Biomedical Research 2012; 23 (3): 357-362 ISSN 0970-938X
Scientific Publishers of India

The *in vitro* effects of a sulphamoylated derivative of 2-methoxyestradiol on cell number, morphology and alpha-tubulin disruption in cervical adenocarcinoma (HeLa) cells.

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Abstract

2-Methoxyestradiol (2ME2) is an endogenous metabolite of estrogen that has both anti-angiogenic and antitumor effects. However, the shortcoming with 2ME2 is that it is rapidly inactivated by 17β-hydroxysteroid dehydrogenase type 2. Thus a bis-sulphamoylated derivative of 2ME2, 2-Methoxyestradiol-bis-sulphamate (2MEBM) was synthesized in order to address the shortcoming of 2ME2. The aim of this *in vitro* study was to investigate the influence of 2MEBM on cell growth, morphology and tubulin structure in a cervical cancer (HeLa) cell line. Dose-dependent studies revealed that 0.55µM of 2MEBM inhibited cell growth by 50%. 2MEBM-treated cells showed an increase in the number of metaphase cells, apoptotic cells, and disrupted tubulin structure after 48 hours of exposure to 0.55µM of 2MEBM. Future studies will be conducted to further investigate the mechanism of action of 2MEBM in cervical carcinoma cells.

Keywords: Cancer, 2-methoxyestradiol-bis-sulphamate, mitotic block, apoptosis

Accepted March 29 2012

Introduction

2-Methoxyestradiol (2ME2) is a naturally occurring estradiol metabolite which is commercially registered as Panzem® by Entremed, Inc. (Rockville, MD) [1, 2]. 2ME2 exerts antiproliferative activity both *in vitro* and *in vivo* [1, 2, 3, 4, 5]. The antiproliferative effects of 2ME2 were tested on various cancer and non-cancer cell lines such as the human breast adenocarcinoma ER+ cell line (MCF-7), non-tumorigenic breast cell line (MCF-12A), human cervical adenocarcinoma (HeLa), human breast adenocarcinoma ER- cell line (MDA-MB-231), human umbilical vein endothelial cells (HUVEC), etc. [1, 2, 6, 7]. In addition to its antiproliferative effects, 2ME2 inhibits angiogenesis via the hypoxia inducible factor-alpha (HIF-α), which plays an important role in the secretion of a potent angiogenic agent called vascular endothelial growth factor (VEGF). 2ME2 can directly inhibit the secretion of VEGF in a dose-dependent manner [8]. Clinical trials have been conducted to evaluate the efficacy of 2ME2 on breast, prostate, and melanoma cancers, however, high oral doses of 2ME2 were required per day to significantly reduce tumour growth [9, 10, 11, 12]. 2ME2 is rapidly inactivated by 17β-hydroxysteroid dehydrogenase type 2, expressed in both the liver and gastrointestinal tract. This leads to a low oral bioavailability of 2 ME2 [13]. A new nanocrystal dispersion formulation of 2ME2 has been developed in an attempt to address the problem of low bioavailability; it has been shown that a nanoparticulate dispersion of 2ME2 leads to higher and more consistent plasma levels of 2ME2, suggesting that therapeutic levels in humans could be achieved using this drug delivery approach, however, the new formulation is still in the test phase [8, 14].

Analogues of 2ME2 are currently being developed, synthesised, and evaluated in an attempt to create compounds with improved anticancer potency, oral bioavailability, and inhibition of tubulin polymerization [9, 14, 15]. 2-Methoxyestradiol-bis-sulphamate (2MEBM) is a sulphamoylated derivative of 2ME2 that is currently being researched as a potential anticancer drug. 2MEBM is also known as STX-140 and it is not commercially available [16]. *In vitro* and *in vivo* studies have found that 2MEBM exerts antiproliferative, as well as anti-angiogenic activity [15, 17, 18, 19, 20, 21, 22].

Suzuki *et al*. (2003) treated doxorubicin as well as mitoxantrone resistant MCF-7 cells with 2MEBM in plastic, collagen I, and Matrigel substrates respectively, and cell proliferation was inhibited in both cell lines [20]. 2MEBM was more potent and had a higher oral bioavail-

ability when compared to 2ME2. Newman *et al*. (2004) examined the ability of 2ME2 and its sulphamyolated derivative to inhibit HUVEC proliferation over a 4-day period. The IC_{50} of 2MEBM was found to be 0.05µM while that of 2ME2 was 3µM; therefore, 2MEBM was 60 fold more potent at inhibiting the proliferation of human umbilical vein endothelial cells (HUVEC) compared to 2ME2 [23]. It has been postulated that the sulphamoylated derivatives of 2ME2 are not easily metabolised because they are sequestered into red blood cells (RBCs), which enables them to transit the liver without undergoing first pass metabolism. They bind reversibly to carbonic anhydrase II within RBCs from where they can be slowly released into the blood stream [19, 21].

2MEBM is thought to act like its parental molecule by binding to the colchicine binding site of tubulin and disrupt microtubule dynamics which in turn leads to inhibition of cell cycle progression and the induction of apoptosis [9, 24, 25]. 2MEBM induced the intrinsic mitochondrial pathway in MDA-MB-231 cell line. A concentration of 0.5µM of 2MEBM caused inner mitochondrial membrane potential depolarization in HUVEC and in MDA-MB-231 cell lines. This is associated with a significant increase in apoptotic cells. Cytochrome *c*, a protein located within the inner mitochondrial membrane, was also dissociated from the inner membrane after 48 hours of treatment with 2MEBM, indicating a collapse of the inner mitochondrial membrane potential which was then followed by apoptosis [9]. 2MEBM also induced the phosphorylation of B-Cell lymphoma-2 (Bcl-2), an anti-apoptotic protein, which resulted in apoptosis. Breast cancer cells (CAL51) were treated with 2MEBM and this induced the activation of caspases 3 and 9, but not caspase 8 [26].

Previous studies on HeLa cells that were exposed to 2ME2 demonstrated that 1µM of 2ME2 causes an increase in Bcl-2-associated X protein (Bax) expression levels; in contrast, no statistically significant effects were observed on Bcl-2 expression levels in cervical carcinoma cells. Bax can be regarded as a pro-apoptotic protein, whereas Bcl-2 is perceived as an anti-apoptotic protein. It has been proposed that an increased ratio of pro-apoptotic Bax to anti-apoptotic Bcl-2 can be associated with apoptosis, thus this study indicated the induction of apoptosis in HeLa cells [6]. However, there have been no previous studies on the effects of 2MEBM on cervical adenocarcinoma cells. Therefore, the aim of this study was to test the *in vitro* effects of 2MEBM on cervical adenocarcinoma cells since it was shown in previous studies that 2MEBM is more potent than the parental molecule [15]. The effects of 2MEBM on cell number, morphology and alpha-tubulin disruption were studied.

Materials and Methods

Cell lines. Human epithelial cervical cell line (HeLa) was purchased through Sterilab Services (Johannesburg, South Africa) from American Tissue Culture Collection (ATCC) (Maryland, United States of America).

Materials.

Penicillin, streptomycin, fungizone and trypsin were obtained from Highveld Biological (Pty) Ltd. (Sandringham, South Africa). Dimethyl sulphoxide (DMSO) was supplied by Sigma-Aldrich Co. (St Louis, United States of America). Haematoxylin, eosin, ethanol and xylol fixative were purchased from Merck (Darmstadt, Germany). Alpha-tubulin, alexafluor 488, and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Biocom biotech (Centurion, Pretoria, South Africa). Dulbecco's Modified Eagle's Medium with glucose, sodium pyruvate and L-glutamine (DMEM), fetal calf serum (FCS), sterile cell culture flasks and plates were obtained from Separations (Randburg, Johannesburg, South Africa). 2MEBM was synthesized by Prof. Vleggaar from the Department of Chemistry (University of Pretoria, Pretoria, South Africa).

Cell culture. Cells were propagated as monolayers in sterile 25cm^2 tissue culture flasks containing DMEM at 37°C in a humidified atmosphere containing 5% CO_2. A stock solution of 2MEBM ($2.0x10^{-3}$M) was prepared in DMSO and stored at -20°C. All experiments included control samples in the form of cells propagated in growth medium, cells treated with the same volume of DMSO used to expose cells with 2MEBM as well as positive controls for apoptosis (0.1µg/ml actinomycin D) and autophagy (cells starved by culturing in 33.3% medium and 66.7% phosphate buffered saline (PBS)). Dose-dependent studies (0.2-1µM) were conducted with analysis of the effects of 2MEBM on cell growth in HeLa cells after 48 hours of exposure. This concentration range was chosen because previous studies have shown that 2MEBM is most efficient as an antiproliferative agent within this range [15, 21, 27].

Determination of cell numbers (Crystal violet staining).

Crystal violet staining was used to study the effects of 2-MeOE2bisMATE on cell numbers. Crystal violet is a DNA stain which allows for fast and reliable quantification of cell numbers, of cells grown in a monolayer culture [28, 29]. Exponentially growing HeLa cells were seeded in 96-well tissue culture plates at a cell density of 5 000 cells per well. Cells were incubated at 37°C for a period of 24 hours to allow for attachment. The medium was discarded and cells were exposed to a dilution series ranging from 0.2-1µM of 2MEBM including controls. After 48 hours of exposure, cells were fixed with 100µl of 1% glutaraldehyde in water for 15 minutes. Crystal violet (100µl) (1%, in water) was added for 30 minutes, after which the culture wells were immersed in running tap water for 15 minutes. The plates were left overnight to dry. Subsequently, 200µl of Triton X-100 (0.2% in water) was added to each well and incubated for 30 minutes.

Following incubation, 100µl of the liquid content was transferred to a new 96-well plate. The absorbance of the samples was analysed using an ELX800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Analytical Diagnostic Products, Weltevreden, SA).

Polarization-optical differential interference contrast (PlasDIC).

PlasDIC was used to observe morphological characteristics (including types of cell death) of HeLa cells after they were exposed to 2MEBM. PlasDIC is an improved differential interference contrast method which provides high-quality imaging of cells grown in plastic cell culture containers [30]. Cells were seeded at 500 000 cells per well in a 6-well plates. After a 24 hour incubation period at 37°C to allow for cell adherence, cells were exposed to 0.55µM of 2MEBM for a period of 48 hours. A concentration of 0.55µM of 2MEBM was selected as focus dosage, since this concentration demonstrated pronounced cell growth inhibition. Appropriate controls were included. PlasDIC images were obtained using the Zeiss Axiovert-40 microscope (Zeiss, Göttingen, Germany).

Light microscopy (Haematoxylin & eosin staining).

Light microscopy was used to reveal morphological changes of the nuclear and cytoplasmic components of HeLa cells that may be affected by 2MEBM. Haematoxylin is a dye that stains the nucleic acids of the cells a blue-purple colour. Eosin stains the cytoplasm of the cells a red-pink colour [31, 32]. Cells (250 000) per well were seeded on heat-sterilized coverslips in 6 well plates and incubated overnight. After 24 hours of attachment, cells were exposed to 0.55µM of 2MEBM for a period of 48 hours. Cells were fixed in Bouin's fixative for 30 minutes. Subsequently, the fixative was discarded and 70% ethanol was added to the coverslips (20 minutes), rinsed in tap water and then left for 20 minutes in Mayer's Hemalum. After rinsing with water for 2 minutes, coverslips were washed with 70% ethanol before being subjected to 1% eosin for 2 minutes. This was followed by rinsing twice for 5 minutes with 70% ethanol, 96% ethanol, 100% ethanol and xylol respectively. Coverslips were then mounted with resin and left to dry before they were evaluated with a Zeiss Axiovert MRs microscope (Zeiss, Göttingen, Germany).

Confocal microscopy- alpha-tubulin assay.

Microtubules form part of the eukaryotic cytoskeleton and are composed of alpha and beta tubulin heterodimers [33, 34]. Confocal microscopy was conducted to observe the effects of 2MEBM on the alpha-tubulin structure of HeLa cells. Exponentially growing HeLa cells were seeded at 250 000 cells per well in 6-well plates on coverslips. After a 24 hour incubation period at 37°C, cells were exposed to 0.55µM of 2MEBM for 48 hours. Cells were fixed in pre-warmed glutaraldehyde fixer for 10 minutes and then permeabilized with a permeabilization buffer for 15 minutes at room temperature. After 15 minutes of incubation, the coverslips were washed in cytoskeletal buffer for 3 minutes. This was followed by rinsing twice for 5 minutes with a wash buffer. Coverslips were incubated for 1.5 hours with each of the following: primary antibody cocktail (Imgenex anti-tubulin alpha), secondary antibody cocktail (alexafluor 488 anti-mouse) and then with nuclear stain DAPI for 30 minutes. Following three 5 minute washes with the wash buffer, the coverslips were mounted in mounting fluid and left to dry before they were evaluated with a Zeiss, LSM 510 Meta confocal microscope at the Electron Microscopy unit at the University of Pretoria (Pretoria, South Africa).

Statistical analysis of data.

PlasDIC, haematoxylin and eosin staining procedure and confocal microscopy provided qualitative data. Quantitative data was supplied by means of cell number determination (crystal violet staining). The ANOVA students'*t*-test was used to determine the analytical variation in experimental procedures and biological variations within each experiment. Cell growth studies were repeated three times with a sample size of 6 in each experiment. A *P*-value of <0.05 was regarded as statistically significant. Means are presented in bar charts, with T-bars referring to standard deviations.

Results

Determination of cell numbers (Crystal violet staining).

The antiproliferative effects of 2MEBM (0.2-1µM) at 48 hours in HeLa cells were evaluated and compared to the controls using the crystal violet DNA staining assay. Control cells as well as cells which were treated with 0.2µM of 2MEBM did not show a significant decline in cell number (Fig. 1). The dosage of 2MEBM which significantly inhibits 50% of HeLa cell proliferation (IC_{50}) was found to be 0.55µM after 48 hours of exposure. This concentration was chosen as the dose of exposure for subsequent experiments.

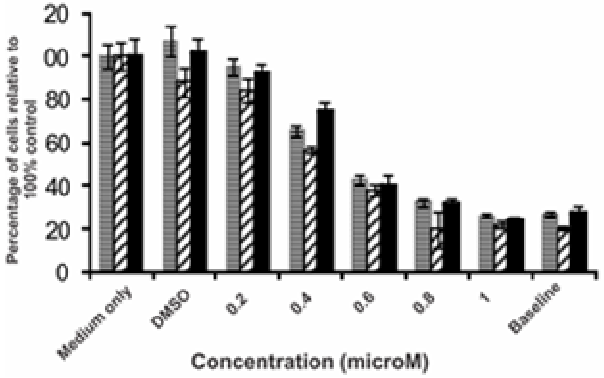

Figure 1. *HeLa cell numbers expressed as a % of cells relative to the control (cells propagated in growth medium only) after being exposed to 2MEBM for 48 hours. Exposure to 0.55µM 2MEBM resulted in pronounced growth inhibition in the HeLa cell line.*

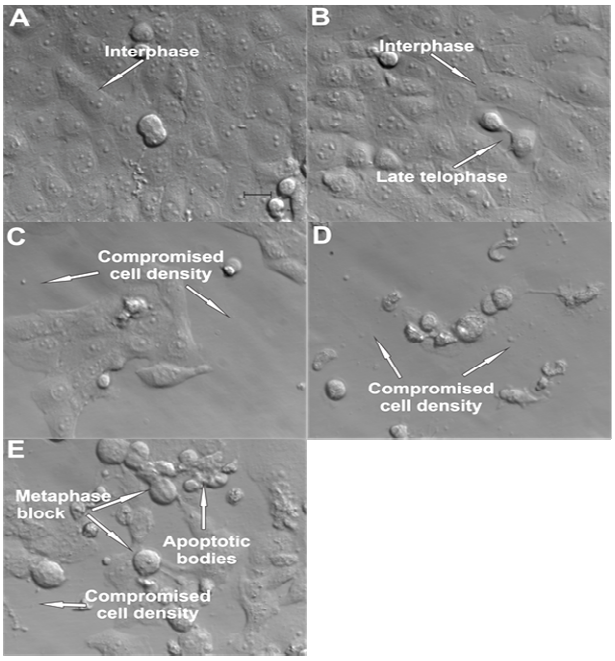

Figure 2. *The morphological effects of HeLa cells A) propagated in growth medium, B) vehicle-control, C) starved, D) actinomycin D and E) 0.55µM 2MEBM-treated were visualised using PlasDIC. In the 2MEBM-treated cells apoptotic bodies and an increase in rounded cells, indicative of a mitotic block, were observed when compared to the vehicle-control cells. (Scale bar: 10µm)*

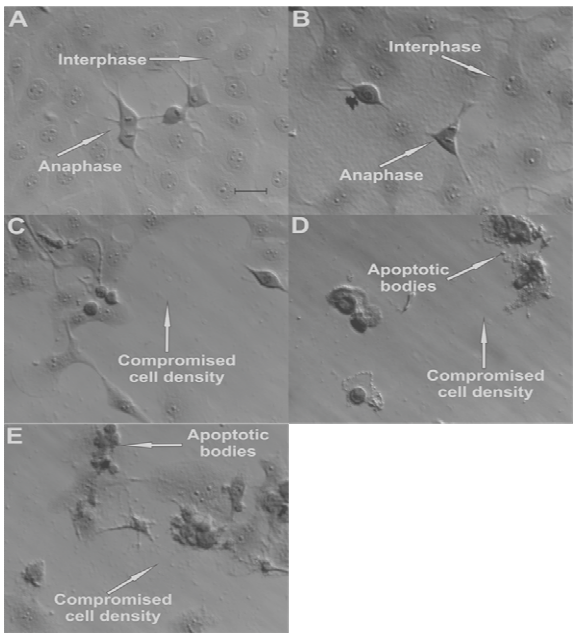

Figure 3. *Haematoxylin & eosin staining images of HeLa cells A) propagated in growth medium, B) vehicle-control, C) starved, D) actinomycin D and E) 0.55µM 2MEBM-treated after 48 hours of exposure. The 2MEBM-treated cells showed a decrease in cell density and the presence of apoptotic bodies* when compared to the vehicle-control cells. (Scale bar: 10µm)

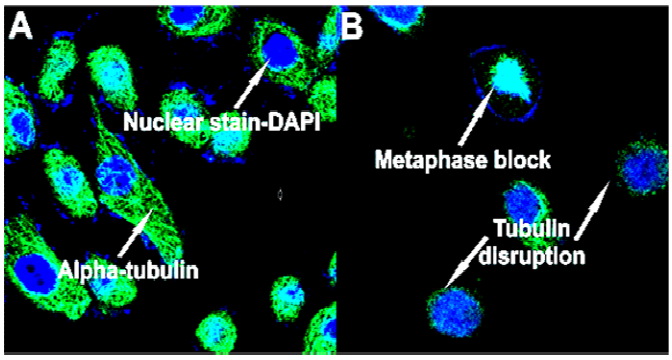

Figure 4. *Immunofluorescent staining with alpha-tubulin in A) vehicle-control and B) 0.55µM 2MEBM-treated HeLa cells. Confocal microscopy revealed the disruption of alpha-tubulin in 2MEBM-treated cells. (Scale bar: 50 pixels)*

Polarization-optical differential interference contrast (PlasDIC). PlasDIC was used to visualise the effects of 0.55µM 2MEBM on morphology of HeLa cells. Vehicle-treated and medium only control cells showed cells with normal cell division and mainly in interphase (Fig. 2A&B). Cell density of treated HeLa cells was compromised when compared to the density of the cells propagated in growth medium and those of the vehicle control (Fig. 2A, B &E). In cells treated with 0.55µM of 2MEBM apoptotic bodies as well an increase in the number of rounded cells, indicative of a mitotic block, were observed (Fig. 2E).

Light microscopy (haematoxylin and eosin staining). Haematoxylin and eosin cell staining technique was conducted in order to reveal morphological changes of nuclear and cytoplasmic components of HeLa cells after 48 hours of exposure to 0.55µM of 2MEBM (Fig. 3). Vehicle-treated and medium only control cells revealed the presence of cells in interphase and normal cell division (anaphase, metaphase & telophase). Cells treated with 2MEBM were rounded and apoptotic features such as membrane blebbing and apoptotic bodies were observed. Treated cell were shrunken when compared to the vehicle control.

Confocal microscopy- alpha-tubulin assay

Tubulin polymerization dynamics within the cell are critical for the process of mitosis and are frequently targeted by agents that induce mitotic arrest. Since the parental molecule of 2MEBM has been shown to be a tubulin poison, the alpha-tubulin detection assay was used to determine the effects of 2MEBM on tubulin activity. Cells treated with 2MEBM had disrupted alpha-tubulin and they were blocked in mitosis (round in appearance) (Fig. 4).

Discussion

In the present investigation the *in vitro* effects of a sulphamoylated derivative of 2-methoxyestradiol namely 2MEBM on HeLa adenocarcinoma cells were investigated. A dose dependent study using 0.2-1µM of 2MEBM was performed to determine the IC_{50} after 48 hours of exposure. Subsequent experiments were conducted using 0.55µM of 2MEBM and an exposure time of 48 hours. The IC_{50} was found to be 0.55µM, and this is consistent with the concentration range found in previous results [16, 23, 27, 35] Day *et al*. (2009) found an IC_{50} of 0.24µM and 1.53µM in MCF-7wt and MCF-7.MR respectively [35]. Newman *et al*. (2008 & 2004) concluded that the IC_{50} in MCF-7wt cells was 0.25µM, although further experiments were conducted using 0.5µM [16, 23]. Research conducted in our laboratory revealed that the exposure of non-tumourigenic (MCF-12A) cell population to 0.4µM of 2MEBM for a period of 48 hours yielded a yielded a 79% growth reduction [27].

In addition to the determination of 2MEBM's influence on cell numbers, PlasDIC, haematoxylin, and eosin cell staining techniques were utilized to demonstrate the effects of 2MEBM on HeLa cell morphology. Treated cells showed a decrease in cell density when compared to the vehicle control. Cells were rounded which indicated a mitotic block. Apoptotic features such as apoptotic bodies, membrane blebbing, and shrunken cells were noted. These results were consistent to those of Raobaikady *et al*. (2003) who found an increase in the number of rounded, shrunken MCF-7 cells, with some cells detaching from the surface [15]. Similarly, it was observed in a study done by Day *et al*. (2003) that 2MEBM had a marked effect on the morphology and growth of prostate and ovarian cells and that the cells that were treated with the sulphamoylated derivative became detached and rounded, displaying a characteristic apoptotic appearance [18].

Various studies have shown the parental molecule, 2ME2, to be a spindle poison that disrupts the polymerization of alpha-tubulin. This induced a metaphase block. Joubert *et al*. (2009) found MCF-7 cells blocked in metaphase after 24 hours of exposure to 1µM of 2ME2 [36]. Disrupting alpha-tubulin polymerization leads to abnormal spindle formation and mitotic accumulation in the G_2/M-phase of cell division [37, 38]. The effects of 2MEBM on alpha-tubulin activity were investigated and the results showed clear disruption of alpha-tubulin in the treated cells. Newman *et al*. (2007) tested the effects of 10µM of 2MEBM on *in vitro* polymerisation of purified bovine brain tubulin which was measured by turbidometry. This study showed that tubulin polymerisation was significantly inhibited by 2MEBM after 5 minutes of exposure [24].

In conclusion, this study demonstrated the *in vitro* effects of 2MEBM on HeLa cells. Changes in cell density, morphology, as well as tubulin structure were observed in cells that were treated with 0.55µM of 2MEBM compared to the controls. Future studies which will include studies to determine possible induction of various types of cell death will be conducted to further elucidate the mechanism of action of 2MEBM on HeLa cells.

Acknowledgements

This research was supported by grants from the Cancer Association of South Africa (AK246), the Medical Research Council (AG374, AK076), National Research Foundation and Struwig Germishuysen Trust (AJ038).

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