

In vitro effects of 2-methoxyestradiol on cell morphology and Cdc2 Kinase activity in SNO oesophageal carcinoma cells

Annie Joubert* and Sumari Marais

Department of Physiology, University of Pretoria, Pretoria, South Africa

The effects of 1×10^{-6} M exogenous 2-methoxyestradiol (2 ME) were determined on cell morphology and cell division cycle (Cdc) 2 kinase activity in SNO oesophageal carcinoma cells. Mitotic indices revealed an increase in metaphase cells (11.2%) when compared to the 0.5% vehicle-treated cells after 18 h of exposure to 2 ME. Vehicle-treated control cells did not show any hallmarks of apoptosis after 18 h of exposure to dimethyl sulphoxide. Only 0.5% of 2 ME-treated cells showed characteristics of apoptosis. Conversely, increased morphological hallmarks of apoptosis were observed in SNO-treated cells after 21.5 h of 2 ME exposure. When compared to the 0.5% in vehicle-treated cells, 4.7% of cells were in apoptosis. Furthermore, 34.1% of cells were blocked in metaphase after 21.5 h of 2 ME exposure compared to 0.6% of vehicle-control cells. In addition, Cdc2 kinase activity was statistically significantly increased (1.3-fold) ($p < 0.005$) in 2 ME-treated cells when compared to vehicle-treated controls. The present preliminary study suggests that the accumulation observed in metaphase cells and the increase in Cdc2 kinase activity caused by 2 ME are consistent with morphological hallmarks of mitotic arrest and disrupted mitotic spindle formation, thus leading to induction of apoptosis in SNO cells.

INTRODUCTION

2-Methoxyestradiol (2 ME) is a 17-beta estradiol metabolite regarded as an anti-mitotic drug and tubulin poison that inhibits growth and induces apoptosis in a large variety of tumour and non-tumour cell lines.¹⁻³ 2 ME exerts both anti-angiogenic and anti-tumour effects regardless of the cell's hormone receptor status and is responsible for mitotic accumulation and abnormal mitotic spindle formation in both estrogen receptor (ER) positive and ER negative cells.⁴⁻⁶ As a consequence, this endogenous estradiol metabolite has emerged as a promising anti-cancer agent.⁵

Recent evidence has implicated 2 ME in the activation of c-Jun NH2-terminal kinase signalling, generation of reactive oxygen species and induction of apoptosis via both the extrinsic and intrinsic pathways. JNK is involved in the phosphorylation and inactivation of the pro-apoptotic protein Bcl-2, thus contributing to the induction of apoptosis.^{7,8} It has been demonstrated that 2 ME is responsible for the upregulation of death receptor 5 expression *in vitro* and *in vivo*, thereby activating the extrinsic pathway of apoptosis. However, the contribution of the extrinsic and intrinsic pathways of apoptosis induction appears to vary depending on the cell type.⁴

Research has shown that 2 ME exhibits a biphasic pattern on cell proliferation at concentrations ranging from 10^{-8} to 10^{-5} M, namely a stimulatory effect at low concentrations and an inhibitory effect at the highest concentration.² Dose-dependent studies conducted in our laboratory have demonstrated that a concentration of 10^{-6} M 2 ME reduced cell numbers by 40% and 60% in SNO and WHCO3 oesophageal carcinoma cells, respectively.⁹ Light and electron microscopy demonstrated cell shrinkage, membrane blebbing, condensed chromatin, nuclear fragmentation and micro-nuclei formation after 24 h of WHCO3 and SNO cells exposure to 10^{-6} M 2 ME. Flow cytometric analysis revealed that 10^{-6} M 2 ME was also responsible for spindle disruption and a G₂/M arrest of these cells thereby preventing them from proceeding through the cell cycle.⁹

Initial preclinical data demonstrated that 2 ME might be used to treat multiple myeloma, sarcoma and other solid tumours thus rendering it as a potential anti-cancer agent when compared to conventional

chemotherapeutic treatments.^{4,10,11} However, the exact action mechanism of 2 ME is complex, still not clearly defined and appears to vary according to cell type.^{4,12} The aim of this preliminary study was to investigate the mechanism of action of 2 ME in an oesophageal carcinoma cell line, by determining the influence of 2 ME on cytoplasmic and nuclear morphology, as well as on cell division cycle (Cdc) 2 kinase activity as a cell cycle regulatory component.

MATERIALS AND METHODS

Materials

2 ME, minimum essential medium eagle with Earle's salts, L-glutamine and NaHCO₃ (MEM), Trypsin-EDTA and Trypan blue were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Heat-inactivated foetal calf serum (FCS), sterile cell culture flasks and plates were obtained through Sterilab Services (Kempton Park, Johannesburg, South Africa). Phosphate buffered saline (PBS) was purchased from GIBCO BRL (USA). Penicillin, streptomycin and fungizone were obtained from Highveld Biological (Sandringham, SA). Haematoxylin, eosin, ethanol, xylol and Entellan® fixative were purchased from Merck (Darmstadt, Germany). The MESACUP Cdc2 Kinase Assay Kit and HCK-gel suspension were purchased from MBL Medical & Biological Laboratories (Nagoya, Japan). Cdc2 reaction buffer, biotinylated MV peptide, peroxidase (POD) conjugated streptavidin, wash concentrate (10x PBS), substrate A (o-phenylenediamine), substrate B (H₂O₂), stop solution (20% H₃PO₄) and phosphorylation stop reagent (PBS containing 50 mM EDTA) were supplied with the kit. ATP, Tris-HCl, EDTA, EGTA, Brij35, PMSF, beta-glycerophosphate, Na₃VO₄, 2-mercaptoethanol and the protease inhibitor cocktail were supplied by Sigma-Aldrich (St. Louis, USA). NaCl was purchased from Associated Chemical Enterprises (Southdale, South Africa), MgCl₂ from Merck (Darmstadt, Germany) and HEPES from BDH Laboratory Supplies (Poole, England). All other chemicals were of analytical grade and supplied by Sigma Chemical Co. (St. Louis, MO, USA). The SNO oesophageal carcinoma cell line was purchased from Highveld Biological (Sandringham, SA). SNO cells are described as a non-keratinizing squamous epithelium cell line.

Cell culture

Cells were grown as monolayers in MEM at 37°C in a humidified atmosphere containing 5% CO₂. Media were supplemented with 10% heat-inactivated FCS, penicillin (100µg/L), streptomycin (100 µg/L) and fungizone (250 µg/L). Non-viable cells were excluded with the Trypan blue staining procedure. Stock solutions of 2 ME were prepared in DMSO at concentrations of 2 x 10⁻³ M and stored at room temperature. The DMSO content of the final dilutions never exceeded 0.1% (v/v). Control experiments showed that 0.1% DMSO had no toxic effects on the SNO cells.

Light microscopy

In an attempt to obtain more insight into the anti-proliferative effect of 2 ME observed in cell growth studies previously conducted in our laboratory,⁹ haematoxylin and eosin cell staining was used to determine the morphological characteristics of cytoplasm and nuclear components of SNO cells after exposure to 10⁻⁶ M 2 ME. The latter concentration was chosen, since it has been revealed that 2 ME displays a biphasic pattern on cell proliferation at concentrations ranging from 10⁻⁸ to 10⁻⁵M, namely a stimulatory effect at low concentrations and an inhibitory effect at the highest concentration. 2 ME caused the strongest inhibitory effect at a concentration of 10⁻⁶ M.² Exponentially growing SNO cells were seeded at 3 x 10⁵ cells per well in 6-well plates on heat-sterilized coverslips. After a 24 h incubation period at 37°C to allow for cell adherence, cells were exposed to 1 x 10⁻⁶M 2 ME and incubated for 18 and 21.5 h, respectively at 37°C. Exposure times of 18 and 21.5 h were chosen to monitor the increase in metaphase cells, since previous results in our laboratory had revealed that peak occurrence of apoptosis occurs after 24 h of exposure to 2 ME in SNO cells.⁹ Cells were fixed in Bouin's fixative for 60 min after exposure to 2 ME and stained by standard haematoxylin and eosin staining procedures. To investigate 2 ME's influence on cell proliferation, mitotic indices of vehicle-treated cells, as well as 2 ME-exposed cells were also determined on the stained slides by counting 1000 cells on every coverslip.

Cell cycle checkpoint regulatory molecule—Cdc2 kinase assay

SNO cells were grown in 75 cm² flasks until 80% confluency and exposed to 1 x 10⁻⁶M 2 ME for 21.5 h. Since the activation of Cdc2 kinase is vital for the initiation of mitosis and since 2 ME caused a prominent block in metaphase after 21.5 h of exposure, the influence of 2 ME was determined on SNO Cdc2 kinase activity after 21.5 h of exposure. Subsequently, cells were harvested and washed (three times) with PBS. Cells (2 x 10⁷) were suspended in 1000 µl sample buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM NaCl, 5mM EDTA, 2mM EGTA, 0.01% Brij35, 1 mM phenyl methyl sulfonyl fluoride, 25 mM beta-glycerophosphate, 1 mM Na₃VO₄, 50 mM mer-captoethanol, 0.08% protease inhibitor cocktail) and lysed by sonication on ice. Cell extracts were centrifuged for 1 h at 100 000g. The protein content was determined by the Bradford method.¹⁴ Twenty microlitres of the HCK-gel was prepared by washing twice with sample buffer before it was incubated with 300 µl of cell extract for 1 h on ice. After incubation, the HCK-gel was washed three times in sample buffer and twice with 2nd washing buffer (25 mM HEPES buffer, pH 7.5 and 10 mM MgCl₂), followed by centrifugation for 5 min at 1500g. The phosphorylation reaction was assembled on ice, by adding 5 µl of the gel sediment, 5 µl 10 x Cdc2 reaction buffer, 5 µl biotinylated MV peptide and 30 µl distilled water. The reaction was initiated by adding 5 µl, 1 mM ATP and incubated for 30 min at 30°C. Subsequently the reaction was terminated by the addition of 200 µl phosphorylation stop solution and the reaction mixture was centrifuged for 20 s at 11 000g. A 100 µl sample of reaction mixture was transferred to the microwell strips coated with monoclonal antibody 4A4, provided with the kit. After 1 h incubation at 25°C, the wells were washed five times with wash solution and 100 µl POD conjugated streptavidin was added to each well. After 30 min incubation at 25°C, the wells were washed five times with wash solution and 100 µl POD substrate solution was added to each well. The microwell strips were incubated for 5 min at 25°C before 100 µl stop solution was added to each well and the absorbance read at 450 nm in an ELx800 Universal Microplate Reader (Bio-Tek Instruments Inc., Analytical Diagnostic Products, Weltevreden, South Africa).

Statistics

Data were obtained from independent experiments. Results obtained are shown as the mean ± SD and were statistically analyzed 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 indicated by an * or number as indicated in the legends.

RESULTS

Light microscopy

The effects of 1 x 10⁻⁶M 2 ME were determined on cell morphology in SNO oesophageal carcinoma cells and compared to vehicle-treated controls after 18 h (Figure 1A,B) and 21.5 h (Figure 2A,B) of exposure, respectively. As already mentioned, exposure times of 18 and 21.5 h were chosen to monitor the increase in metaphase cells, since previous results in our laboratory had revealed that peak occurrence of apoptosis occurs after 24 h of exposure to 2 ME in SNO cells.⁹

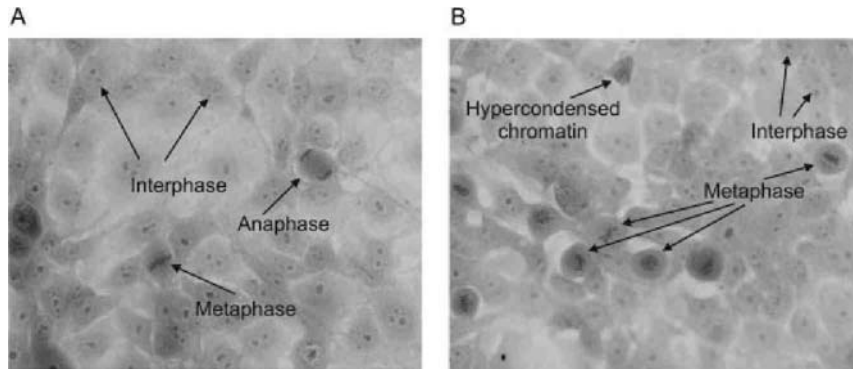


Figure 1. Haematoxylin and Eosin staining of SNO cells after 18h of exposure to vehicle (A) and 1×10^{-6} M 2 ME (B), respectively (400x magnification). Control SNO cells showed normal cell division including a cell in metaphase and anaphase. Interphase cells were also observed (A). Treated cells showed an increase in metaphase cells and cells with hypercondensed chromatin (B). Interphase cells were also observed (B)

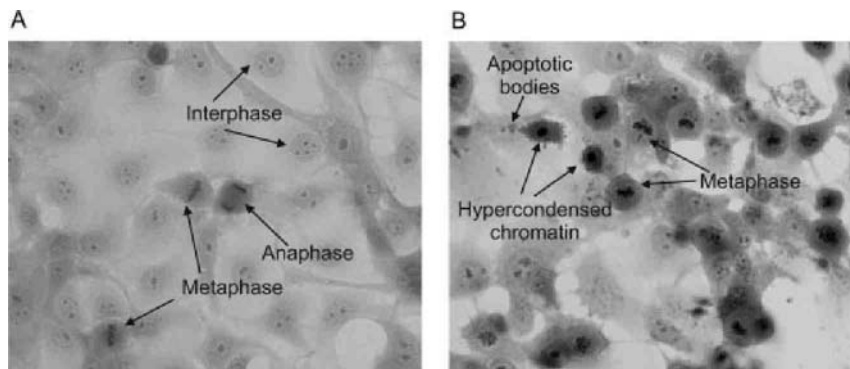


Figure 2. Haematoxylin and Eosin staining of SNO cells after 21.5 h of exposure to vehicle (A) and 1×10^{-6} M 2 ME (B), respectively (400x magnification). Control SNO cells showed interphase cells and normal cell division including a cell in metaphase and anaphase (A). Treated cells showed an increase in metaphase cells, cells with hypercondensed chromatin and apoptotic bodies (B)

The SNO control showed normal cell division including a cell in metaphase and anaphase after 18 h of exposure to DMSO. Interphase cells were also observed (Figure 1A). Treated cells showed an increase in metaphase cells and revealed hypercondensed chromatin after 18 h of exposure (Figure 1B). Interphase cells were also observed (Figure 1B). The SNO control revealed interphase cells and normal cell division including a cell in metaphase and anaphase after 21.5 h of exposure to DMSO (Figure 2A). Treated cells showed an increase in metaphase cells, cells with hypercondensed chromatin and apoptotic bodies after 21.5 h of exposure (Figure 2B). Mitotic indices demonstrated an increase in metaphase cells (11.2%) when compared to the 0.5% vehicle-treated cells after 18 h of exposure to 2 ME (Figure 3). Vehicle-treated control cells did not show any hallmarks of apoptosis after 18 h of exposure to dimethyl sulphoxide. Only 0.5% of 2 ME-treated cells showed characteristics of apoptosis (Figure 3). Conversely, increased morphological hallmarks of apoptosis were observed in SNO-treated cells after 21.5 h of 2 ME exposure. Mitotic indices demonstrated an increase in apoptotic cells (4.7%) when compared to the 0.5% vehicle-treated cells after 21.5 h of exposure to 2 ME (Figure 4). Furthermore, 34.1% of cells were blocked in metaphase after 21.5 h of 2 ME exposure compared to 0.6% of vehicle-control cells (Figure 4).

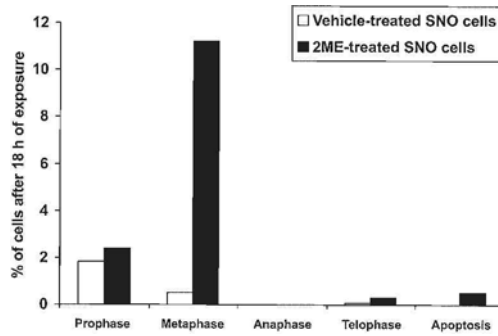


Figure 3. Mitotic indices of SNO cells after 18 h of exposure to vehicle and 2 ME, respectively, expressed as a percentage of a thousand cells counted. An increase in metaphase cells (11.2%) was observed when compared to the 0.5% vehicle-treated cells. SNO control cells did not show any hallmarks of apoptosis. Only 0.5% of 2 ME-treated cells showed characteristics of apoptosis

Cell cycle checkpoint regulatory molecule—Cdc2 kinase assay

Since activation of Cdc2 kinase is fundamental for the initiation of mitosis and since 2 ME caused a prominent block in metaphase after 21.5 h of exposure, the influence of 1×10^{-6} M 2 ME was determined on SNO Cdc2 kinase activity after 21.5 h of exposure and compared to vehicle-treated controls. Cdc2 kinase activity was statistically significantly increased (1.3-fold) ($p < 0.005$) in 2 ME-treated cells when compared to vehicle-treated controls (Figure 5).

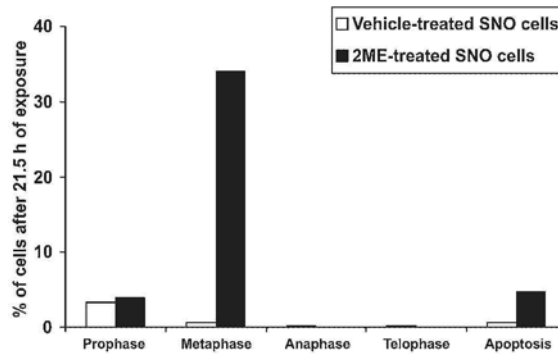


Figure 4. Mitotic indices of SNO cells after 21.5h of exposure to vehicle and 2 ME, respectively, expressed as a percentage of a thousand cells counted. SNO-treated cells showed an increase in apoptotic cells (4.7%) when compared to the 0.5% vehicle-treated cells. In metaphase, 34.1% of 2 ME-exposed cells were blocked compared to 0.6% of vehicle-control cells

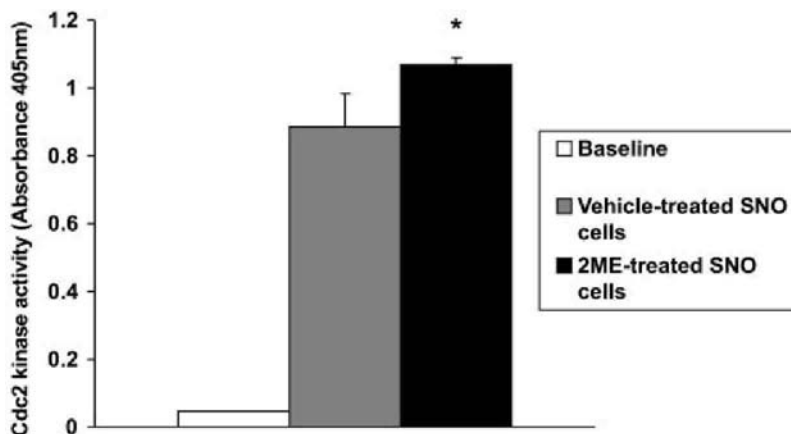


Figure 5. Cdc2 kinase activity of vehicle- and 2 ME-treated SNO cells, respectively, after 21.5 h of exposure. 2 ME caused a statistically significant increase (1.3-fold) when compared to vehicle-treated controls. The baseline represents a background reading of the Cdc2 kinase assay performed as described without cells in growth medium. p -Values < 0.05 were regarded as statistically significant and indicated by an on the graph

DISCUSSION

Several researchers have demonstrated 2 ME's biological activities in endothelial cells and in a diverse variety of tumour and non-tumour cell types.^{1,5,6,9,15,16} We have previously confirmed antiproliferative effects and morphological hallmarks of apoptosis in 2 ME-exposed HeLa, as well as WHCO3 squamous oesophageal carcinoma cells and revealed that 2 ME was responsible for an altered ratio of Bax/ Bcl-2 in favour of Bax suggesting that this could lead to the induction of apoptosis in both these cells.^{17,18} However, the exact action mechanism of 2 ME is complicated, still not clearly defined and appears to vary according to cell type.

In this pilot study, we investigated the influence of 2 ME on cytoplasmic and nuclear morphology, as well as on Cdc 2 kinase activity as a cell cycle regulatory component in the SNO oesophageal carcinoma cell line. 2 ME was responsible for an accumulation in metaphase cells and an increase in Cdc2 kinase activity, consistent with morphological hallmarks of mitotic arrest and disrupted mitotic spindle formation. Wang *et al.* stated that an accumulation of cells with condensed chromatin is characteristic of a mitotic block.⁹ Hypercondensed chromatin was also observed in the 2 ME-treated SNO cells. Similarly Attalla *et al.* revealed that 2 ME was responsible for a metaphase block in Jurkat cells.

2 ME-treated SNO cells showed a high percentage of dividing cells, but most of these cells were abnormal and blocked in metaphase. Several studies have demonstrated that mitotic arrest precedes the induction of apoptosis, thus explaining the increase in apoptotic cells concomitant with the increase in the amount of cells blocked in metaphase.^{21,22} However, Golebiewska *et al.* revealed that osteosarcoma cells exposed to 1×10^{-6} M 2 ME were arrested in G1 thereby supporting the hypothesis that the action mechanisms of 2 ME are dependent on cell type.²¹

Activation of Cdc2 is vital for the initiation of mitosis, while inactivation of Cdc2 is required for mitotic exit. Prolonged Cdc2 activity can maintain the cell in the mitotic state for an extended period until specified conditions are met for mitotic exit.²³ Consequently, the increased Cdc2 activity observed in 2 ME-treated SNO cells is consistent with the observed morphological hallmarks of mitotic arrest. Prolonged mitotic arrest may result in apoptosis. Previous research has reported an increase in Cdc2 kinase activity during apoptosis.^{24–26} Van Zijl reported an increase in Cdc2 kinase activity, as well as an increase in the amount of apoptotic cells in the tumourigenic MCF-7 cell line after exposure to 2 ME. However, in contrast to the latter finding, only a slight elevation of Cdc2 kinase activity, as well as a less pronounced increase in the amount of apoptotic cells were observed in the 2 ME-exposed non-tumourigenic MCF-12A cell line.¹⁶

Although the exact mechanism of 2 ME's action and the role of key molecules influenced in oesophageal carcinoma cells require further investigation, the present study suggests that the accumulation observed in metaphase cells and the increase in Cdc2 kinase activity caused by 2 ME are consistent with morphological hallmarks of mitotic arrest and disrupted mitotic spindle formation, thus leading to induction of apoptosis in SNO cells.

REFERENCES

1. Ghosh R, Ott AM, Seetharam D, Slaga TJ, Kumar AP. Cell cycle block and apoptosis induction in a human melanoma cell line following treatment with 2-methoxyoestradiol: therapeutic implications? *Melanoma Res* 2003; 13: 119–127.
2. Lippert C, Seeger H, Mueck AO. The effect of endogenous estradiol metabolites on the proliferation of human breast cancer cells. *Life Sci* 2003; 72: 877–883.
3. Tinley TL, Leal RM, Randall-Hlubek DA, et al. Novel 2-methoxyestradiol analogues with antitumor activity. *Cancer Res* 2003; 63: 1538–1549.
4. Mooberry SL. Mechanism of action of 2-methoxyestradiol: new developments. *Drug Resist Updat* 2003; 6: 355–361.
5. Mueck AO, Seeger H, Huober J. Chemotherapy of breast cancer-additive anticancerogenic effects by 2-methoxyestradiol? *Life Sci* 2004; 75: 1205–1210.
6. Seegers JC, Aveling ML, Van Aswegen CH, Cross M, Koch F, Joubert WS. The cytotoxic effects of estradiol-17 beta, cate-cholestradiols and methoxyestradiols on dividing MCF-7 and HeLa cells. *J Steroid Biochem* 1989; 32:

- 797–809.
7. Basu A, Castle VP, Boziane M, Bhalla K, Haldar S. Crosstalk between extrinsic and intrinsic cell death pathways in pancreatic cancer: synergistic action of estrogen metabolite and ligands of death receptor family. *Cancer Res* 2006; 66: 4309–4318.
 8. Bu S, Bluakat A, Fu X, Heldin NE, Landstrom M. Mechanisms for 2-methoxyestradiol-induced apoptosis of prostate cancer cells. *FEBS Lett* 2002; 531: 141–151.
 9. Thaver V. Mechanism(s) of apoptosis induction by 2-methoxyestradiol, an estrogen metabolite, in SNO and WHCO3 esophageal carcinoma cell lines. M.Sc. Dissertation, 2003; Department of Physiology, University of Pretoria, Pretoria, South Africa.
 10. Mooberry SL. Mechanism of action of 2-methoxyestradiol: new developments. *Drug Resist Updat* 2003; 6: 355–361.
 11. Sweeney C, Liu G, Yiannoutsos C, et al. 2-ME2 in multiple tumor types phase II multicenter, randomized, double-blind, safety trial assessing the pharmacokinetics, pharmacodynamics, and efficacy of oral 2-methoxyestradiol capsules in hormone-refractory prostate cancer. *Clin Cancer Res* 2005; 11: 6625–6633.
 12. Pribluda VS, Gubish ER, Jr, LaVallee TM, Treston A, Swartz GM, Green SJ. 2-Methoxyestradiol: an endogenous antiangiogenic and antiproliferative drug candidate. *Cancer Metastasis Rev* 2000; 19: 173–179.
 13. Seegers JC, Bohmer LH, Kruger MC, Lottering M-L, De Kock M. A comparative study of ochratoxin A-induced apoptosis in hamster kidney and HeLa cells. *Toxicol Appl Pharmacol* 1994; 129: 1–11.
 14. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 1976; 72: 248–254.
 15. Dingli D, Timm M, Russell SJ, Witzig TE, Rajkumar SV. Promising preclinical activity of 2-methoxyestradiol in multiple myeloma. *Clin Cancer Res* 2002; 8: 3948–3954.
 16. Van Zijl MC. In vitro effects of 2-methoxyestradiol, an endogenous estrogen, on MCF-12A and MCF-7 cell cycle progression. M.Sc. Dissertation, 2006; Department of Physiology, University of Pretoria, Pretoria, South Africa.
 17. Joubert A, Maritz C, Joubert F. Influence of prostaglandin A₂ and 2-methoxyestradiol on Bax and Bcl-2 expression levels in cervical carcinoma cells. *Biomed Res* 2005; 26: 87–90.
 18. Joubert A, Maritz C, Joubert F. Bax/Bcl-2-expression levels of 2-methoxyestradiol-exposed esophageal cancer cells. *Biomed Res* 2005; 26: 131–134.
 19. Wang X, Jin D-Y, Wong YC, et al. Correlation of defective mitotic checkpoint with aberrantly reduced expression of MAD2 protein in nasopharyngeal carcinoma cells. *Carcinogenesis* 2000; 21: 2293–2297.
 20. Atalla H, Ma'kela TP, Adlercreutz H, Anderson LC. 2-Methoxyestradiol arrests cells in mitosis without depolymerising tubulin. *Biochem Biophys Res Commun* 1996; 228: 467–473.
 21. Golebiewska J, Rozwadowski P, Spodnik JH, Knap N, Wakabayashi T, Wozniak M. Dual effect of 2-methoxyestradiol on cell cycle events in human osteosarcoma 143B cells. *Acta Biochim Pol* 2002; 49: 59–65.
 22. Lin H-L, Liu T-Y, Chau G-Y, Lui W-Y, Chi C-W. Comparison of 2-methoxyestradiol-induced, docetaxel-induced, and paclitaxel-induced apoptosis in hepatoma cells and its correlation with reactive oxygen species. *Cancer* 2000; 89: 983–994.
 23. Andreassen PR, Margolis RL. Microtubule dependency of p34cdc2 inactivation and mitotic exit in mammalian cells. *J Cell Biol* 1994; 127: 789–802.
 24. Burke DJ. Complexity in the spindle checkpoint. *Curr Opin Genet Dev* 2000; 10: 26–31.
 25. Canman JC, Sharma N, Straight A, Shannon KB, Fang G, Salmon ED. Anaphase onset does not require the microtubule-dependent depletion of kinetochore and centromere-binding proteins. *J Cell Sci* 2002; 115: 3787–3795.
 26. Lundberg AS, Weinberg RA. Control of cell cycle and apoptosis. *Eur J Cancer* 1999; 35: 531–539.