (apoptotic cells) was measured with a FACS FC500 System flow cytometer (Beckman Coulter South Africa (Pty) Ltd) equipped with an air-cooled argon laser excited at 488nm. Data from at least 30 000 cells were analyzed with cyflogic version 1.2.1 software (Pertu Therho, Turko, Finland)

STATISTICS

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) and mitotic indices. Data obtained from three independent experiments (each conducted in six replicates) are shown as the mean ±SD and data for mitotic indices were obtained by counting 1000 cells (repeated three times) on each slide of the biological replicates. 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-12A cells were investigated by means of PlasDIC. Slightly rounded MCF-12A 2-MeOE2bisMATE-treated cells indicated an increase in the number of cells occupying metaphase were observed when compared to the vehicle-treated controls and to cells propagated in growth medium (Fig. 1A, 1B and 1C). However, effects were not prominent and cell density was not compromised when compared to the vehicle-treated controls and to cells propagated in growth medium.

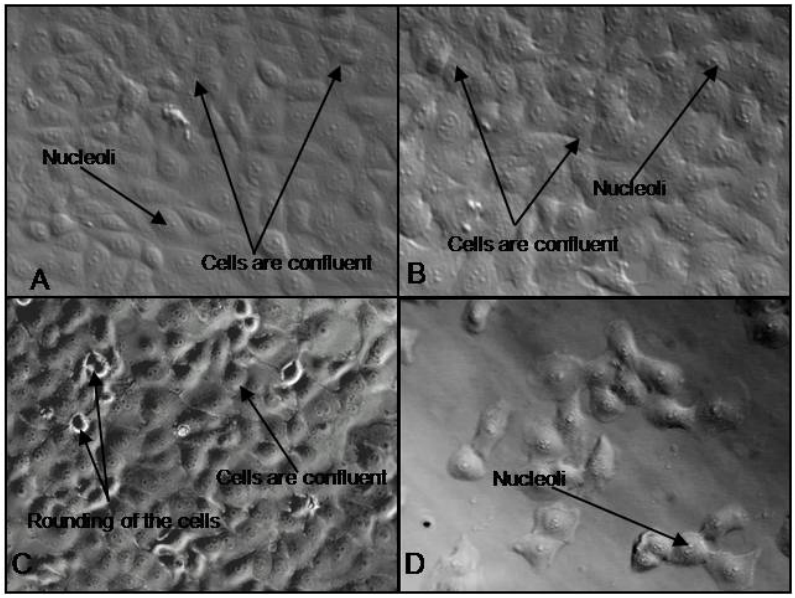

Fig. 1. PlasDIC micrographs of MCF-12A cells propagated in growth medium (A) and vehicle-treated cells (B) after 48 h of exposure presented confluent cells displaying no signs of distress where nucleoli were clearly visible (20X magnifiction). PlasDIC of MCF-12A 2-MeOE2bisMATE–treated cells IS illustrated in figure C and D. Figure C revealed a slight increase of cells in metaphase with unaffected cell density when compared to vehicle-treated control and cells propagated in growth medium.

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 (0.4µM) resulted in a decrease of cell growth to 79% (Fig. 2B). Neither the 0.02%, nor the 0.05% vehicle-treated cells revealed significant influences on cell numbers.

Previous data obtained in our laboratory revealed that the IC_{50} of 2-MeOE2bisMATE on the tumorigenic MCF-7 cell line was found to be at 48 h of exposure and a concentration a 0.4 µM (data not shown). All subsequent experiments were performed at 48 h exposure period with a 0.4 µM 2-MeOE2bisMATE concentration in the MCF-12A cell line. The vehicle control did not reveal significant effects on cell numbers.

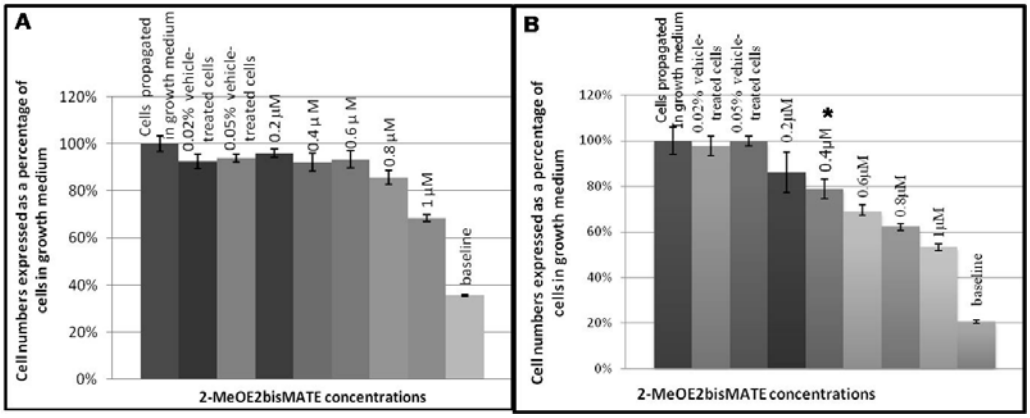

Fig. 2. Crystal violet staing during the 24 h exposure of the MCF-12A cell line to 2-MeOE2bisMATE resulted in slight inhibition of cell growth (A). 0.4 µM 2-MeOE2bisMATE treatment decreased cell numbers to 92% in the MCF-12A cell line. However, the decrease in cell numbers was not statistically significant. The 48 h exposure of the 0.4µM 2-MeOE2bisMATE-treated cells had an inhibitory growth effect of 21% when compared to cells in growth medium (B). An * indicates a statistical 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 production was measured in the medium after exposure by conducting spectrophotometry at a wavelength of 450nm (reference wavelength of 630nm). A slight increase in the LDH (Fig. 3) production 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).

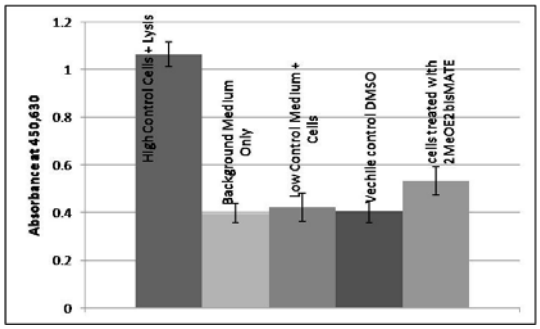

Fig. 3. LDH production measured by means of spectrophotometry revealed a slight increase in 2-MeOE2bisMATE-treated cells after 48 h of exposure when compared to the vehicle-treated control cells. The increase found in the exposed cells was, however, not statistically significant (P> 0.05).

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 4B). In addition, there was no difference in cell density between the 2 -MeOE2bisMATE-treated MCF-12A cells when compared to the vehicle-treated cells.

Mitotic indices were obtained by counting 1000 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%.

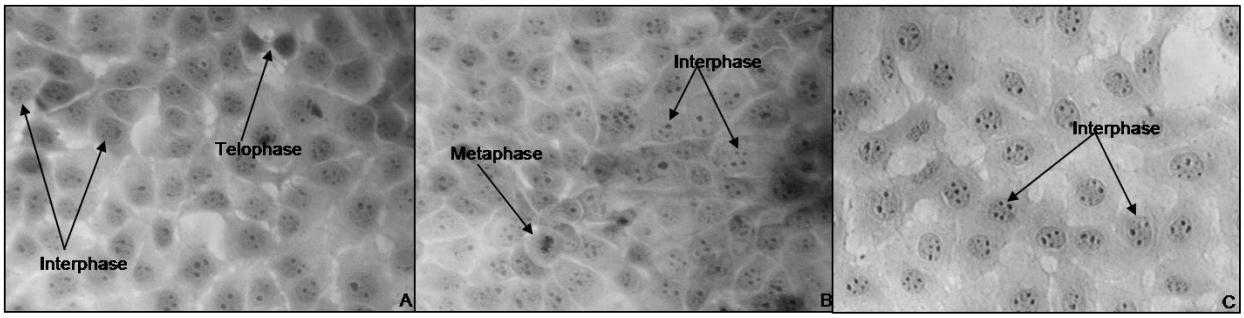

Fig. 4. Light microscopy revealed confluent vehicle-treated MCF-12A cells (A) showed no signs of cell distress and 48 h 0.4μM 2-MeOE2bisMATE MCF-12A-treated cells revealed no hypercondensed chromatin (B and C). Several cells were observed in interphase and cells in metaphase were also found.

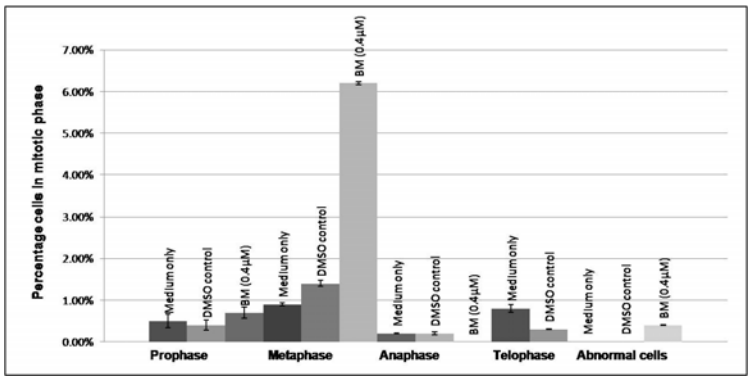

Fig. 5. Mitotic indices of MCF-12A cells propagated in growth medium, DMSO vehicle-treated cells and 2-MeOE2bisMATE- treated cells (indicated in the figure by BM (0,4µM)). The 2-MeOE2bisMATE-treated cells revealed an increase in metaphase cells when compared to cells propagated in growth medium and vehicle-treated cells. Mitotic indices were determined by counting 1000 cells on each slide (3 repeats) of the biological replicates and calculating which percentage of the cells in mitosis and interphase. Distinction was made between normal mitotic cells (included anaphase, metaphase, anaphase and telophase), cells in interphase and abnormal cells which included cells 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 by means of 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 6B).

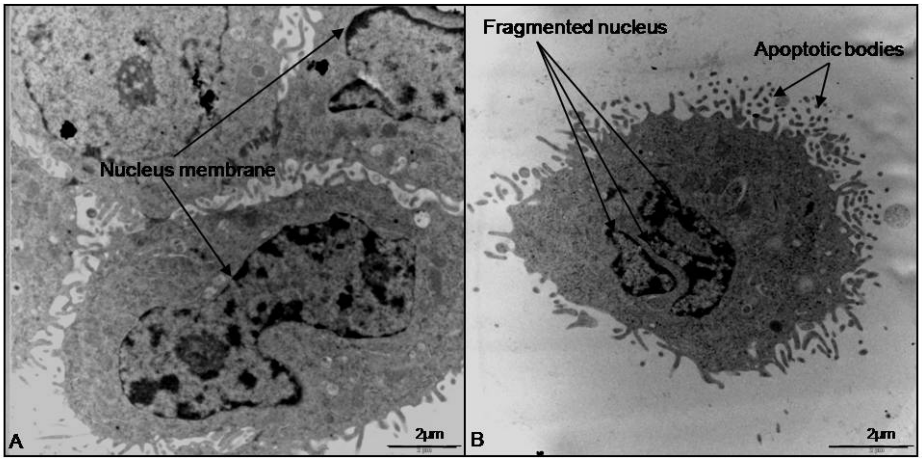

Fig. 6. Transmission electron microscopy (TEM) demonstrated vehicle-treated cells with no signs of distress (6000X magnification) (A). 0.4µM 2-MeOE2bisMATE-treated cells exposed for 48 h revealed nuclear fragmentation, extensions of the cell membrane and apoptotic bodies (6000 X magnification) (B). TEM allows for the illustration of the cell at a higher magnification. Morphology 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 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 indicates autophagy. Propidium iodide can only penetrate cells where the cell membrane has been compromised. Minor increased lysosomal staining was observed in the 2-MeOE2bisMATE-exposed cells when compared to the vehicle-treated cells (Fig. 7A and 7B). No propidium iodide staining was found in neither the treated cells, nor the vehicle control cells, revealing the absence of necrosis. Necrosis is a 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, causing 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.

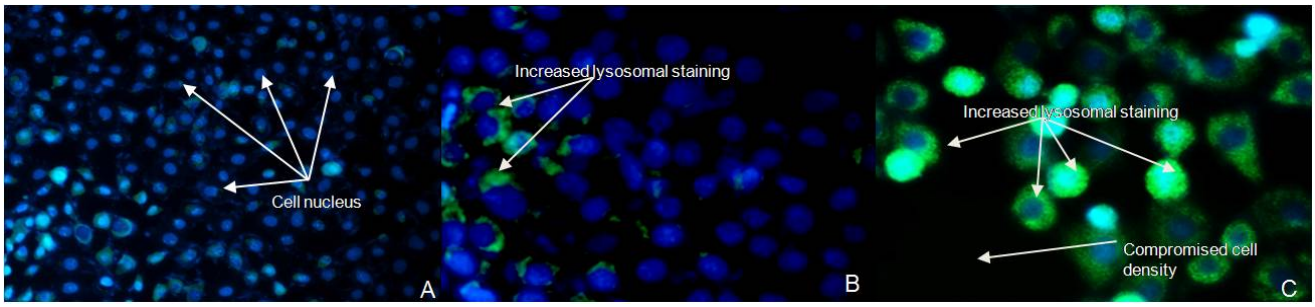

Fig. 7. Vehicle-treated control cells were confluent (A). The nucleus can be distinguished as Hoechst 33342 stains the DNA. Minimal lysosomal staining was observed indicating the presence of minimal autophagic activity. (10X magnification). Figure B is an representation of MCF12A cells treated with 2-MeOE2bisMATE for 48 h, lysosomal staining was observed and cell density was minimally affected. 2-MeOE2bisMATE-treated cells after 48 h of exposure are also diplayed in Figure C revealed decreased cell density when compared to the vehicle-treated cells. Minor increased lysosomal staining was indicating the presence of increased acidic vesicles suggesting the presence of autophagy. However, no propidium iodide staining was observed revealing the absence of necrosis (10X magnification). The cell density of the 2-MeOE2bisMATE-treated cells was affected and confirmed by TEM.

Apoptosis detection: Annexin V-FITC

The presence of apoptosis was investigated by means of flow cytometry and Annexin V-FITC. After exposure to 0.4µM 2=MeOE2bisMATE for 48 h, 76% of the cells were viable when compared to the 98% of the vehicle control (Fig. 8A and B). In addition, 10,9% of the 0.4µM 2-MeOE2bisMATE-treated cells were found in early apoptosis, 9% in late apoptosis and 4% in necrosis.

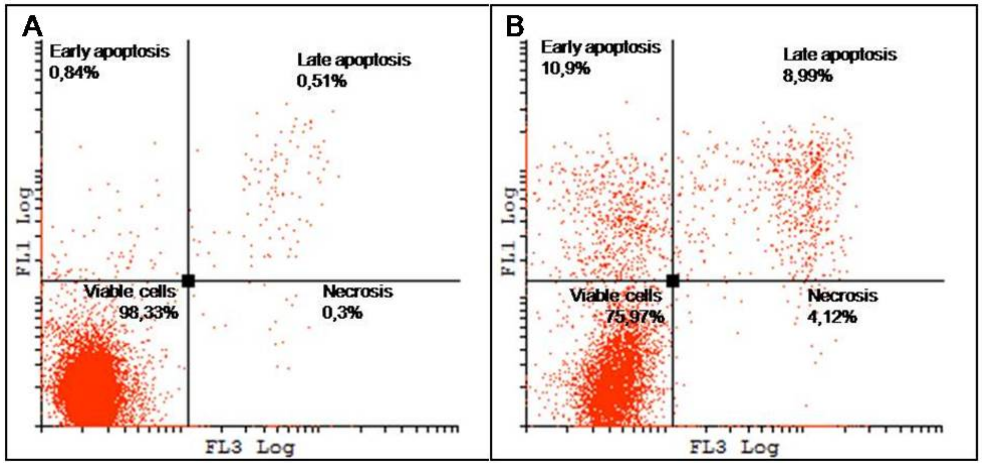

Figure 8: Investigation by means of flow cytometry revealed that vehicle-treated cells (A) had cell viability of 98% when compared to 2-MeOE2bisMATE-treated MCF-12A cells (B) that had decreased cell viability of 76%, 11% were found in early apoptosis, 9% in late apoptosis and 4% in necrosis.

DISCUSSION

Previous data collected in our own laboratory and by other researchers illustrated that 2-MeOE2bisMATE possesses antiproliferative activity in tumorigenic cell lines [1,2,3,8,9,11,12,13,14,15,16,17,18,19,20]. No publications has been published previously reporting the *in vitro* effects of 2-MeOE2bisMATE on non-yumorigenic cell lines. When

compared to *in vitro* effects in tumorigenic cell line, it is clear that tumorigenic cell lines are more susceptible than non-tumorigenic cell lines to 2-MeOE2bisMATE (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 the present study we have demonstrated the dose-dependent (0.2-1.0 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 the cell line derived from the bone marrow of an individual with myelodysplastic syndrome (MDS-RAEB MUTZ-1 cell line) (e) and the human promyelocytic leukaemia cell line (HL-60 cell line) [26, 27]. Nonetheless, the relationship between lactate dehydrogenase and apoptosis remains elusive and literature involving 2-methoxyestradiol and 2-MeOE2bisMATE influence on LDH are limited.

Membrane blebbing, membrane budding, minor vacuoles and nuclear fragmentation were revealed by conducting qualitative morphological studies in 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. Mitotic indices demonstrated the presence of a mitotic block in 2-MeOE2bisMATE-treated cells. However, these results revealed that the influence of 2-MeOE2bisMATE are not as pronounced when compared to the effects on tumorigenic cell lines [1,2,3,8,9,11,12,13,14,15,16,17,19,20]. The latter indicates that 2-MeOE2bisMATE possesses specificity to effect tumorigenic cell lines more severely when compared to non-tumorigenic cell lines.

It has been reported that STS inhibition can be considered for a possible treatment of cancer. STS is one of twelve sulphatases that have been characterized in human cells and regulates the formation of oestrone from oestrone sulphate [12]. STS mRNA levels are detected across the non-tumorigenic breast cancer MCF-10A cell line. STS Studies have shown that STS mRNA expression is higher in malignant than in normal breast tissue and is found in 74% of all breast cancer biopsies [28]. High levels of STS mRNA expression in breast tumors are associated with a poor prognosis. [29]. A recently published study revealed that mRNA STS, oestradiol sulfotransferase (EST) and 17-β-hydroxysteriod dehydrogenase II are elevated in breast carcinomas. This suggests regulation by a common metabolite, possibly oestradiol. Furthermore, oestradiol has recently been associated with apoptosis in tumorigenic breast cancer cells [30].

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 have been met. The present study 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 are limited. For this reason the signaling events and effects exerted by 2-MeOE2bisMATE warrants further investigation.

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REFERENCES

1. Dubey, R.K. and Jackson, E.K. Potential vascular actions of 2-methoxyestradiol. **Trends. Endocrinol. Metab**. 20(8) (2009) 374-379.

2. Pribluda, V.S., Gubish, E.R., La Vallee, T.M., Treston, A., Swartz, G.M and Green, S.J. 2-Methylestradiol: An endogenous antiangiogenic and antiproliferative drug candidate. **Cancer. Metastasis. Rev.** 19 (2000) 173-179.

3. Bhati, R., Gokmen-Polar, Y., Sledge, G.W., Fan, C., Nakshatri, H., Ketelsen, D., Borchers, C.H., Dial, M.J., Patterson, C. and Klauber-DeMore, N. 2-Methoxyestradiol inhibits the anaphase-promoting complex and protein translation in human breast cancer cells. **Cancer. Res.** 67 (2007) 702-708.
4. Banerjee, S.N., Sengupta, K., Banerjee, S., Saxene, N.K. and Banerjee, S.K. 2-Methoxyestradiol exhibits a biphasic effect on VEGF-A in tumor cells and upregulation is mediated through ER-α: a possible signaling pathway associated with the impact of 2-ME_2 on proliferating cells. **Neoplasia.** 5(5) (2003) 417-426.
5. Funakoshi, T., Birsner, A.E. and D'Amato, R.J. Antiangiogenic effect of oral 2-methoxyestradiol on choroidal neovascularization in mice. **Exp. Eye. Res.** 83 No 5 (2006) 1102-1107.
6. Matei, D., Schilder, J., Sutton, G., Perkins, S., Breen, T., Quon, C. and Sidor, C. Activity of 2 methoxyestradiol (Panzem® NCD) in advanced, platinum-resistant ovarian cancer and primary peritoneal carcinomatosis: A hoosier oncology group trial. **Gynaecol. Oncol**. 115 No 1 (2009) 90-96.
7. Lakka, S.S. and Rao, J.S. Angiogenic therapy in brain tumors**. Expert. Rev. Neurother.** 8 No 10 (2008) 1457-1473.
8. Sutherland, T.E., Anderson, R.L., Hughes, R.A., Altmann, E., Schuliga, M., Ziogas, J. and Stewart, A.G. 2-Methoxyestradiol- a unique blend of activities generating a new class of anti-tumour/anti-inflammatory agents. **Drug. Discov. Today.** 12(13/14) (2007) 577-584.
9. Thaver, V., Lottering, M.L., Van Papendorp, D. and Joubert, A. In vitro effects of 2-Methoxyestradiol on cell numbers, morphology, cell cycle progression, and apoptosis induction in oesophageal carcinoma cells. **Cell. Biochem. Funct.** 27 (2009) 205-210.
10. Lakhani, N.J., Lepper, E.R., Sparreboom, A., Dahut, W.L., Venitz, J. and Figg, W.D. Determination of 2-methxyestradiol in human plasma, using liquid chromatography/tandem mass spectrometry. **Rapid. Commun. Mass. Spectrom.** 19 (2005) 1176-1182.
11. Foster, P.A., Ho, Y.T., Newman, S.P., Kasprzyk, M.P., Leese, M.P., Potter, B.V., Reed, M.J. and Purohit, A. 2-MeOE2bisMATE and 2-EtE2bisMATE induce cell cycle arrest and apoptosis in breast cancer xenografts as shown by a novel ex vivo technique. **Breast. Cancer. Res. Treat.** 111 (2008) 251-260.
12. Reed, M.J., Purohit, A., Woo, L.W.L., Newman, S.P. and Potter, B.V.L. Steroid Sulfatase: Molecular Biology, regulation, and inhibition. **Endocr. Rev.** 26(2) (2005) 171-202.
13. Kirches, E. and Wraich-Kirches, M. 2-Methoxyestradiol as a potential cytostatic drug in gliomas? **Anticancer. Agents. Med. Chem.** 9(1) (2009) 55-65.
14. Utsumi, T., Leese, M.P., Chander, S.K., Gaukroger, K., Purohit, A., Newman, S.P., Potter B.V. and Reed, M.J. The effects of 2-methoxyoestrogen sulphamates on the in vitro and in vivo proliferation of breast cancer cells. **J. Steroid. Biochem. Mol. Biol.** 94 (2005) 219-227.
15. Tagg, S.L., Foster, P.A., Leese, M.P., Potter, B.V., Reed, M.J., Purohit, A. and Newman, S.P. 2-methoxyoestradiol-3,27-O,O-bis-sulphamate and 2-deoxy-D-glucose in combination: a potential treatment for breast and prostate cancer. **Br. J. Cancer.** 99(11) (2008) 1842-1848.
16. Ireson, C.R., Chander, S.K., Purohit, A., Perera, S., Newman, S.P., Parish, D., Leese, M.P., Smith, A.C., Potter, B.V. and Reed, M.J. Pharmacokinetics and efficacy of 2-methoxyoestradiol and 2-methoxyoestradiol-bis-sulphamate in vivo in rodents. **Br. J. Cancer.** 90 (2004) 932-937.
17. Suzuki, R.N., Newman, S.P., Purohit, A., Leese, M.P., Potter, B.V. and Reed, M.J. Growth inhibition of multi-drug-resistant breast cancer cells by 2-methoxyoestradiol-bis-sulphamate and 2-ethyloestradiol-bis-sulphamate. **J. Steroid. Biochem. Mol. Biol.** 84 (2003) 269-278.
18. Foster, P.A., Newman, S.P., Leese, M.P., Bernetiere, S., Diolez, C., Camara, J., Hacher, B., Baronnet, M.M., Ali, T., Potter, B.V., Reed, M.J. and Purohit, A. A new micronized formulation of 2-methoxyestradiol-bis-sulphamate (STX140) is therapeutically potent against breast cancer. **Anticancer. Res.** 28 (2008) 577-581.
19. Wood, L., Leese, M.P., Mouzakiti, A., Purohit, A., Potter, B.V., Reed, M.J. and Packham, G. 2-MeOE2bisMATE induces caspase-dependent apoptosis in CAL51 breast cancer cells and overcomes resistance to TRAIL via cooperative activation of caspases. **Apoptosis.** 9 (2004) 323-332.
20. Newman, S.P., Leese, M.P., Purohit, A., James, D.R.C., Rennie, C.E., Potter, B.V. and Reed, M.J. Inhibition of in vitro angiogenesis by 2-Methoxy- and 2-ethyl-estrogen sulphamates**. Int J Cancer** 109 **(**2004) 533-540.
21. Joubert, A.M., Marais, S. and Maritz, C. Influence of 2-methoxyestradiol on MCF-7 cells: An improved differential interference contrasting technique and Bcl-2 and Bax protein expression levels. **Biocell.** 33(1) **(**2009) 67-70.
22. Gillies, R.J., Didier, N. and Denton, M. Determination of cell number in monolayer cultures. **Anal. Biochem.** 159 **(**1986) 109–113.
23. Grever, M.R., Schepartz, S.A. and Chabner, B.A. The National Cancer Institute: cancer drug discovery and development program. **Semin. Oncol.** 19 **(**1992) 622-638.

24. Kusuzaki, K., Murata, H., Takeshita, H., Hashiguchi, S., Nozaki, T., Emoto, K., Ashihara, T. and Hirasawa, Y. Intracellular binding sites of acridine orange in living osteosarcoma cells. **Anticancer. Res**. 20(2A) (2000) 971-975.
25. Elmore, S. Apoptosis: a Review of programmed cell death. **Toxicol. Pathol.** 35 (2007) 495-516.
26. Xia, GH., Chen, B.A., Shao, Z.Y., Lu, H.X., Konstanze, D. and Hartmut, D. Mechanism of 2-methoxyestradiol-induced apoptosis in myelodysplastic syndrome MUTZ-1 cell line. **Zhongguo. Shi. Yan. Xue. Ye. Xue. Za. Zhi.** 15(2) (2007) 296-301.
27. Kachadourian, R., Liochev, S.I., Cabelli, D.E., Patel, M.N., Fridovich, I. and Day, B.J. 2-Methoxyestradiol does not inhibit superoxide dismutase. **Arch. Biochem. Biophys.** 392(2) (2001) 349-353.
28. Fu, J., Weise, A.M., Falany, J.L., Falany, C.N., Thibodeau, B.J., Miller, F.R., Korarek, T.A. and Runge-Morris, M. Expression of estrogenicity genes in a lineage cell culture model of human breast cancer progression. **Breast. Cancer. Res. Treat** 120 (2010) 35-40.
29. Stanway, S.J., Delavault, P., Purohit, A., Woo, L.W., Thurieau, C., Potter, B.V. and Reed, M.J. Steroid sulfatase: a new target for the endocrine therapy of breast cancer. **Oncologist** 12 (2007) 370-374.
30. Yoshimura, N., Harada, N., Bukholm, I., Karesen, R., Borresen-Dale, A. and Kristensen, V.N. Intratumoural mRNA expression of genes from the oestradiol metabolic pathway and clinical and histopathological parameters of breast cancer. **Breast. Cancer. Res**. 6(2) (2004) R46-R55.