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The effects of 2-substituted oestrogen sulphamates on the growth of prostate and ovarian cancer cells☆

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Abstract

The human endogenous metabolite 2-methoxyoestradiol (2-MeOE2) has been shown to inhibit the proliferation of breast cancer cells. We have previously shown that sulphamoylation of a series of 2-substituted oestrogens greatly enhances their ability to inhibit breast cancer cell proliferation and induce apoptosis. In this study, we have investigated the ability of a number of 2-substituted oestrogens and their sulphamoylated derivatives to inhibit the proliferation of two prostate cancer cell lines, an ovarian cancer cell line and its drug-resistant derivatives. 2-Methoxyoestrone, 2-ethyloestrone and 2-ethyloestradiol had little effect on the growth of the cell lines tested ($IC_{50} > 10\ \mu M$). 2-MeOE2 did inhibit the growth of the cells ($IC_{50} < 10\ \mu M$), but to a lesser extent than any of the sulphamoylated derivatives tested ($IC_{50} < 1.0\ \mu M$). Cells treated with the sulphamoylated derivatives became detached and rounded, displaying a characteristic apoptotic appearance. FACS analysis revealed induced G_2/M cell cycle arrest. Treatment of cells and subsequent drug removal indicated that the effects of the drugs on the cells were irreversible. Immunoblot analysis indicated that apoptosis may be induced by phosphorylation of BCL-2. From these studies, 2-substituted oestrogen sulphamates are emerging as a potent new class of drug that may be effective against AR+/AR− prostate and ovarian tumours, and against tumours that are resistant to conventional chemotherapeutic regimens.

Keywords: Prostate cancer; Ovarian cancer; 2-Methoxyoestradiol; Sulphamates

1. Introduction

Both prostate and ovarian cancers are heterogeneous: the tumours can include many cell types and are often made up of both androgen/oestrogen receptor positive and negative cells [1,2]. Although both are initially responsive to the treatment regimens used they often recur within a few years. Prostate cancer is the third highest cause of cancer-related death in men. Tumours can develop and go unnoticed over several years and as such usually present at an advanced stage in older men. By this stage they are usually androgen-independent and far more resistant to treatment than early stage tumours, which are usually hormone-dependent. The level of prostate specific antigen (PSA) in serum is used as a marker to test for prostate cancer and its response to treatment [3]. There is little consensus on the best treatment, particularly when diagnosed at the later androgen-independent stages, as treatments available are often ineffective by this time. Treatments achieving some level of success include diethylstilboestrol [4], and mitoxantrone, both alone and in combination with secondary hormonal therapies [3].

Ovarian cancer is the fifth most fatal type of cancer, with a 5-year survival rate of around 30% [2]. As with prostate cancer, this low survival rate is due to late stage detection of most ovarian cancers. For many years the standard treatment against ovarian cancer has been aggressive surgery followed by chemotherapy. Initially alkylating agents were used in the 1970s, but their use was replaced or combined with that of platinum-based compounds, such as cisplatinum and carboplatin, in the 1980s. More recently, microtubule disrupting and other chemotherapeutic agents have been used, either alone or in combination with those already mentioned [5]. Although these advances have lead to higher response rates and prolonged survival times, there has been little change in overall mortality rate. Initial response rates are high (70–80%), but the tumours often recur in a drug-resistant form [6]. To improve survival rates for ovarian, prostate and other cancers, microarray analysis of

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differentially expressed genes is now being used to identify markers to detect the cancer at an earlier stage [7].

The human endogenous metabolite 2-methoxyoestradiol (2-MeOE2) has been shown to inhibit the proliferation of ER+/ER− breast cancer cells in vitro [8] and the growth of mammary tumours, B16 melanomas and Meth A sarcomas in mice [9,10]. It causes G_2/M cell cycle arrest and apoptosis in prostate cancer cell lines, and inhibits prostate tumour progression in a transgenic mouse model [11]. 2-MeOE2 also has anti-angiogenic properties [10]. We have previously shown that sulphamoylation of a series of 2-substituted oestrogens greatly enhances their ability to inhibit breast cancer cell proliferation and to induce apoptosis in MCF-7 cells [12,13]. In addition, 2-methoxyoestrone-3-*O*-sulphamate (2-MeOEMATE) caused regression of NMU-induced mammary tumours in intact rats [14]. In the present study, we have extended our initial investigations to examine the ability of a number of 2-substituted oestrogens and their sulphamoylated derivatives to inhibit the proliferation of prostate and ovarian cancer cells.

The prostate cell lines tested include an androgen receptor positive cell line, LNCaP [15], and an androgen receptor negative cell line, PC3 [16]. The ovarian cell lines include a parent cell line, A2780, and the adriamycin- and cisplatin-resistant cell lines derived from these cells, A2780cis and A2780adr [17,18]. The initial studies have shown that the sulphamoylated derivatives are also effective in inhibiting the growth of these cells.

2. Materials and methods

2.1. Culture of cell lines

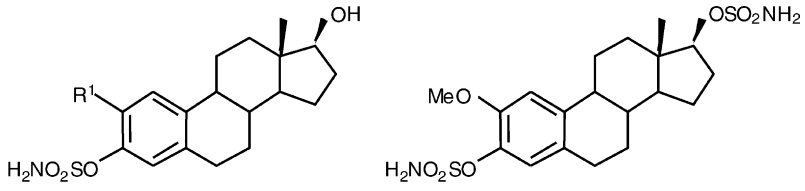
Androgen-responsive LNCaP prostate carcinoma cells, androgen-independent PC3 prostate carcinoma cells, the A2780 ovarian carcinoma cell line and its adriamycin- and cisplatin-resistant derivatives, were purchased from the European Collection of Cell Cultures. The LNCaP cells were grown in RPMI supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine and 1 mM sodium pyruvate; the PC3 cell line maintained in Coon's modified Ham's F12 supplemented with 10% FBS and 2 mM L-glutamine; and the A2780 cell lines were grown in RPMI supplemented with 10% FBS and 2 mM L-glutamine. The derivatised cell lines were treated with either 0.1 μM adriamycin once a week or 1 μM cisplatinum every two–three passages respectively. All cell culture media and supplements were from Sigma. The cells were maintained at 37 °C in a humidified atmosphere at 5% CO_2.

2.2. Drug synthesis

2-MeOE1 was synthesised from E1 (protected at the 3- and 17-positions as the methoxymethyl ether and ethylene ketal, respectively) by *ortho*-lithiation at the 2-position followed by DMF quench. The synthesis was completed by Baeyer–Villiger oxidation, alkylation and deprotection. 2-EtE1 was synthesised from E1 in a similar manner with *ortho*-lithiation at the 2-position followed by ethyl iodide quench and acidic deprotection. Estrones were reduced to estradiols with sodium borohydride in methanol/THF solution. Sulfamoylated estrogens were synthesised by reaction of the parent estrogen with sulfamoyl chloride in dimethyl acetamide (DMA) solution [19]. Thus, 2-MeOE1 was treated with sulfamoyl chloride in DMA to give 2-MeOEMATE which could then be reduced to 2-MeOE2MATE by treatment with sodium borohydride in methanol/THF solution. All compounds gave satisfactory analytical data. Full details of the synthetic procedures will be reported elsewhere. The structures are shown in Fig. 1.

2.3. Proliferation assay

Logarithmically growing LNCaP cells were plated onto 96 well plates (Falcon, Marathon Lab Supplies, London, UK) at a density of 1×10^4 cells per well, PC3 cells at a density of 2.5×10^3 cells per well, and A2780 cells (both parent and derivatives) at a density of 5×10^3 cells per well. Four hours later, the cells were treated with 10, 1 or 0.1 μM of either two known anti-cancer agents, taxol or colchicine, or 2-MeOE2, 2-EtE2, or their 3-*O*-sulphamate or 3,17-*O*,*O*-bis-sulphamate derivatives. Control cells received only the vehicle, THF. After 4 days of incubation with the compounds the CellTiter96 Aqueous One assay (Promega, Hampshire, UK) was used to measure proliferation of the cells. After 2–4 h at 37 °C the absorbance at 495 nm was measured.



R^1 = MeO 2-MeOE2MATE

R^1 = Et 2-EtE2MATE

2-MeOE2-*bis*-sulphamate

Fig. 1. The structures of 2-MeOE2MATE, 2-EtE2MATE and 2-MeOE2-bis-sulphamate.

2.4. Flow cytometric analysis

Cells were plated at 60–70% confluency in T75 flasks (Triple Red, Oxfordshire, UK). After 24 h they were treated with 1 μM of either 2-methoxyoestradiol bis-sulphamate or 2-ethyloestradiol bis-sulphamate. Control cells were untreated or treated with THF vehicle only. After a further 24 or 48 h, the cells were harvested by trypsinisation. All media and washings were collected. The cells and washings were pelleted by centrifugation at 1500 rpm, washed twice with PBS, fixed in cold 70% ethanol, treated with 100 μg/ml RNase for 5 min, stained with 50 μg/ml propidium iodide and analysed using a flow cytometer (FACScan, Becton Dickinson, Oxfordshire, UK).

2.5. Morphology studies

Cells were photographed after 48 h of treatment with vehicle (THF) alone, 1 μM 2-MeOE2bisMATE (2MbM), or 1 μM 2-EtE2bisMATE (2EbM). The photographs were taken on a Kodak DC120 digital camera with an Olympus CK2 microscope and were processed using Adobe Photoshop 5.0 LE.

2.6. Reversibility studies

Cells were plated at 60–70% confluency in T25 flasks (Triple Red, Oxfordshire, UK) and after 24 h were treated in triplicate with 1 μM of the bis-sulphamoylated derivatives of either 2-MeOE2 or 2-EtE2. Control cells were treated with THF vehicle only. After 3 days the medium was aspirated and the cell layer washed. The cells in one flask for each drug treatment were harvested and prepared for FACS analysis as described above. Of the remaining two flasks, one was treated again with 1 μM of the respective compound, and one with vehicle alone. Further, 3 days later the remaining cells were harvested and prepared for FACS analysis.

2.7. BCL-2 immunoblot analysis

A2780 cells were treated with 1 μM of either two known anti-cancer agents, taxol or colchicine, or 2-MeOE1, 2-MeOE2, or their sulphamated derivatives for 24 h. Control cells were untreated. Cells were harvested, lysed and immunoblotted with C124 (DAKO Ltd., Cambridgeshire, UK), an antibody to BCL-2, as previously described [12].

3. Results

3.1. Inhibition of cell proliferation

Prostate cancer cell lines, LNCaP and PC3, and ovarian cancer cell lines, A2780, A2780adr and A2780cis, were treated with three concentrations of the 2-substituted oestrogens, 2-MeOE2 and 2-EtE2, and their sulphamoylated derivatives over 4 days. The cells were also treated with three concentrations of known proliferation inhibitors, taxol and colchicine. Inhibition of growth of the various cell lines in the presence of the treatments is shown in Fig. 2.

The proliferation of both the prostate and the ovarian cell lines was inhibited by all the compounds tested. At high concentrations (>0.1 μM) the A2780 and A2780cis ovarian cell lines are more sensitive to the compounds than either of the two prostate lines, however, at 0.1 μM the ovarian cell lines were not significantly growth inhibited ($P > 0.05$) in contrast to the prostate cell lines. The A2780adr cell line, the adriamycin-resistant ovarian cancer line, is more resistant to the treatments than the other cell lines, and this is reflected in the response of these cells to the known inhibitors, taxol and colchicine.

The IC_{50} value of each of the tested compounds on each of the cell lines was calculated (Table 1). The natural metabolite of oestrogen, 2-MeOE2, is a more effective inhibitor of proliferation than 2-EtE2. Sulphamoylation of either of these two compounds greatly increases their ability to inhibit proliferation in all the cell lines tested, but additional sulphamoylation to form the bis-sulphamated derivatives does not result in any noticeable enhancement of this effect. The two prostate cell lines, LNCaP and PC3, are more sensitive to the sulphamoylated 2-EtE2-based compounds with IC_{50} values around 40 to 25% of those treated with the 2-MeOE2-based compounds. This effect is not seen in the ovarian cells lines where the IC_{50} values for the sulphamoylated 2-methoxy and 2-ethyl oestradiol-based compounds are not statistically different to each other.

Table 1
Cells were treated with 0.1, 1 or 10 μM of 2-MeOE2 (2M), 2-MeOE2MATE (2MM), 2-MeOE2bisMATE (2MbM), 2-EtE2 (2E), 2-EtE2MATE (2EM) or 2-EtE2bisMATE (2EbM)

	LNCaP	PC3	A2780	A2780adr	A2780cis
2M	5.72 ± 2.06	4.55 ± 1.76	0.60 ± 0.10	7.83 ± 2.77	2.06 ± 0.13
2MM	0.51 ± 0.14	0.41 ± 0.14	0.29 ± 0.01	1.32 ± 0.98	0.34 ± 0.01
2MbM	0.53 ± 0.13	0.40 ± 0.28	0.33 ± 0.04	0.87 ± 0.44	0.38 ± 0.01
2E	>10 ± n.d.	>10 ± n.d.	5.08 ± 2.55	>10 ± n.d.	>14 ± n.d.
2EM	<0.32 ± n.d.	<0.10 ± n.d.	0.23 ± 0.01	0.68 ± 0.20	0.29 ± 0.01
2EbM	0.38 ± 0.34	<0.16 ± n.d.	0.36 ± 0.07	0.81 ± 0.42	0.35 ± 0.05

After 4 days proliferation was measured using the CellTiter96 Aqueous One assay (Promega). The IC_{50} values of the compounds in the various cell lines were calculated from two triplicate experiments using Prism software (values shown are the mean ± S.D.; n.d., not determined).

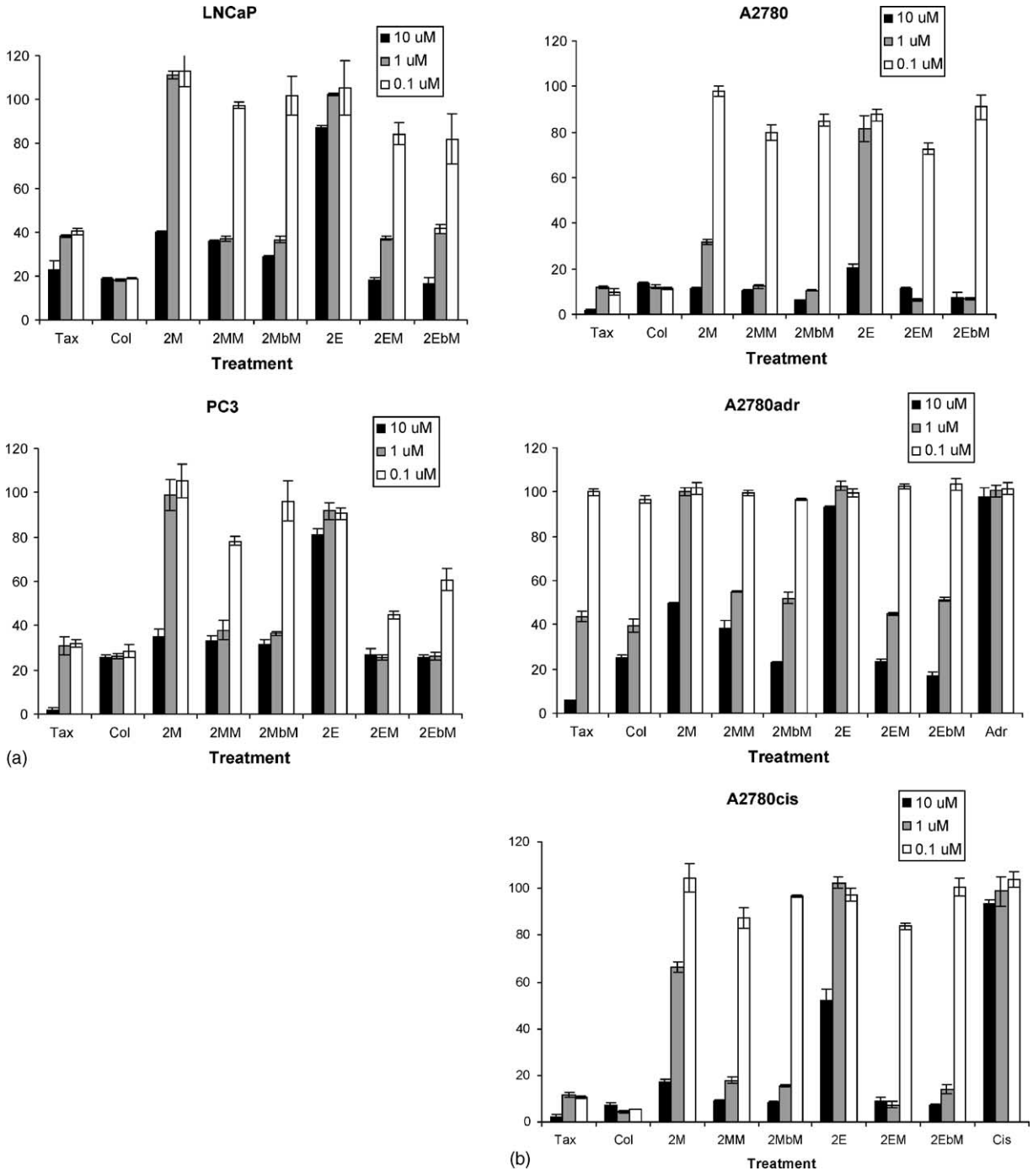


Fig. 2. (a) The effects of various treatments on the proliferation of two prostate cancer cell lines, LNCaP and PC3. Cells were treated in triplicate with 0.1, 1 or 10 μM of taxol (Tax), colchicine (Col), 2-MeOE2 (2M), 2-MeOE2MATE (2MM), 2-MeOE2bisMATE (2MbM), 2-EtE2 (2E), 2-EtE2MATE (2EM) or 2-EtE2bisMATE (2EbM). After 4 days proliferation was measured using the CellTiter96 Aqueous One assay (Promega). Results are expressed as a percentage of the proliferation of the control cells and are representative of two separate experiments. Bars, S.D. (b) The effects of various treatments on the proliferation of A2780, an ovarian cancer cell line, and two of its drug-resistant derivatives, A2780adr and A2780cis. Cells were treated in triplicate with 0.1, 1 or 10 μM of taxol (Tax), colchicine (Col), 2-MeOE2 (2M), 2-MeOE2MATE (2MM), 2-MeOE2bisMATE (2MbM), 2-EtE2 (2E), 2-EtE2MATE (2EM) or 2-EtE2bisMATE (2EbM). After 4 days proliferation was measured using the CellTiter96 Aqueous One assay (Promega). Results are expressed as a percentage of the proliferation of the control cells and are representative of two separate experiments. Bars, S.D.

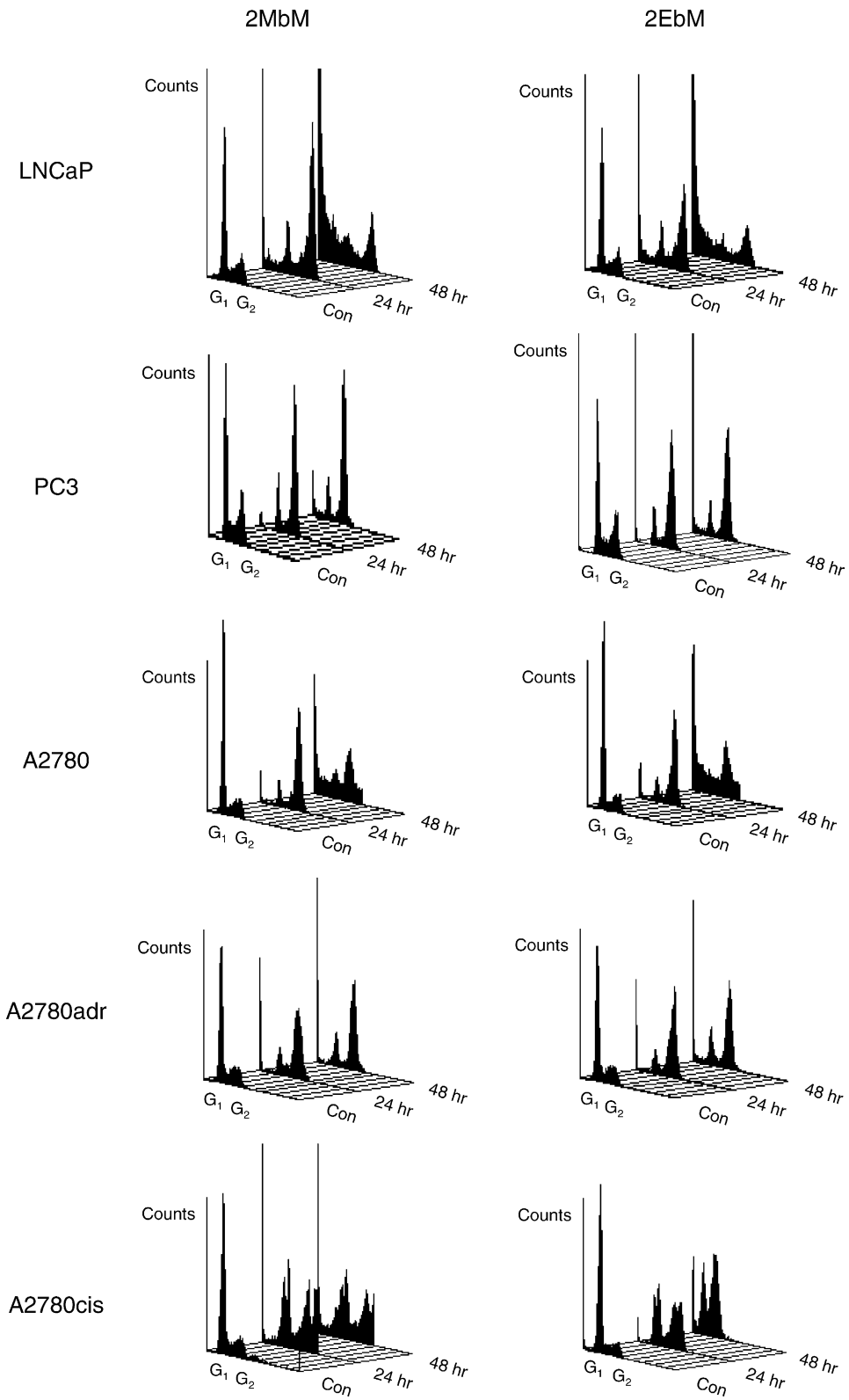


Fig. 3. DNA profiles of cells treated with 1 μM of either 2-MeOE2bisMATE (2MbM) or 2-EtE2bisMATE (2EbM) for 48 h. The cells were washed in PBS, fixed in ethanol, stained with propidium iodide and analysed by FACS.

3.2. Flow cytometric analysis of treated cells

Flow cytometry was used to assess whether the sulphamoylated derivatives cause arrest of the cells in the G_2/M phase of the cell cycle as has been reported for 2-MeOE2 [11]. In all five cell lines, a marked increase in the G_2/M peak could be seen within the first 24 h of treatment with 1 μM of either 2-MeOE2bisMATE or 2-EtE2bisMATE (Fig. 3). A sub-G_1 peak also becomes apparent within 24 h, and this increases in size over a further 24 h of treatment.

3.3. Morphology

The effect of these drugs on cell morphology was also examined by light microscopy. Photographs of the cells after 48 h of treatment with 1 μM of either 2-MeOE2bisMATE or 2-EtE2bisMATE are shown in Fig. 4. Cells treated with the sulphamoylated derivatives become detached and rounded and display the characteristic appearance of cells undergoing apoptosis. These effects were more pronounced than those seen with the natural oestrogen metabolite, 2-MeOE2 (results not shown).

3.4. Reversibility assay

The DNA profile of cells treated with 1 μM of either 2-MeOE2bisMATE or 2-EtE2bisMATE followed by subsequent drug removal was compared with untreated and continuously treated cells (Fig. 5). The cycle profile after removal of the drug resembled the profile of the continuously treated cells and was not restored to that of the control profile in any of the cell lines, with the majority of cells remaining in the G_2 or sub-G_1 phases. This indicates that the effects of the sulphamoylated 2-substituted oestrogen derivatives are irreversible in all the cell lines tested.

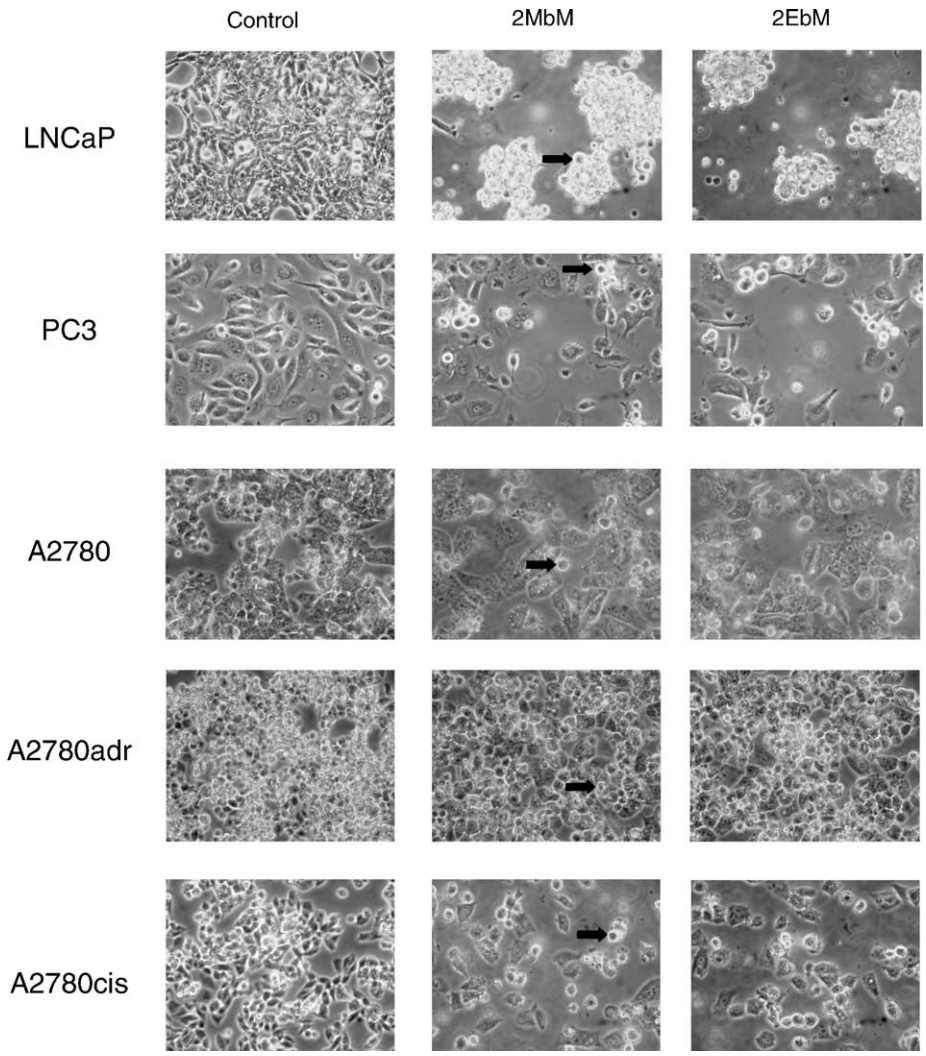


Fig. 4. Morphology of each of the cell lines after 48 h of treatment with vehicle (THF) alone, 1 μM 2-MeOE2bisMATE (2MbM), or 1 μM 2-EtE2bisMATE (2EbM). Arrows indicate apoptotic-like cells.

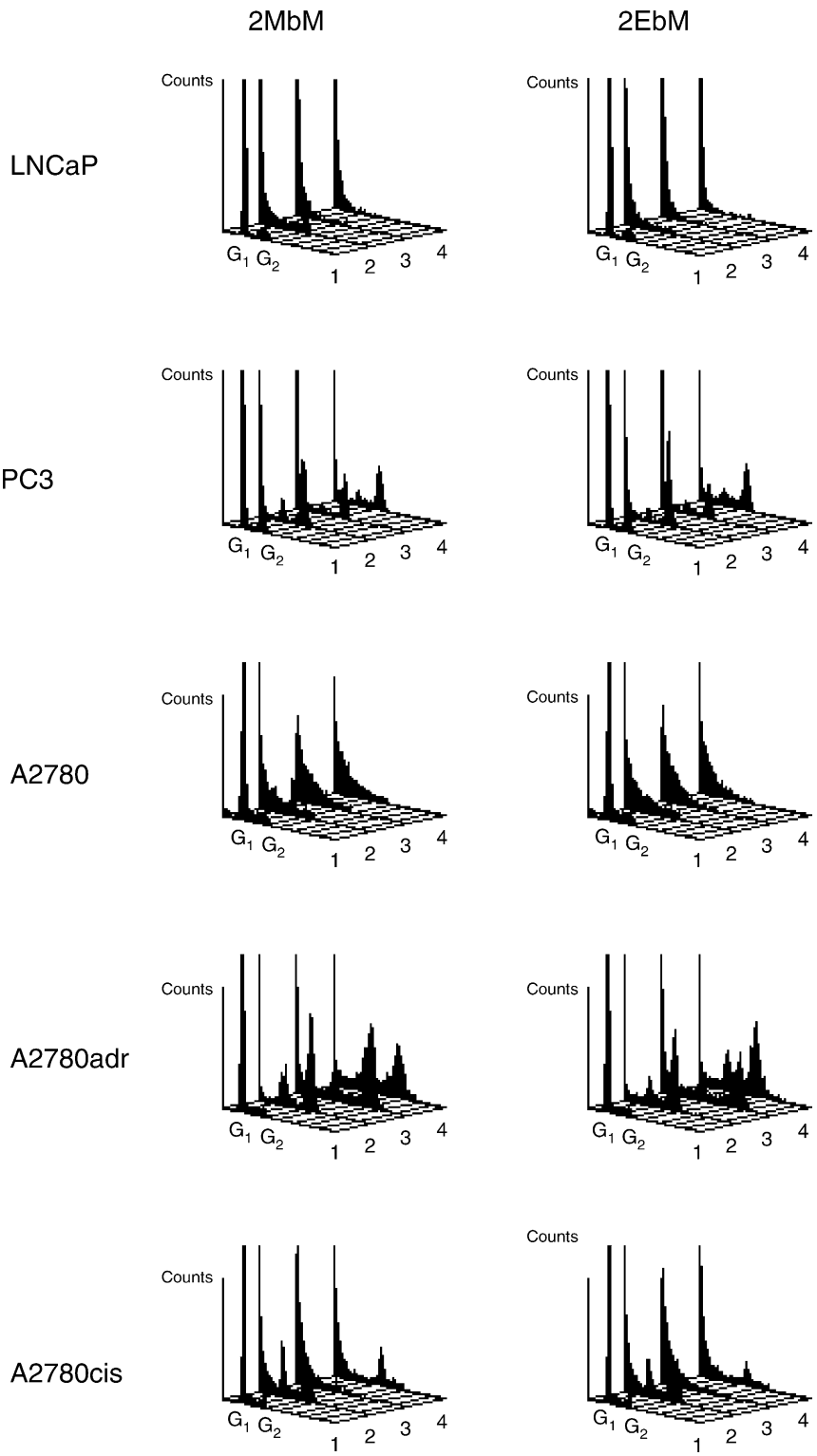


Fig. 5. DNA analysis of cells treated with vehicle (THF) alone for 3 days (Profile 1), 1 μM of either 2-MeOE2bisMATE (2MbM) or 2-EtE2bisMATE (2EbM) for 3 (Profile 2) or 6 (Profile 3) days. Cells treated for 3 days and then washed and cultured in untreated medium for a further 3 days are shown in Profile 4. The cells were harvested by trypsinisation, washed in PBS, fixed in ethanol, stained with propidium iodide and analysed by FACS.

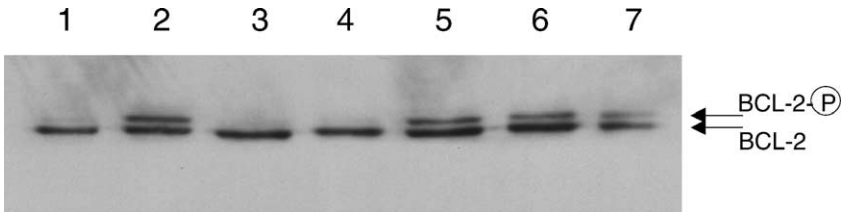


Fig. 6. Expression and phosphorylation of BCL-2 in the A2780 ovarian cancer cell line. Cells were treated with 1 μM taxol (2), cisplatin (3), 2-MeOE1 (4), 2-MeOEMATE (5), 2-MeOE2 (6), or 2-MeOE2MATE (7) for 24 h. Untreated cells were used as a control (1). Expression and phosphorylation of BCL-2 was determined by lysing the cells and immunoblotting with C124 (DAKO).

3.5. BCL-2 immunoblot analysis

As sulphamoylated oestrogen derivatives are potent inducers of apoptosis in breast cancer cell lines [12] their effect on BCL-2 phosphorylation, which is involved in the regulation of apoptosis, was examined in A2780 ovarian cancer cells. These compounds may act as antimicrotubule agents and so their effect on BCL-2 phosphorylation was compared with another antimicrotubule agent, taxol, which is known to induce BCL-2 phosphorylation. As shown in Fig. 6, cisplatin and 2-MeOE1 did not induce BCL-2 phosphorylation. However, taxol, 2-MeOEMATE, 2-MeOE2 and 2-MeOE2MATE all induced phosphorylation of BCL-2 as determined by the appearance of an isoform which migrates more slowly on the gel.

4. Discussion

These initial proliferation studies indicate that of the compounds tested the sulphamoylated derivatives of 2-EtE2 are the most efficient inhibitors of proliferation of both the prostate cancer cell lines, PC3 and LNCaP, and that at 1 μM they are as effective as taxol and colchicine. The ovarian cell line proliferation is inhibited by the sulphamoylated and bis-sulphamoylated derivatives of both 2-MeOE2 and 2-EtE2, again at 1 μM as efficiently as when treated with taxol or colchicine. The cisplatin-resistant A2780-derived cell line and the parent line are highly sensitive to the treatments in comparison to the other cell lines. In contrast the adriamycin-resistant A2780-derived cell line is most resistant to all treatments.

The variation in sensitivity between the A2780-derived cell lines may be due to expression of a multidrug resistance protein (MDR) in the A2780adr cell line. Multidrug resistance proteins were first recognised in the 1970s with the discovery of P-glycoprotein, a 170 kDa transporter glycoprotein in the cell membrane of a colchicine-resistant CHO cell line [20]. Several other multidrug resistance transporter proteins known to have a broad range of substrates have now been isolated and characterised including multidrug resistance associated protein (MRP1) [21] and breast cancer resistance protein (BCRP) [22]. A2780-derived cell lines resistant to various chemotherapeutic agents have been shown to express these proteins: BCRP is over-expressed in an A2780-derived cell line resistant to camptothecin [23], and although the original data for the A2780adr cell line submitted to the European Collection of Cell Cultures suggested that these cells are not P170 glycoprotein positive, some studies have reported the presence of this protein in adriamycin-resistant A2780 cells [24–26].

2-MeOE2 has been shown to induce G_2/M cell cycle arrest in various cell types [27,28], including prostate cell lines [1,11]. These effects were also seen when MCF-7 breast cancer cells were treated with sulphamoylated oestradiol derivatives [14]. In this study, FACS analysis indicates that treatment with the sulphamoylated oestradiol derivatives also causes G_2/M arrest within 24 h in both the prostate and the ovarian cell lines. During treatment a sub-G_1 peak, indicative of apoptotic cells, also becomes apparent and increases in size. Cell cycle arrest continues after subsequent washing of the cells, indicating that the observed effects of the treatments are irreversible. This is in contrast to the effects of 2-MeOE2 which are known to be reversible in prostate cell lines [11]. During treatment the cells display a characteristic apoptotic appearance, rounding up and becoming detached from each other. The cell cycle arrest and the morphological changes observed over several days of treatment suggest that these drugs induce apoptosis in these cell lines.

Cell death caused by the compounds may be mediated via their effects on apoptosis regulators such as BCL-2, as has been previously found to occur in MCF-7 cells [12]. BCL-2 opposes apoptosis and its inactivation via phosphorylation is induced in K562 leukaemia cells treated with 2-MeOE2 leading to subsequent apoptosis [29]. In this study, the phosphorylated form of BCL-2 is seen after treatment of the A2780 cell line with 2-MeOE2 and the sulphamoylated derivatives of 2-MeOE1 and 2-MeOE2. This response appears to be specifically due to microtubule damage as phosphorylation of BCL-2 is also seen after treatment with taxol, an anti-cancer drug known to exert its effect by microtubular binding [30].

From these studies it is apparent that 2-substituted oestrogen sulphamates are emerging as a potent new class of drug that may be effective against androgen receptor positive and negative prostate and ovarian tumours in vivo. Furthermore, they are also effective in ovarian cell lines which are resistant to known anti-cancer agents indicating that the

2-substituted oestrogen derivatives may also have a role in the treatment of tumours that have become resistant to conventional chemotherapeutic regimens.

Acknowledgements

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