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The effects of 2-methoxy oestrogens and their sulphamoylated derivatives in conjunction with TNF-α on endothelial and fibroblast cell growth, morphology and apoptosis

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Abstract

2-Methoxyoestradiol (2-MeOE2) is a potent anti-angiogenic agent. Its 3- and 17-sulphamoylated derivatives have been demonstrated to induce G_2-M cell cycle arrest and apoptosis in breast cancer cells in vitro as well as tumour regression in rats in vivo with greater potency than the parent oestrogen. To determine whether the anti-cancer properties of these derivatives can be synergistically enhanced with low-dose TNF-α co-treatment, we investigated the effects of these treatments in adult human fibroblasts and human umbilical vein endothelial cells (HUVECs). Treatment of fibroblasts with 0.1 μM 2-methoxyoestradiol-3,17-bis sulphamate (2-MeOE2bisMATE) but not 2-MeOE2 caused a reversible morphology change and induced G_2-M arrest (from 12 to 33%) but not subsequent apoptosis. In contrast, treatment of HUVECs did not induce morphology change or G_2-M arrest. Using a nucleosomal ELISA assay, we showed that TNF-α (20 ng/ml) combination treatment synergistically increases 0.1 μM 2-MeOE2bisMATE-induced but not 0.1 μM 2-MeOE2-induced apoptosis in HUVECs. These results suggest that TNF-α co-treatment may be a beneficial method of increasing the potency of 2-substituted oestrogens as anti-angiogenic agents through synergistic induction of apoptosis in endothelial cells while maintaining low cytotoxicity to fibroblasts.

Keywords: Fibroblast cell growth; Morphology; Sulphamoylated

1. Introduction

The endogeneous oestrogen metabolite 2-methoxyoestradiol (2-MeOE2) inhibits proliferating cells in vitro and suppresses the growth of certain murine tumours in vivo. These oestrogen receptor-independent anti-cancer properties arise from the ability of 2-MeOE2 to disrupt microtubule dynamics [1,2] resulting in cell cycle arrest and induction of apoptosis. Current research is focused on maximising the potential of the steroid nucleus as a target for the development of anti-proliferative agents which are less toxic than existing compounds that interact with tubulin.

2-Methoxy oestrogens have been shown to inhibit cell proliferation and angiogenesis [2,3], and induce apoptosis in breast cancer cell lines in vitro [4]. D'Amato et al. [1] have shown that 2-MeOE2 competitively inhibits colchicine binding to β-tubulin in vitro, albeit weakly ($K_i = 22\,\mu M$), thereby inhibiting tubulin assembly and inducing mitotic arrest. However, Attalla et al. [5] have shown that at concentrations that block mitosis, 2-MeOE2 does not cause depolymerisation of tubulin filaments. The inference from these data is that 2-MeOE2 exerts its anti-tumour properties not through gross inhibition of tubulin polymerisation, but through the suppression of microtubule dynamics and kinetic stabilisation of mitotic spindle assembly [6].

Alteration of microtubule stability by colchicine and paclitaxel has been shown to have downstream effects on BCL-2 phosphorylation [6,7] and increased p53 protein stability [8]. Stabilisation of wild-type p53 in human lung cancer cells was reported by Mukhopadhyay et al. [8] following treatment with 2-MeOE2, resulting in apoptosis.

2-MeOE2 inhibits vascular endothelial cell proliferation in vitro in normal bovine vascular endothelial cells [9], in concurrence with data published by Fotsis et al. [3]. This vascular endothelial cell cytotoxicity of 2-MeOE2 is thought to be responsible for its anti-angiogenic activity. In addition 2-MeOE2 induces apoptosis in the MCF-7 breast cancer cell line but not in normal human fibroblasts [10].

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2-MeOE2MATE induces irreversible G_2-M arrest and subsequent apoptosis in MCF-7 cells after 12 h of treatment [11]. In a panel of breast cancer cell lines, sulphamoylation of 2-substituted oestrogens increased their potency as inducers of cell cycle arrest and apoptosis compared to the parent compound. MacCarthy-Morrogh et al. demonstrated that 2-EtEMATE, a synthetic sulphamoylated oestrogen derivative, increased BCL-2 phosphorylation in MCF-7, CAL51 and CAMA1 breast cancer cells at concentrations that inhibit proliferation [4].

TNF-α is a major immune response-modifying cytokine produced mainly by macrophages and activated T-cells. It also enhances apoptosis in cancer cells in vitro [12]. However, its cytotoxicity limits in vivo use, but its efficacy may be increased if used in conjunction with other chemotherapeutic agents. TNF-α and EMATE co-administration caused a remission of NMU-induced mammary tumours in rats [13]. In a rat sarcoma model, TNF-α exhibited synergistic anti-tumour effects when administered with doxorubicin [14] using an isolated limb perfusion method.

In TNF-α and doxorubicin co-treatment experiments with soft tissue sarcoma and osteosarcoma cells, van der Veen et al. [14] used a final TNF-α concentration of 0–10 μg/ml. However, observations in our laboratory suggested lower TNF-α doses in the 1–100 ng/ml range were effective. In order to distinguish any effects of TNF-α co-treatment with the compounds, a sub-toxic dose of 20 ng/ml was used, along with 0.1 μM 2-MeOE2 or 2-MeOE2bisMATE (Fig. 1) which would induce modest mitotic arrest and apoptosis in cells at the time-points investigated.

In order to assess the impact of TNF-α on the anti-angiogenic properties of 2-methoxy-substituted oestrogens, their effects on cell cycle arrest and induction of apoptosis were investigated in cells responsible for vascular formation. HUVECs are routinely utilised as vascular precursor cells in in vitro angiogenic models and induction of apoptosis and perturbation of the cell cycle is thought to reflect the effects of these compounds in vivo.

In our model of breast cancer, we attempt to elucidate the cell-type specificity of combination treatment using TNF-α and/or 2-MeOE2 and 2-MeOE2bisMATE (Fig. 1). These investigations include the effects of compounds on cells involved in angiogenesis; endothelial cells which respond to angiogenic factors by migration, proliferation and formation of vessels [15] and fibroblasts, which form the stromal component surrounding the tumour, that can be stimulated to produce various factors, such as vascular endothelial growth factor (VEGF), which influence angiogenesis.

Dermal fibroblasts are used in a well established commercially available in vitro angiogenesis model (TCS Cellworks, UK) [16]. This system utilises a co-culture of fibroblasts and endothelial cells where the endothelial cells proliferate, migrate and form vessel-like structures within the culture matrix. Based on this model, we examined the effects of treatments on induction of apoptosis and cell cycle arrest in each of the two cells types in isolation.

2-MeOE2 exerts anti-angiogenic effects by inhibiting endothelial cell migration [2,3] and enhancing apoptosis [9] and here we present preliminary data to support the hypothesis that co-treatment with 2-substituted oestrogens and TNF-α synergistically induces HUVEC apoptosis without being cytotoxic to fibroblasts and potentiates its anti-angiogenic activity.

2. Materials and methods

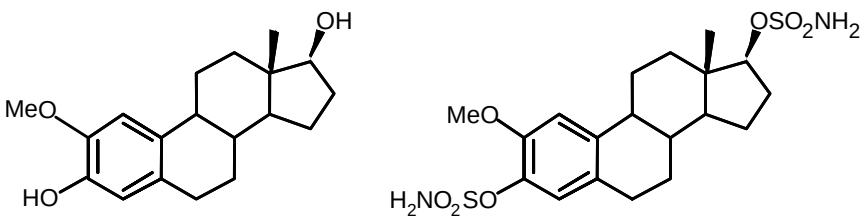
2.1. Compounds

All compounds used were synthesised at the Department of Pharmacy and Pharmacology, University of Bath. All compounds were analytically pure and exhibited spectroscopic data in accordance with their structure. Full details of their synthesis will be published elsewhere.

2.2. Cell culture

Adult human dermal fibroblasts and HUVECs were procured from TCS Cell Works and cultured in manufacturer-supplied medium.

All cells which were analysed with flow cytometry were cultured in 25 mm^2 tissue culture flasks and were seeded at a density of 5×10^5 cells per flask. Experiments were initiated when the cells were 50–70% confluent, as some cell lines were contact-inhibited and this would have an impact on the cell cycle profiles investigated.



2-MeOE2 2 -MeOE2 bis sulphamate

Fig. 1. The structures of 2-MeOE2 and 2-MeOE2bisMATE.

2.3. Flow cytometry analysis

Cells were cultured in the presence of 2-MeOE2, 2-MeOE2bisMATE with or without TNF-α for 24–48 h before being harvested for flow cytometric analysis using a FACScalibur (BD Biosciences, UK). To prepare cells for flow cytometric DNA analysis, cells were trypsinised (0.25% trypsin 0.05% EDTA), washed with PBS and fixed with 70% ethanol. The cells were collected by centrifugation and re-suspended in PBS (1–2 × 10^6 cells/ml) before being treated with RNase A (0.1 mg/ml) and stained with propidium iodide (0.05 mg/ml) for 5 min at room temperature before being analysed. The fraction of cells in G_2-M phase of the cell cycle was defined as the proportion of cells with twice the normal DNA content. A total of 1×10^5 singlet cells were analysed.

2.4. Nucleosomal ELISA

The number of free nucleosomes was quantified using an ELISA based on DNA affinity mediated capture of free nucleosomes followed by their detection by anti-histone 3 antibodies (Oncogene Research Products, Cambridge, MA). Briefly, cells were seeded into T-25 flasks, at approximately 2.5×10^5 cells per flask in 5 ml of cell specific medium and incubated at 37 °C in a 5% CO_2, humidified incubator. After 24 h the compounds were added and the plates incubated for a further 72 h. Both floating cells and adherent cells were harvested and lysed. The assay was then carried out in accordance with the manufacturer's protocol. The absorbance at 405 nm was measured spectroscopically using a plate reader. A standard curve was plotted using values obtained from manufacturer-supplied standards and used to calculate nucleosome concentration.

2.5. Statistical methods

A two-tailed Student's *t*-test assuming unequal variance was performed using Microsoft Excel to determine if there was any significant difference in the induction of apoptosis between treated and untreated cells.

3. Results

3.1. Effect of 2-methoxy oestrogens and TNF-α on cell cycle progression

We treated adult human dermal fibroblasts and HUVECs with TNF-α and/or 2-MeOE2 and 2-MeOE2bisMATE in order to determine the effect of TNF-α co-treatment on cell cycle arrest and/or cell death and the differential sensitivity of cell types.

Exposure of adult human dermal fibroblasts to 0.1 μM of 2-MeOE2bisMATE for 24 h resulted in a dramatic morphology change (Fig. 2) from the native elongated fibroblast morphology to a markedly different 'cobblestone' appearance, independent of co-treatment with 20 ng/ml TNF-α. This was not observed during treatment with 0.1 μM of 2-MeOE2 ± TNF-α. This morphological change was not accompanied by visible apoptotic bodies or rounded up cells and upon changing to drug free-medium after 14 days, was found to be completely reversible.

Flow cytometric analysis of cell cycle status of drug-treated fibroblasts revealed that although morphological changes were observed as quickly as 24 h after treatment, cell cycle arrest was maximal after 48 h (24 h data not shown). At 48 h, there was increased G_2-M arrest (from 12 to 33%) (Fig. 3 and Table 1) and a decreased number of cells in G_1-S phase (from 46 to 23%) (Fig. 3 and Table 1) of 0.1 μM 2-MeOE2bisMATE-treated fibroblasts compared to untreated controls. The cell cycle profile of TNF-α alone and 2-MeOE2 ± TNF-α-treated cells did not differ significantly from control cells (Fig. 3 and Table 1), demonstrating that morphology changes and the induction of G_2-M arrest were due specifically to the actions of 2-MeOE2bisMATE.

Twenty-four-hour treatment of HUVECs with 2-MeOE2 or 2-MeOE2bisMATE did not significantly affect cell cycle arrest (Fig. 4 and Table 1). However, co-treatment with 20 ng/ml TNF-α caused a decrease in G_2-M arrested cells compared to control and an increase in the proportion of the sub-G_1 population, indicative of apoptotic cells (Fig. 4 and Table 1). TNF-α treatment alone caused a very small increase in the sub-G_1 population (21 to 29%). Taken together,

Table 1
Tabulation of flow cytometric analyses of cell cycle parameters of adult human dermal fibroblasts and HUVECs exposed to 20 ng/ml TNF-α and 0.1 μM drug co-treatment for 48 and 24 h respectively as presented in Figs. 3 and 4

	Adult human dermal fibroblasts				Human umbilical vein endothelial cells			
	<G_1	G_1-S	S	G_2-M	<G_1	G_1-S	S	G_2-M
Control	24	46	6	12	21	52	6	17
TNF-α	19	61	5	11	29	52	5	11
2-MeOE2	23	57	5	9	14	56	6	19
2-MeOE2 + TNF-α	18	59	6	12	42	41	5	9
2-MeOE2bisMATE	29	23	9	33	14	63	3	16
2-MeOE2bisMATE + TNF-α	25	30	9	29	45	40	3	9

The proportion of cells in various phases of the cell cycle is shown as a percentage of total cells counted. Results presented are representative of two experiments, each performed in duplicate ($n = 2$). <G_1, G_1-S, S, G_2-M, <G_1, G_1-S, S, G_2-M are all cell cycles.

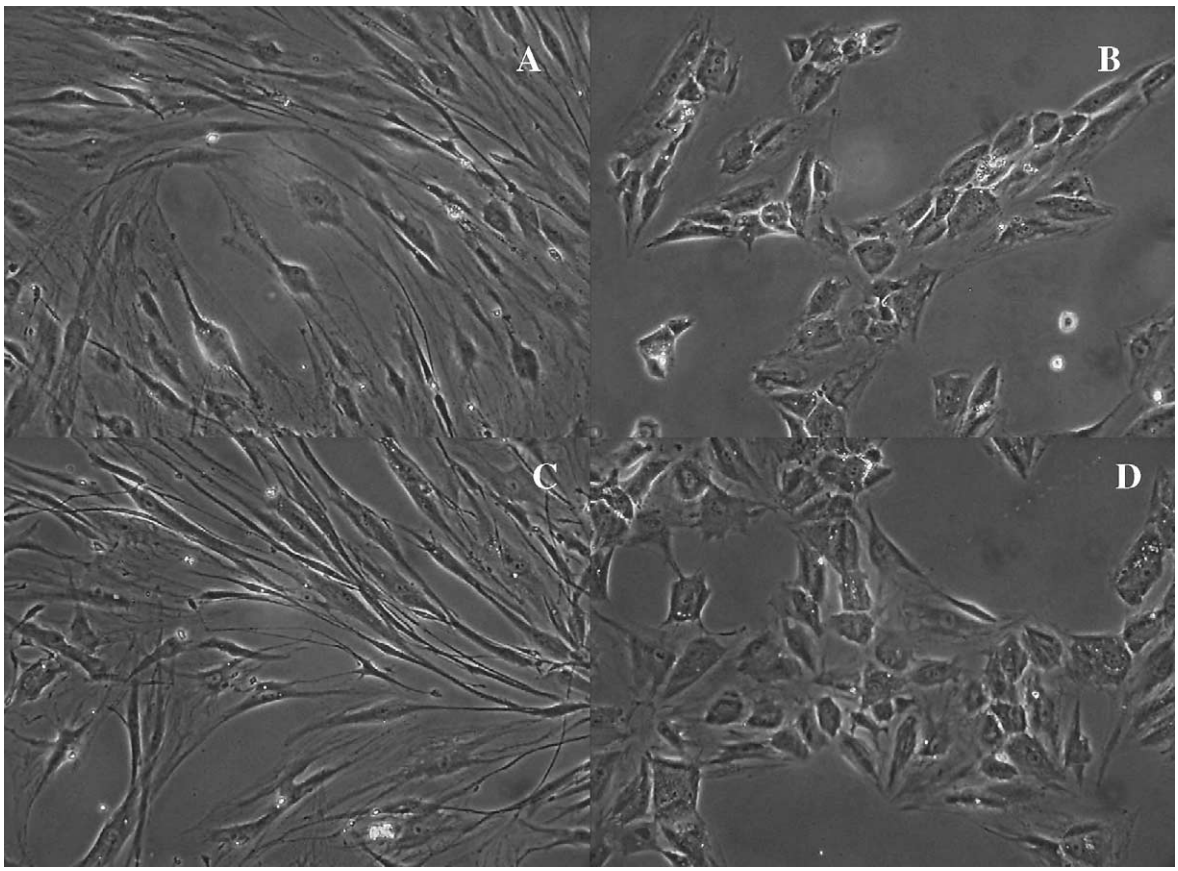


Fig. 2. Morphology change in adult human dermal fibroblasts which have been treated with compounds for 24 h. (A) Control fibroblasts in normal culture media; (B) 0.1 μM 2-MeOE2bisMATE; (C) 20 ng/ml TNF-α; (D): 0.1 μM 2-MeOE2bisMATE and 20 ng/ml TNF-α.

these results suggest that TNF-α co-treatment sensitises HUVECs to apoptosis induced by 2-MeOE2bisMATE and its parent compound.

These data demonstrate the variable sensitivity of different cell types to 2-substituted sulphamated oestrogens and TNF-α induced mitotic arrest. Adult human dermal fibroblasts were the most sensitive cell type with regards to induction of G_2-M arrest (Table 1). Treatment of HUVECs with 2-MeOE2bisMATE did not result in significant G_2-M arrest, but co-treatment with TNF-α increased the number of sub-G_1 cells (Table 1), demonstrating a synergy between the TNF-α and 2-MeOE2bisMATE.

3.2. Analysis of TNF-α and 2-MeOE2bisMATE induction of apoptosis in HUVECs

Flow cytometric cell cycle analysis of drug-treated cells revealed that HUVECs showed potential synergistic induction of apoptosis by a combination of 2-MeOE2bisMATE and TNF-α. To further quantify and confirm the efficacy of TNF-α in increasing the sensitivity of drug-induced apoptosis, a nucleosomal ELISA assay was performed. The concentrations of compounds and TNF-α were identical to those used in the cell cycle analysis, but the cells were harvested after 72 h, as this time-point was determined to give optimal apoptosis for the given conditions. As cell cycle analysis of treated fibroblasts did not exhibit the increase in sub-G_1 population characteristic of apoptosis, they were excluded from this study.

TNF-α treatment alone raised the endogeneous apoptosis in HUVECs by 10% ($P < 0.05$) and was therefore slightly cytotoxic at the concentrations used. 2-MeOE2 and 2-MeOE2bisMATE induced an appreciable increase in apoptosis which was significantly different from control cells ($P < 0.01$) (Fig. 5). Co-treatment of HUVECs with 0.1 μM 2-MeOE2bisMATE and 20 ng/ml TNF-α resulted in a synergistic 33% increase in apoptosis ($P < 0.001$) compared to cells treated only with 0.1 μM 2-MeOE2bisMATE (Fig. 5).

4. Discussion

The endogeneous metabolite 2-MeOE2 has been shown to possess anti-cancer activity in vivo and in vitro and is being investigated as a novel agent for the treatment of cancer. Sulphamoylation of the 3- and 17-positions of the steroid nucleus of 2-MeOE2 has been demonstrated to enhance the

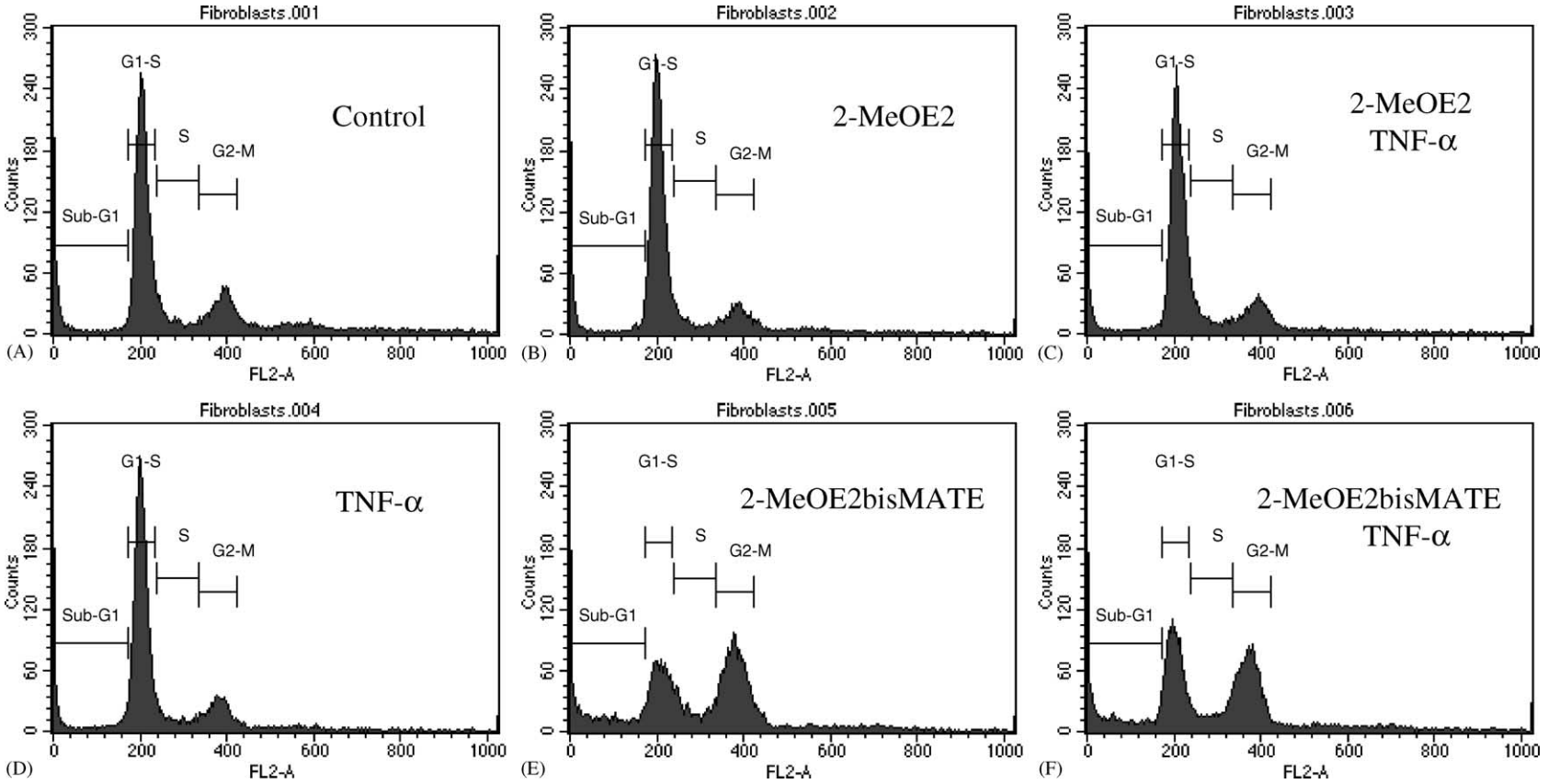


Fig. 3. Induction of G_2-M arrest in adult human dermal fibroblasts by the bis-sulphamoylated derivative of 2-MeOE2. Cells were treated for 48 h with vehicle (A), 0.1 μM 2-MeOE2 (B), 0.1 μM 2-MeOE2 and 20 ng/ml TNF-α (C), 20 ng/ml TNF-α (D), 0.1 μM 2-MeOE2bisMATE (E) and 0.1 μM 2-MeOE2bisMATE and 20 ng/ml TNF-α (F) before being stained with PI for flow cytometric cell cycle analysis. The markers indicate the different stages of the cell cycle. Results presented are representative of two experiments, each performed in duplicate ($n = 2$).

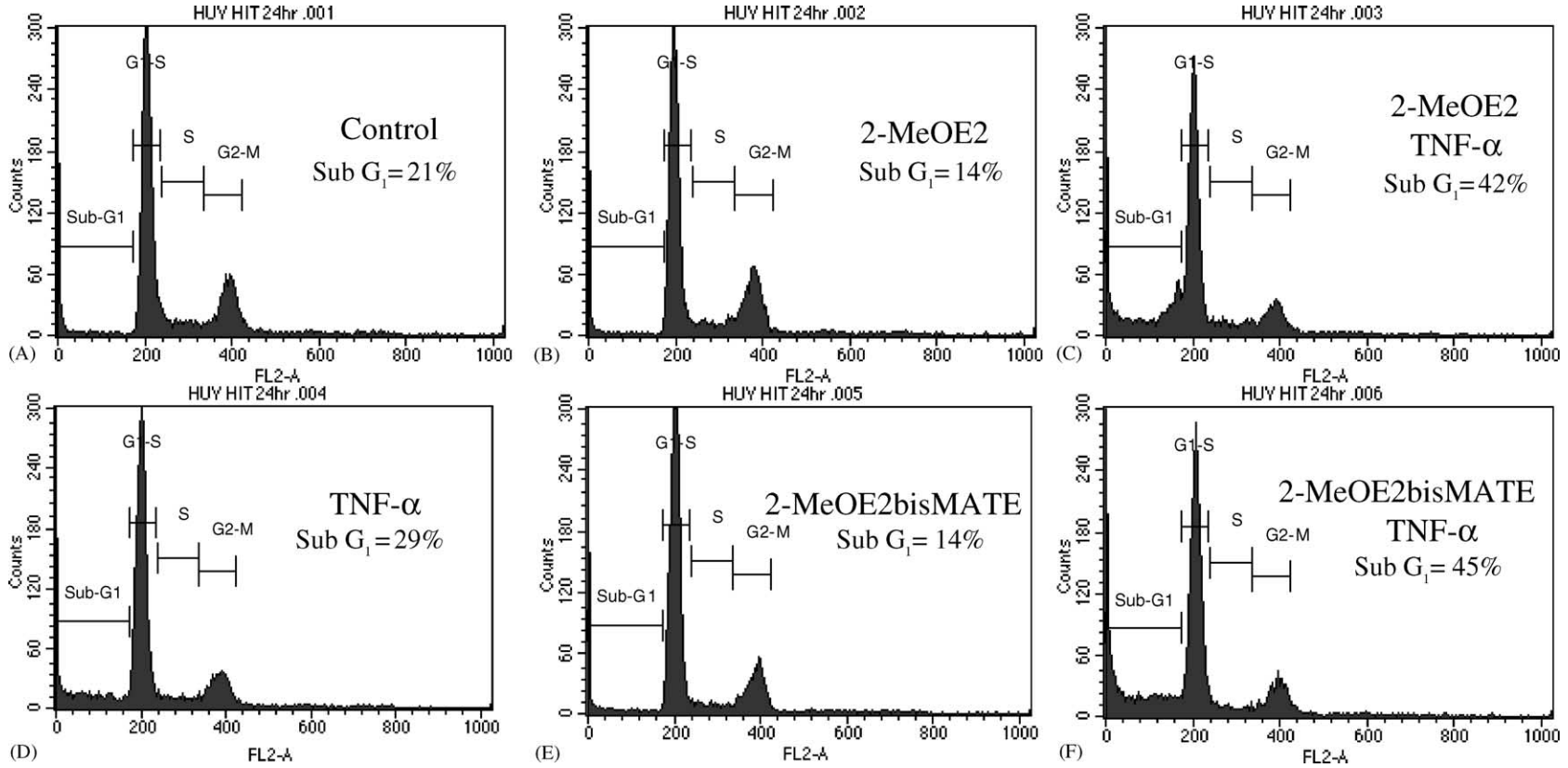


Fig. 4. Synergistic induction of apoptosis in HUVECs through co-exposure to TNF-α and 2-MeOE2 and its bis-sulphamoylated derivative. Cells were treated for 24 h with vehicle (A), 0.1 μM 2-MeOE2 (B), 0.1 μM 2-MeOE2 and 20 ng/ml TNF-α (C), 20 ng/ml TNF-α (D), 0.1 μM 2-MeOE2bisMATE (E) and 0.1 μM 2-MeOE2bisMATE and 20 ng/ml TNF-α (F) before being stained with PI for flow cytometric cell cycle analysis. The markers indicate the different stages of the cell cycle and the percentages quoted represent the fraction of total cells which possess sub-G_1 DNA content, which is indicative of apoptosis. Results presented are representative of two experiments, each performed in duplicate ($n = 2$).

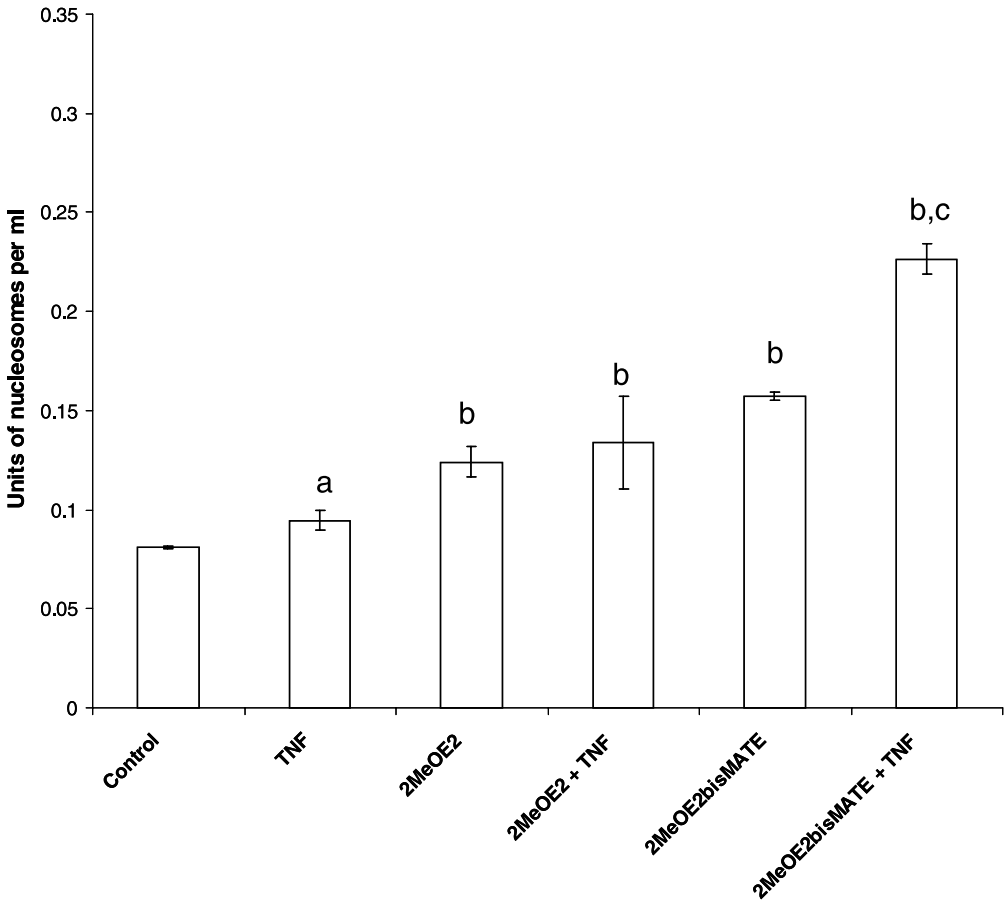


Fig. 5. Synergistic induction of apoptosis in HUVECs by drug and TNF-α co-treatment as measured by nucleosome production. 5×10^5 cells were treated with 20 ng/ml of TNF-α and/or 0.1 μM compound for 72 h before being harvested and used in the nucleosomal ELISA kit. (a) Denotes values that are significantly different ($P < 0.05$) compared to control, and (b) indicates significant difference ($P < 0.01$) compared to control and (c) indicates significant difference ($P < 0.01$) compared to cells treated with compound only, as measured by a two-tailed Student's *t*-test assuming unequal variance. Results presented are representative of three experiments performed in triplicate ($n = 3$).

anti-cancer properties of the parent compound [4]. Our laboratory is interested in the possibility of combination therapy of 2-substituted oestrogens and TNF-α which has been shown to have potent anti-cancer properties but is too toxic to administer alone at an effective dose. It is thought that co-treatment with TNF-α, or induction of its endogeneous upregulation will allow for lower doses of the cytokine to be administered, thus reducing any indiscriminate cytotoxic side effects.

Cell cycle analysis revealed that exposure of fibroblasts to 2-MeOE2bisMATE, but not 2-MeOE2, induces G_2-M arrest and a morphological change, and that co-treatment with TNF-α alone does not have any significant effect. The morphological change and G_2-M arrest were reversible upon removal of the drug. These results suggest that normal human fibroblasts are susceptible to mitotic arrest brought about by treatment with 2-MeOE2bisMATE, and that this arrest does not trigger apoptosis. In contrast, induction of apoptosis in HUVECs by 2-MeOE2 and 2-MeOE2bisMATE is enhanced by low dose TNF-α co-treatment (Fig. 5).

Here we demonstrate that TNF-α co-treatment enhances the efficacy of 2-MeOE2bisMATE as a cytotoxic and cytostatic agent. The combination of the reversible mitotic arrest of fibroblasts and cytotoxic effect on HUVECs makes low dose TNF-α and 2-MeOE2bisMATE treatment a potentially potent anti-angiogenic therapy. To study this hypothesis further, we have carried out preliminary experiments utilising a novel mixed fibroblast/endothelial in vitro model of angiogenesis (TCS CellWorks, UK) and in this assay, 2 ng/ml TNF-α alone completely inhibited angiogenesis. We are currently investigating the increased sensitivity of this assay to TNF-α to allow for further drug combination therapy studies.

To conclude, low dose TNF-α co-treatment increases the levels of apoptosis induced by 2-substituted oestrogens in HUVECs but not in fibroblasts. In addition, 2-MeOE2bisMATE but not 2-MeOE2 treatment induced marked G_2-M arrest in fibroblasts independent of TNF-α co-treatment. The combination of cytostatic effects on fibroblasts and HUVEC cytotoxicity brought about by 2-MeOE2bisMATE and

TNF-α co-treatment offer a novel means of enhancing the anti-angiogenic potential of 2-substituted oestrogens in treatment of various cancers.

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