PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 84 (2003) 269–278

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Growth inhibition of multi-drug-resistant breast cancer cells by 2-methoxyoestradiol-bis-sulphamate and 2-ethyloestradiol-bis-sulphamate☆

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Abstract

There is currently considerable interest in the use of the endogenous oestrogen metabolite, 2-methoxyoestradiol (2-MeOE2) for the treatment and prevention of breast cancer. We have previously shown that sulphamoylation of 2-MeOE2 and related derivatives greatly enhances their ability to inhibit the proliferation of ER+ and ER− breast cancer cells. In this study, we have compared the abilities of 2-methoxyoestradiol-bis-sulphamate (2-MeOE2bisMATE) and 2-ethyloestradiol-bis-sulphamate (2-EtE2bisMATE) with that of 2-MeOE2 to inhibit the proliferation of breast cancer cells when grown on three different substrata: plastic, collagen I and Matrigel. The human breast cell line MCF-7 was utilised for these studies together with its doxorubicin resistant variant, MCF-7 DOX40 and mitoxantrone resistant variant, MCF-7 MR, as a longitudinal model of in vitro drug resistance. On a plastic substratum all three cell lines were sensitive to the effects of 2-MeOE2bisMATE and 2-EtE2bisMATE whereas MCF-7 cells and the MCF-MR variant cells were resistant to the effects of 2-MeOE2 at 1 μM. The sensitivity of the cell lines to those compounds also remained significant when grown on more physiological substrata. All of the drugs tested arrested cells in the G2/M phase of the cell cycle. The finding that breast cancer cells that are resistant to conventional chemotherapeutic agents remain sensitive to 2-substituted oestrogen sulphamates offers considerable potential for the treatment of women with drug-resistant breast cancer.
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Keywords: Breast cancer; Oestrogens; Multi-drug resistance; 2-Methoxyoestradiol; Oestrogen sulphamates

1. Introduction

Breast cancer is reported to be one of the commonest cancers, accounting for almost 20% of all malignancies world-wide, and over half a million women develop breast cancer every year [1]. For advanced breast cancer (recurrence and metastasis), the aim of drug treatments is to shrink the existing breast cancers, or to decrease the rate at which they grow or spread. For metastasis affecting the liver or lungs, chemotherapy is used as the first line of treatment. It is also applied if patients do not respond to hormone therapy. Many patients with metastatic breast cancer, however, develop recurrences after an initial response to chemotherapy. In such patients, drug resistance is a common phenomenon. When they no longer respond to chemotherapeutic cytotoxic agents, there is currently little therapeutic alternative available. More than three quarters of patients with metastatic breast cancers die within 5 years of diagnosis [2]. Discovery of new therapeutic regimens for such patients is, therefore, most urgently required.

In order to identify novel therapeutic candidates for advanced breast cancer, 2-methoxyoestradiol-bis-sulphamate (2-MeOE2bisMATE) and 2-ethyloestradiol-bis-sulphamate (2-EtE2bisMATE) were investigated in comparison with the human endogenous metabolite 2-methoxyoestradiol (2-MeOE2) (Fig. 1). Previously, 2-MeOE2 has been shown to inhibit the growth of breast cancer cell lines in vitro [3,4] and it has been suggested that this metabolite may be an endogenous inhibitor of breast cancer [5]. The sulphamoylated oestrogen derivatives were initially developed as steroid sulphatase inhibitors but it became apparent that they also inhibited cell proliferation. They induce a mitotic arrest and apoptosis in cells which is thought to result from their anti-microtubule activity [4].

☆ Poster paper presented at the 15th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology, "Recent Advances in Steroid Biochemistry and Molecular Biology", Munich, Germany, 17–20 May 2002.

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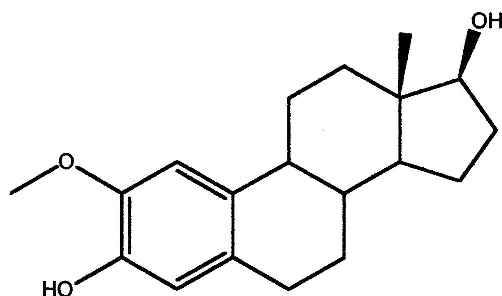
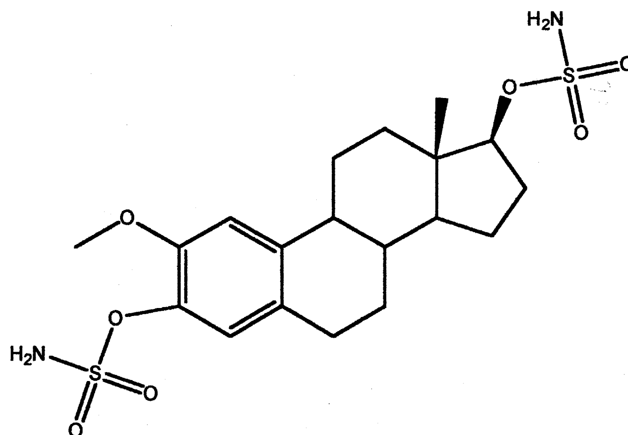
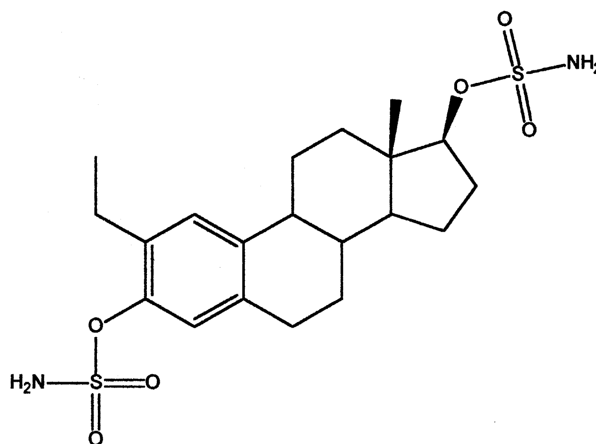
0960-0760/03/$ – see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0960-0760(03)00035-9



2-Methoxyoestradiol (2-MeOE2)



2-Methoxyoestradiol-3,17-*O,O*-bissulphamate (2-MeOE2bisMATE)



2-Ethyloestradiol-3,17-*O,O*-bissulphamate (2-EtE2bisMATE)

Fig. 1. Chemical structures of 2-EtE2bisMATE, 2-MeOE2bisMATE and 2-MeOE2.

In immunodeficient mice oral administration of 2-MeOE2 inhibited the growth of MDA-MB-435 tumours, Meth-A sarcomas and B16 melanomas [6,7]. In addition to its anti-proliferative effects, 2-MeOE2 is also a potent inhibitor of in vitro and in vivo angiogenesis [6,7]. In the present study, the human breast cancer cell line MCF-7, its doxorubicin resistant variant, MCF-7 DOX40, and mitoxantrone resistant variant, MCF-7 MR, were utilised as a longitudinal model of drug-resistant breast cancer in vitro. MCF-7 DOX40 is a typical multi-drug-resistant cell line in that it expresses P-glycoprotein and is resistant not only to doxorubicin (75-fold) but also to other chemotherapeutic agents [8]. MCF-7 MR is highly resistant to mitoxantrone (over 1200-fold) and, to a lesser extent, also to other agents. It is P-glycoprotein negative but expresses breast cancer resistance protein (BCRP) [9]. The expression levels of

oestrogen receptors in these cell lines are unknown at present.

Using these cell lines, the pharmacological impacts of test drugs on cellular growth were examined on three different substrata: plastic, collagen I and Matrigel. Changes in the cell cycle were investigated using propidium iodide (PI) labelling and analysed using a Fluorescence Activated Cell Sorter (FACS).

2. Materials and methods

2.1. Drugs

2-MeOE2, 2-MeOE2bisMATE and 2-EtE2bisMATE were synthesised at the Department of Pharmacy and Pharmacology, University of Bath. All compounds exhibited spectroscopic and analytical data in accordance with their structure. Full details of their synthesis will be reported elsewhere.

2.2. Cells

MCF-7 cells were obtained from the American Tissue Culture Collection (ATCC). Both MCF-7 DOX40 and MCF-7 MR were kindly donated by Dr. G.L. Scheffer (Department of Pathology, Free University Hospital, Amsterdam, The Netherlands). Cells were cultured in RPMI 1640 (R7388, Sigma, Irvine, England) with 10% foetal bovine serum (F524, Sigma), L-glutamine (G75L3, Sigma) and antibiotic/antimycotic solution (A9909, Sigma) containing 100 IU penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B. Cells were maintained at 37 °C under 5% CO_2 and 95% air atmosphere. In order to maintain the drug resistance of MCF-7 DOX40 and MCF-7 MR, the cells in growth phase were cultured for a minimum of three days a month in 400 nM doxorubicin (D1515, Sigma) or 80 nM mitoxantrone (M6545, Sigma), respectively.

2.3. Growth assays

2.3.1. On plastic

Cells were plated at 10^5 per 25 cm^2 tissue culture flask. When the cells reached 30% confluence (24–48 h) the spent medium was replaced with experimental conditions as follows: untreated control, 1 μM 2-MeOE2, 1 μM 2-MeOE2bisMATE or 1 μM 2-EtE2bisMATE. Cells were cultured for a further 5 days. On the sixth day, as the confluency of the untreated control reached 80%, the spent medium was discarded and the cells were gently washed twice with phosphate buffered saline (PBS) (14040-091, GibcoBRL, via Life Technologies, Paisley, Scotland). After aspirating until no PBS remained in the flask, 2 ml of Hepes buffer and then 200 μl of Zaponin (Coulter Electronics Ltd., Luton, England) were added. After 10 min incubation at room temperature, the nuclei of the lysed cells were suspended in 25 ml of Isoton II (Beckman Coulter). Twenty millilitres of the nuclei suspension was counted three times per flask using a Coulter cell counter (Coulter Electronics Ltd., Luton, England). The reading of each flask was represented by the mean of the three readings. Each experimental condition was represented in triplicate and each experiment was conducted at least twice.

2.3.2. On collagen I

Cells were plated at 2×10^4 per well of collagen I coated cellware 24-well plates (Biocoat Cell Environments, Becton Dickinson, Bedford, USA). When the cells reached 30% confluence (24–48 h) experimental conditions were introduced for 5 days as follows: control, 1 μM 2-MeOE2, 1 μM 2-MeOE2bisMATE or 1 μM 2-EtE2bisMATE. After 6 days, as the confluency of the untreated control reached 80%, the cells were counted. The cell counting procedure was the same as above except that 33 μl of Hepes, 33 μl of Zaponin and 18 ml of Isoton II were applied per well. The cell number of each well was represented by the average of three readings, and each experimental condition was represented in duplicate. The experiment was conducted three times.

2.3.3. On Matrigel

Each well of 24 multiwell plates was coated with 500 μl of growth factor reduced Matrigel matrix (Biocoat Cell Environments), using pre-chilled pipettes (−20 °C) on ice. The Matrigel was incubated at room temperature for 30 min and at 37 °C for further 30 min. Cells were plated at 10^4 per well. After 24 h, as the cells reached 30% confluence, the cells were introduced to 5 day treatments with control, 1 μM 2-MeOE2, 1 μM 2-MeOE2bisMATE or 1 μM 2-EtE2bisMATE. On day 6, as the confluency of the untreated control reached 80%, the spent medium was aspirated. The cells were gently washed with PBS twice and fixed and stained by 1 min incubation in Accustain (WG-16, Sigma). The stain was removed, and the cells were washed with de-ionised water twice. Numerical analysis of the morphology of the three-dimensional structures in each well was represented by images obtained from three visual fields at 10× magnification. The experiments were conducted three times. From these images, numbers of spheres that were equal to and larger than 150 μm in diameter as well as numbers of oblong structures equal to and longer than 200 μm in length were manually counted.

2.4. Acquiring images

Images were captured using Olympus CK2 microscope (OLYMPUS OPTICAL Co. UK Ltd., London, England) and Kodak MDS120 (Eastman, Kodak Company, New York, USA) and processed using Adobe PhotoShop.

2.5. Analysis of data and production of figures

The means, standard deviations and P values (student's *t*-test) of the results were obtained using Microsoft Excel.

2.6. Cell cycle analysis

Cells were plated on plastic substratum at 2×10^5 per 75 cm^2 flask (Falcon, Becton Dickinson, Oxon, UK). When the cells reached 50–60% confluence, experimental conditions were introduced as follows: control, 1 μM 2-MeOE2, 1 μM 2-MeOE2bisMATE or 1 μM 2-EtE2bisMATE. The cells were treated for 24 h, after which they were swiftly washed twice with Versene 1:5000 (15040-033, Gibco, via Life Technology) and trypsinised (T3924, Sigma) for 1 min at 37 °C. Trypsin was inactivated by addition of the growth medium. The spent medium, Versene that washed the cells and the single cell suspension were pooled into a single 50 ml Falcon tube to collect floating cells as well as adherent cells. The tubes were centrifuged at $400 \times g$ for 10 min at 10 °C. The cell pellets were re-suspended in PBS and centrifuged twice to remove the medium. The cell pellets were then vortexed gently while being fixed with cold 70% ethanol (−20 °C). The fixed cells were stored at 4 °C for up to 7 days. Before analysis, the cells were centrifuged at 2000 rpm for 5 min to remove ethanol, the pellets were re-suspended in PBS and centrifuged twice. The cell pellets were then incubated in 100 μl of 100 μg/ml ribonuclease (R4642, Sigma) for 5 min at room temperature and stained with 400 μl of 50 μg/ml PI (Sigma). Samples were analysed using a FACSCalibar (Becton Dickinson, Oxford, UK) at 488 nm excitation, gating out doublets and clumps and collecting fluorescence above 620 nm. The experiments were carried out in triplicate. The cell cycles figures were prepared using Adobe PhotoShop.

3. Results

3.1. Growth on plastic substratum

Prior to the main cell growth assays, the cell lines were examined for the expected sensitivity and resistance to the chemotherapeutic agents (Fig. 2). When treated with 400 nM doxorubicin or 80 nM mitoxantrone for 5 days, the parental cell line MCF-7 showed obvious sensitivity to both agents. In contrast, the doxorubicin resistant variant MCF-7 DOX40 was completely resistant to both of these drugs, showing no difference between the end-point cell densities in the treated and untreated populations. The mitoxantrone resistant variant MCF-7 MR was resistant to mitoxantrone. However, it was sensitive to doxorubicin, albeit not as much as the parental MCF-7 cells.

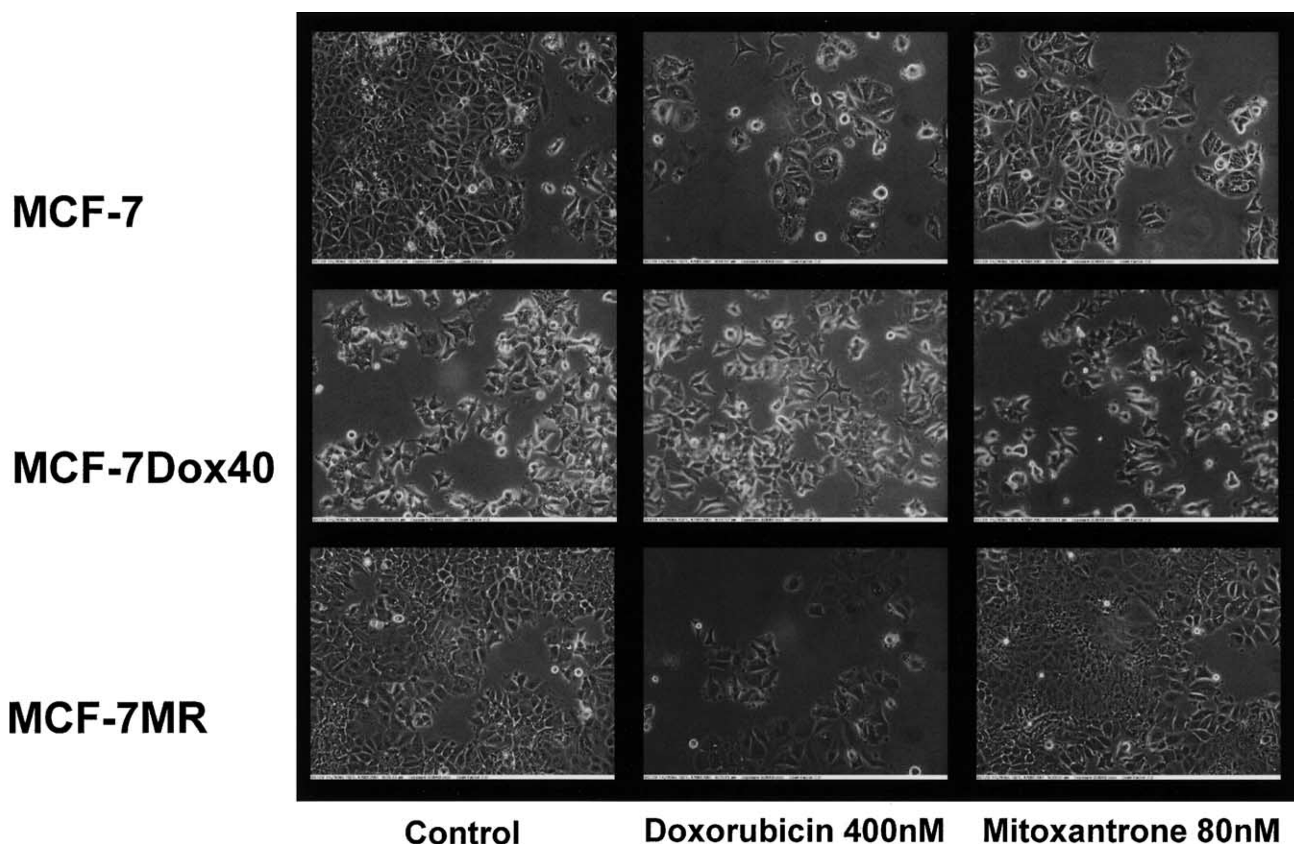


Fig. 2. The sensitivity and resistance of MCF-7, MCF-7 DOX40 and MCF-7 MR to doxorubicin and to mitoxantrone. Cells were treated for 5 days with or without the chemotherapeutic agent during growth phase. The images of untreated populations show that all the cell lines form a monolayer of epithelial morphology with no irregular over lapping or clumping of the cells. MCF-7 was sensitive to both doxorubicin and mitoxantrone. MCF-7 DOX40 was resistant to both of these agents, while MCF-7 MR was sensitive to the former but resistant to the latter.

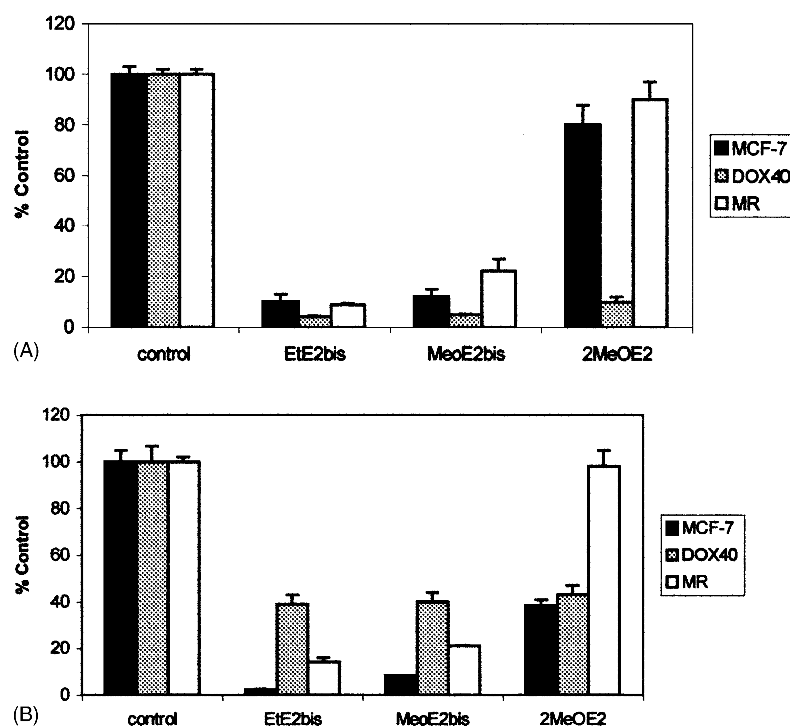


Fig. 3. Growth assays examining the effects of 2-EtE2bisMATE, 2-MeOE2bisMATE and 2-MeOE2 on MCF-7, MCF-7 DOX40 and MCF-7 MR. Cells were grown on plastic (A) or on collagen I (B) for 5 days with or without each compound at 1 μM. At the end of the experiments, cell numbers were counted using a Coulter cell counter. The end-point cell numbers in the treated populations are presented as the percentage of the end-point cell numbers in the untreated control populations (*Y*-axis). Error bars represent standard deviations.

For the growth study on plastic substratum, the monolayer of cells in the untreated control populations was allowed to grow exponentially from about 30–80% confluence over a 5-day period. When treated with 2-EtE2bisMATE, both of the drug-resistant variants MCF-7 DOX40 and MCF-7 MR were growth inhibited as much as the parental MCF-7 cells (Fig. 3A). Compared to the untreated control populations, the end-point cell numbers were 12% (MCF-7), 6% (MCF-7 DOX40) and 11% (MCF-7 MR). MCF-7 and MCF-7 DOX40 showed equal sensitivity to 2-MeOE2bisMATE with the end-point cell numbers being 12 and 6% of the control, respectively. In contrast, MCF-7 MR was less sensitive to 2-MeOE2bisMATE. The end-point cell number was 23% of the control, which was significantly higher ($P < 0.001$) than the result obtained with 2-EtE2bisMATE. Striking differences between the cell lines were found with 2-MeOE2. MCF-7 and MCF-7 MR were relatively resistant to 2-MeOE2 with the end-point cell numbers being 81 and 93% of the control, respectively. The growth inhibition was not significant in either case. However, MCF-7 DOX40 was almost as sensitive to 2-MeOE2 as 2-EtE2bisMATE and 2-MeOE2bisMATE, with the end-point cell number being 8% of the control.

3.2. Growth on collagen I

Cells were grown on collagen I, one of the principle components of the interstitial tissue (Fig. 3B). The cells were plated at 2×10^4 per well of collagen I coated cellware 24-well plates, and the experimental conditions were introduced after 24 h (when the cells had fully attached). The patterns of sensitivity observed in MCF-7 and MCF-7 DOX40 on collagen I were, in part, different from those on the plastic substratum. For instance, MCF-7 was more sensitive to 2-MeOE2 on collagen I than on plastic. The end-point cell number was 10% of the untreated control population on collagen I, compared to 81% on plastic. MCF-7 DOX40 was less sensitive to any of the compounds on collagen I than on plastic. The end-point cell number in the treated populations were all in the region of 40% of the control on collagen I, whereas on plastic they were approximately 10%. In contrast, MCF-7 MR showed equivalent levels of sensitivity to 2-EtE2bisMATE and to 2-MeOE2bisMATE on collagen I as on plastic. This line was resistant to the effect of 2-MeOE2 on both collagen I and plastic.

3.3. Cell growth and morphology on Matrigel

Cells were plated as a single cell suspension on semi-solidified Matrigel. After 24 h, the cells were treated for 5 days with 2-MeOE2bisMATE, 2-EtE2bisMATE or 2-MeOE2. On day 6, numbers of spheres that were equal to and larger than 150 μM in diameter as well as numbers of oblong structures equal to and longer than 200 μM in length were counted from three visual fields (10× magnification) per experiment, and the experiment conducted in triplicate.

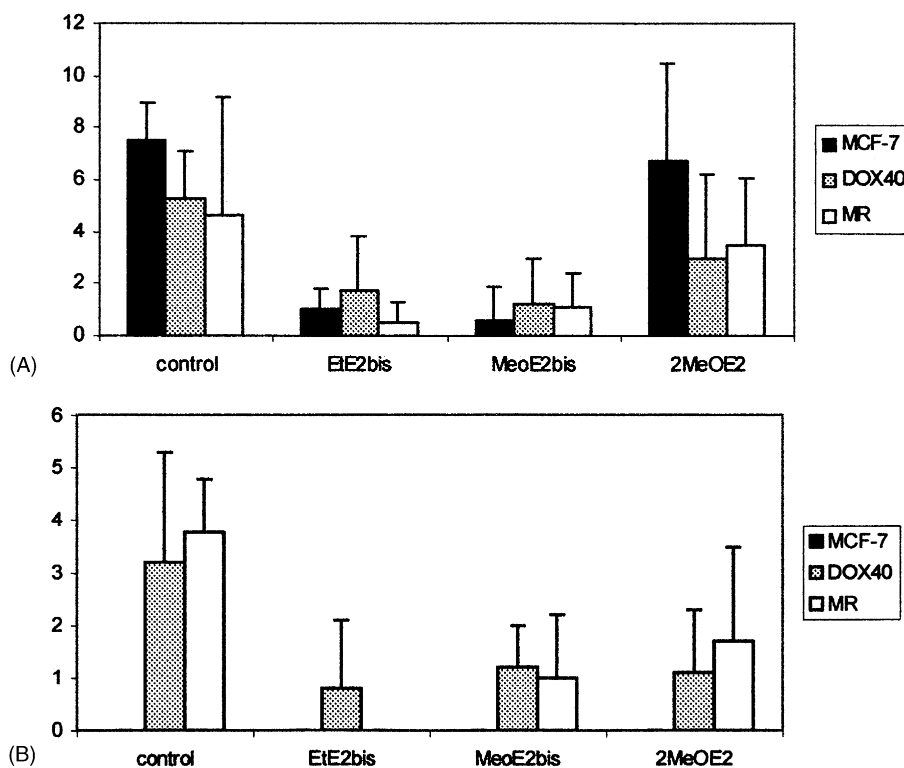


Fig. 4. Growth assays on Matrigel examining the effects of 2-EtE2bisMATE, 2-MeOE2bisMATE and 2-MeOE2 on MCF-7, MCF-7 DOX40 and MCF-7 MR. Cells were plated as single cells on Matrigel and left to attach overnight. The cells were grown for 5 days with or without each compound at 1 μM. On day 6, the cells were fixed, stained and examined with a microscope. Three visual fields (at 10× magnification) were used per experiment, and the experiment was repeated three times. The *Y*-axis represents the mean number of defined structures per visual field, and the error bars represent standard deviation: (A) the numbers of large spheres (150 μm in diameter) on Matrigel; (B) the number of oblong structures (200 μm in length) on Matrigel.

Morphological structures on Matrigel are generally highly variable. Hence, quantitative examination on this substratum yielded relatively large standard deviations (Fig. 4). Nevertheless, some changes were statistically significant, and these are described below. The morphological variations in each condition on Matrigel can be observed in Figs. 5–7.

On Matrigel, MCF-7 cells in the untreated control population grew from single cells to spheres, the majority of which were greater than 150 μM in diameter (Figs. 4A and 5). When treated with 2-MeOE2bisMATE or 2-EtE2bisMATE, the MCF-7 cells remained either as single cells or small spheres. In these populations, the number of large spheres (>150 μM) decreased significantly ($P < 0.002$) in comparison to the control. In contrast, treatment with 2-MeOE2 had no effect.

Both MCF-7 DOX40 (Fig. 6) and MCF-7 MR (Fig. 7) formed a mixture of spheres and oblong structures. Some of the oblong structures were branched out in different directions. In Fig. 4A, these two lines score lower than MCF-7 for the number of large spheres (>150 μM) in the untreated control populations. However, it is important to point out that in the drug-resistant variants, some of the structures were counted as spheres and some as oblongs. If both of these structures were taken into consideration, the drug-resistant variants exhibited more vigorous growth characteristics on Matrigel than the parental MCF-7. The sizes of the spheres and oblong structures were markedly greater in the doxorubicin resistant variant MCF-7 DOX40 than MCF-7, and this is best illustrated in the images provided in Figs. 5 and 6. Fig. 4B illustrates that MCF-7 DOX40 and MCF-7 MR formed oblong structures much more readily than MCF-7 (which did not form single oblong structures). In comparison with the control, MCF-7 DOX40 formed significantly ($P < 0.001$) less oblong structures when treated with 2-EtE2bisMATE, 2-MeOE2bisMATE or 2-MeOE2. It was also noted that when treated with 2-EtE2bisMATE or 2-MeOE2bisMATE, many of the MCF-7 DOX40 cells remained as single cells (Fig. 6). It appeared as if the cell–cell adhesion was affected, and hence even the spheres and oblong structures consisted of relatively loose clusters of cells (see 40× magnification in Fig. 6). In case of MCF-7 MR (Fig. 7), 2-EtE2bisMATE virtually eradicated oblong structure formation, while 2-MeOE2bisMATE significantly ($P < 0.001$) decreased their number, compared to the control. When treated with 2-MeOE2, the number of oblong structures in MCF-7 MR seemed to decrease also, but this did not achieve statistical significance.

3.4. Cell cycle analysis

In all of the cell lines examined in the present study, the 24 h treatment with either 2-MeOE2bisMATE or

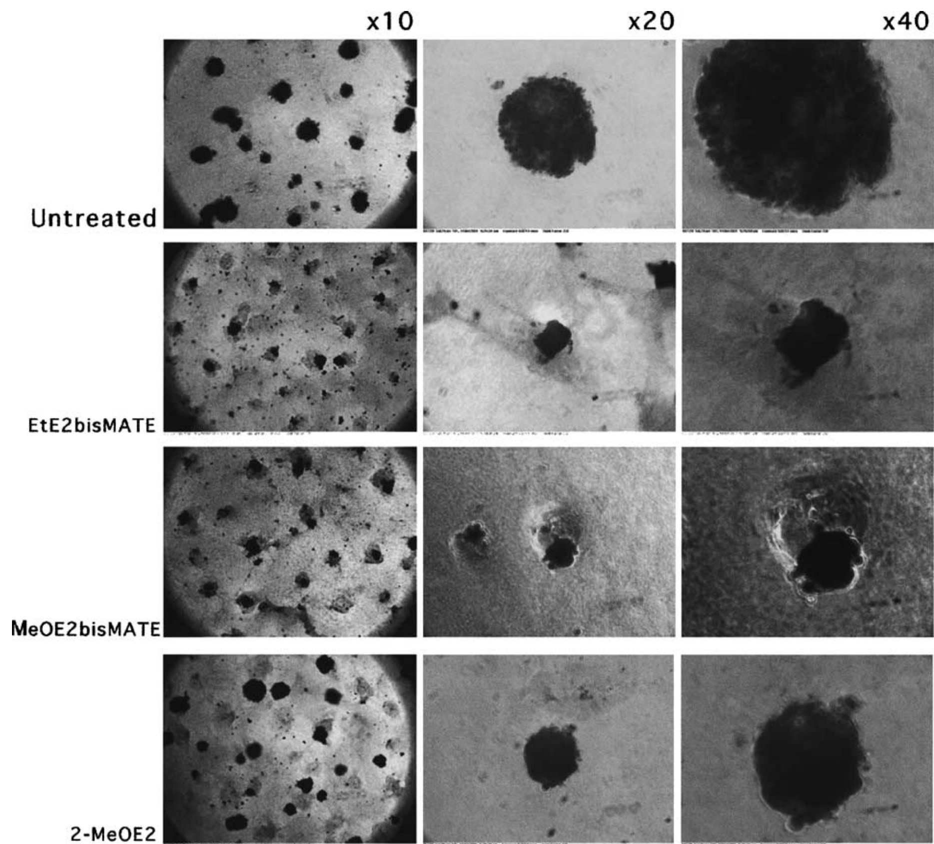


Fig. 5. Morphology of MCF-7 with or without 2-EtE2bisMATE, 2-MeOE2bisMATE and 2-MeOE2 on Matrigel. Cells were treated as above. On day 6, photos were taken at 10×, 20× and 40× magnifications for analysing changes in the morphology.

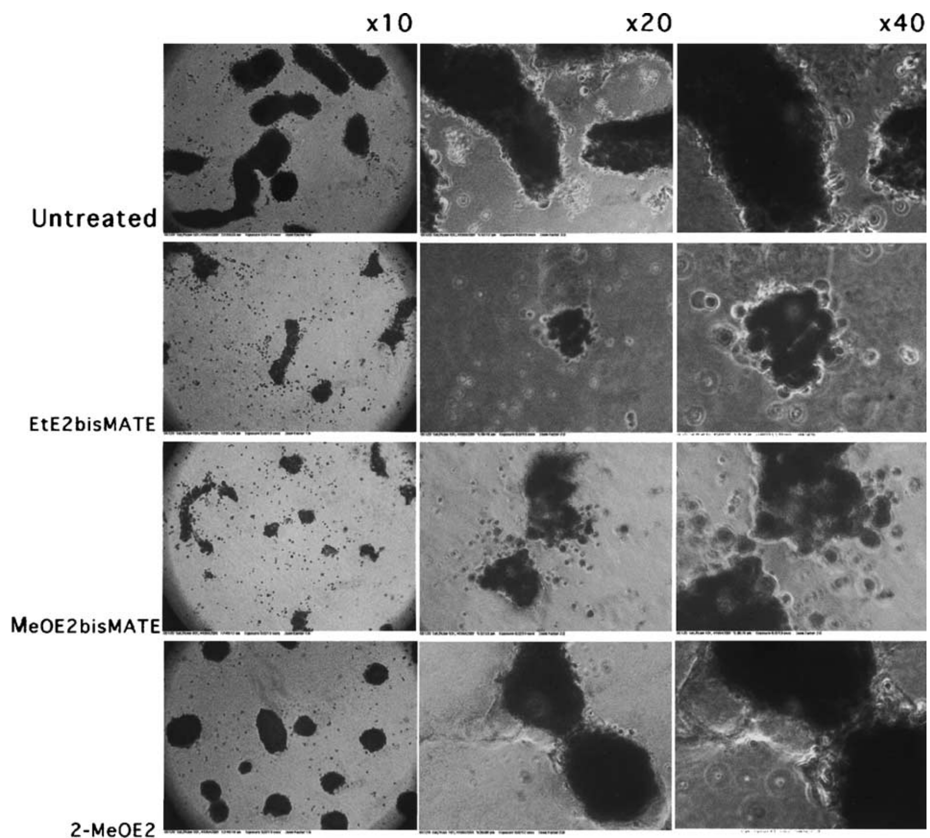


Fig. 6. Morphology of MCF-7 DOX40 with or without 2-EtE2bisMATE, 2-MeOE2bisMATE and 2-MeOE2 on Matrigel.

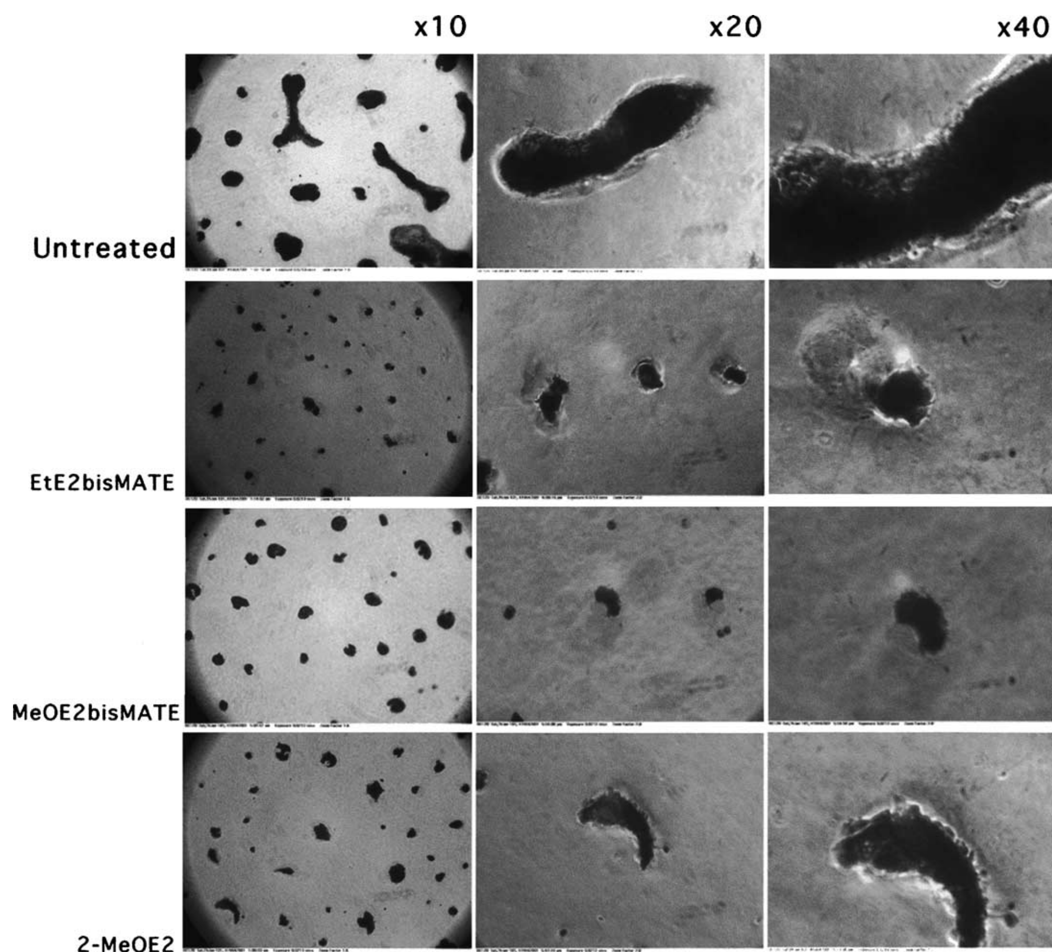


Fig. 7. Morphology of MCF-7 MR with or without 2-EtE2bisMATE, 2-MeOE2bisMATE and 2-MeOE2 on Matrigel.

2-EtE2bisMATE at 1 μM induced a marked decrease in the G1 peak and increase in the G2/M peak. 2-MeOE2, in contrast, produced no change under the same conditions (Fig. 8).

4. Discussion

To identify novel therapeutic candidates for drug-resistant breast cancer, the present study investigated two sulphamoylated 2-substituted oestrogens, 2-MeOE2bisMATE and 2-EtE2bisMATE. The effects of these compounds were compared with a human endogenous metabolite of oestrogen 2-MeOE2.

The present study used a longitudinal model of drug-resistant breast cancer which allows direct comparisons between the parental breast cancer cell line (MCF-7) and its drug-resistant variants (MCF-7 DOX40, MCF-7 MR). This type of in vitro model is particularly useful for correlation studies, since all cell lines stem from a single donor, and hence, it is not influenced by donor-donor variables (e.g. MCF-7 versus MDA-MB-231).

In vitro it has been observed that cancer cells can develop resistance to a broad range of structurally unrelated drugs, displaying multi-drug resistance (MDR). MCF-7 DOX40 and MCF-7 MR are well-characterised examples of such a phenomenon [8]. For instance, MCF-7 DOX40 is resistant to doxorubicin and also cross-resistant to other agents such as the tubulin binding drugs vincristine and verapamil. Nonetheless, the present study demonstrated that this line remained exquisitely sensitive to 2-MeOE2bisMATE and to 2-EtE2bisMATE as well as to 2-MeOE2. MCF-7 DOX40 expresses P-glycoprotein which is capable of pumping out the cytotoxic agents such as those mentioned above. It is possible that the modified oestrogens are able to bypass this mechanism and thereby exert powerful growth inhibitory effects. Further analysis on drug accumulation and functional assays on P-glycoprotein in these cells may prove useful.

In contrast, the mitoxantrone resistant variant MCF-7 MR was sensitive to 2-MeOE2bisMATE and to 2-EtE2bisMATE, but it was resistant to 2-MeOE2 at 1 μM. This line is P-glycoprotein negative but expresses BCRP [8]. Although enforced expression of BCRP in MCF-7 has been shown to confer chemo-resistance [9], its protein expression was found to be low or undetectable in a panel of drug treated and untreated breast cancer samples [10]. Thus, the significance of functional BCRP remains uncertain. An explanation of

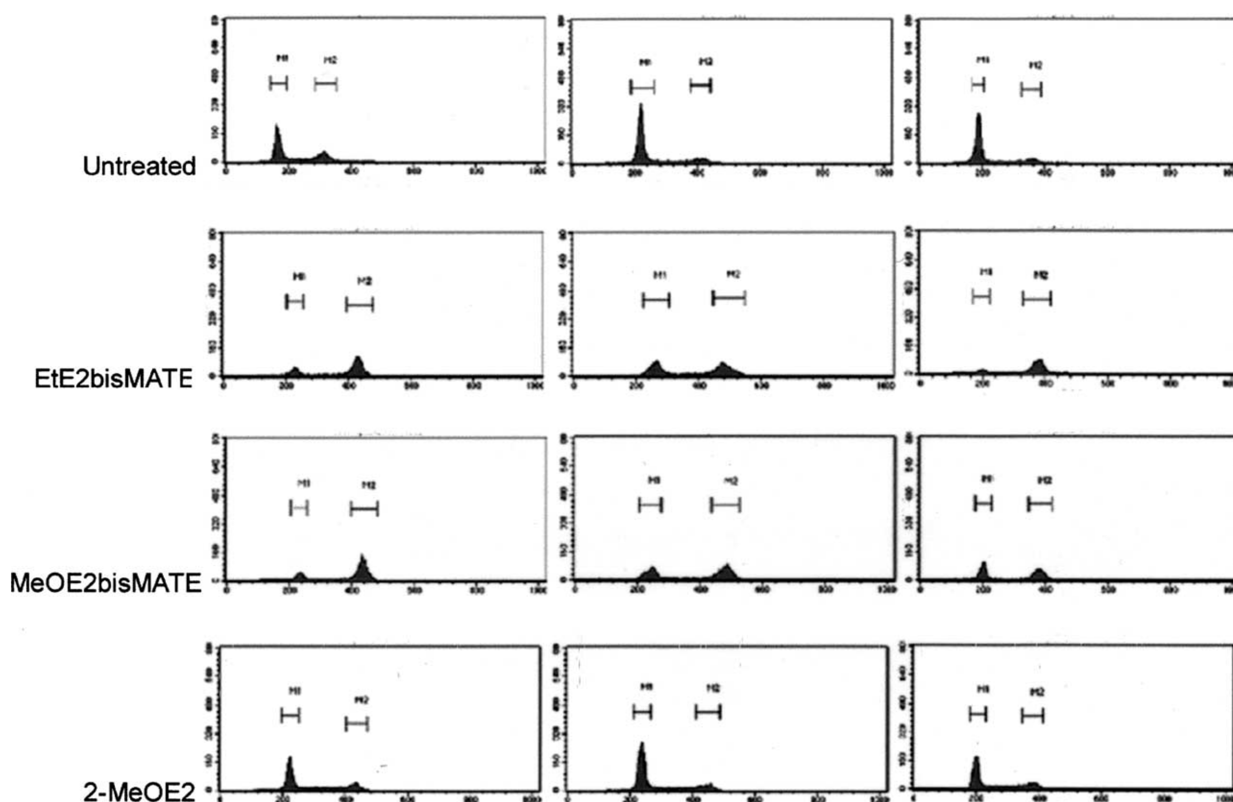


Fig. 8. PI labelling examining the effects of 2-EtE2bisMATE, 2-MeOE2bisMATE and 2-MeOE2 on the cell cycle of MCF-7, MCF-7 DOX40 and MCF-7 MR. Cells in the growth phase were treated with or without the compound, after 24 h, both the floating cells and adherent cells were collected, fixed in cold ethanol, washed, stained with PI and analysed using FACSCalibar at 488 nm excitation, gating out doublets and clumps and collecting fluorescence above 620 nm.

the potent growth inhibitory effects of 2-MeOE2bisMATE or 2-EtE2bisMATE is also unclear and requires further investigation.

The present study conducted growth assays on three different substrata: plastic, collagen I and Matrigel. Plastic is by far the most commonly used substratum in vitro, and it is a convenient surface for routine cell culture. However, it can be argued that plastic is a poor representation of the in vivo situation. Since the primary aim of the present study was to identify new anti-cancer agents, collagen I and Matrigel were considered particularly relevant. The former is one of the principle components of the interstitial tissue through which malignant cancer cells invade and grow. The latter represents an experimental "basement membrane" that compartmentalises normal breast epithelial cells from the interstitial tissue and is broken down when breast cancer cells become invasive. In the present study, the sensitivity to 2-MeOE2bisMATE or 2-EtE2bisMATE remained significant on these more physiological substrata.

Some researchers have proposed that the level of differentiation and transformation can be deduced from the three-dimensional morphogenesis on Matrigel [11–17]. Petersen et al. [15] demonstrated that normal breast epithelial cells were capable of forming duct and gland-like structures with internal lumina. Once they reached a size equivalent to the in vivo mammary acini, proliferation stopped. This self-regulation of growth was absent in two out of three primary breast cancer cells and six out of six breast cancer cell lines, which continued to grow and formed much larger colonies of tightly fused cells without lumina. Other researchers have further defined the morphogenesis of breast cancer cell lines on Matrigel. Briefly, their morphologies can be categorised as follows: well-circumscribed round colonies of tightly fused cells, loose clusters of spherical cells or branching structures. Tight clusters were associated with a relatively differentiated phenotype and expression of adhesion molecules, whereas branching structures were typically observed in breast cancer cell lines with highly invasive properties in vitro [12,13,16,17]. In the present study, MCF-7 formed round colonies of tightly fused cells, while MCF-7 DOX40 and MCF-7 MR formed additional oblong structures. It is uncertain whether these oblong structures are equivalent to structures which other researchers have previously associated with highly invasive properties. Nevertheless, treatment with 2-MeOE2bisMATE and 2-EtE2bisMATE decreased the number of the oblong structures, and it will be interesting to find out if these compounds do indeed decrease the aggressiveness of breast cancer cells by further investigations such as invasion and migration assays [12,13,16,17].

The cell cycle analysis demonstrated that the cell lines treated by these compounds were growth arrested at G2/M. The accumulation of cells in the G2/M phase was obvious by 24 h. Preliminary data in our laboratory have shown that by 48 h, dead cells start to accumulate before the G1 peak, and by 72 h both the G1 and G2/M peaks have been replaced by a sub G1 peak.

The present study provides a new opening to the future treatment of advanced breast cancer. It is possible that even at the post-chemotherapeutic stage, and even when patients have become unresponsive to the conventional cytotoxic agents, they could be treated with the 2-substituted oestrogen derivatives. Further studies to dissect the mechanism(s) of action and animal studies will reveal the potential of these novel therapeutic candidates.

Acknowledgements

We thank Professor Pinedo and Dr Scheffer (Free University Hospital, The Netherlands) for the donation of the drug resistant breast cancer cell lines.

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