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Reduction of the Anti-metabolic and Anti-proliferative Effects of Methotrexate by 17β-Oestradiol in a Human Breast Carcinoma Cell Line, MDA-MB-436

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Abstract—*We have investigated the modifying influence of 17β-oestradiol on the anti-metabolic and growth inhibitory actions of methotrexate (MTX) in a human breast cancer cell line, MDA-MB-436. This cell line contains detectable oestrogen receptors but is progesterone receptor-negative. Furthermore, 17β-oestradiol (10^{-10}–10^{-6}M) failed to influence DNA synthetic rate as assessed by [^{3}H]-TdR or [^{3}H]-UdR incorporation and cell proliferative rate was similarly unaffected. Although by these criteria 17β-oestradiol failed to elicit a biological response in the MDA-MB-436 cell line, 10^{-6}M 17β-oestradiol significantly reduced the anti-metabolic and anti-proliferative actions of MTX. In the presence of 17β-oestradiol approximately twice the concentration of MTX was required to inhibit cell proliferation to the same extent as was observed following exposure to MTX alone. This partial reversal of MTX effects was accompanied by a 20% reduction in the steady-state intracellular MTX concentration when cells were exposed to the drug in the presence of 10^{-6}M 17β-oestradiol.*

INTRODUCTION

BREAST cancer has proved responsive to a variety of chemotherapeutic and endocrine manipulative regimes. The response rate for oestrogen therapy can be as high as 85% if patients are selected for treatment on the basis of the oestrogen and progesterone receptor content of their tumours [1]. One of the more useful cytotoxic drugs used in the management of breast cancer is methotrexate (MTX), with objective responses of up to 34% being recorded where MTX has been used as a single agent [2].

However, the results of clinical trials designed to determine whether hormone receptor status affects response to cytotoxic drug therapy have been equivocal [3, 4], as have the results of investigations of the value of combined hormone–cytotoxic drug therapy [5, 6].

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The establishment of several human breast cancer cell lines varying in their ability to synthesise and respond to a number of hormones has greatly facilitated the study of drug-hormone interactions. Thus Weichselbaum *et al.* [7] demonstrated that physiological doses of 17β-oestradiol enhanced the cytotoxicity of cytosine arabinoside towards MCF-7 human breast cancer cells, whilst higher doses failed to influence proliferation kinetics. These results were interpreted as reflecting the ability of low doses of 17β-oestradiol to increase the proportion of cells in S phase, thus potentiating the cell population's sensitivity to a cell cycle phase-specific anti-cancer drug.

Human breast cancer almost certainly consists of cell populations heterogeneous with respect to steroid hormone receptor content [8]. We have therefore investigated the influence of 17β-oestradiol on the anti-metabolic and growth inhibitory effects of methotrexate towards the human breast cancer cell line MDA-MB-436, a cell line which differs from MCF-7 in its hormone sensitivity and receptor content.

MATERIALS AND METHODS

Cell culture conditions

MDA-MB-436 [9–11] were obtained from Flow Laboratories Ltd (Irvine, U.K.) and routinely cultured in L-15 (modified) medium supplemented with 5% foetal calf serum, 100 I.U. penicillin and 100 μg/ml streptomycin in a humidified atmosphere at 37°C. Cells were mycoplasma-free on routine testing [12]. Cells were transferred to L-15 supplemented with 5% 'hormone-free' serum for 4 days before treatment with MTX or 17β-oestradiol to ensure removal of any residual intracellularily bound oestrogens. Foetal calf serum was stripped of endogenous hormones by treatment with dextran T-40- and Norit-A-activated charcoal (Sigma Chemical Co., London, U.K.) at 58°C for 60 min. 'Hormone-free' serum contained $<10^{-11}$M 17β-oestradiol.

Cytotoxicity assays

Incorporation of precursors into DNA and cell population growth rate studies were carried out as previously described [13].

Hormone receptor assays were carried out using the dextran-coated charcoal method for separation of free from receptor bound [^{3}H]-17β-oestradiol [14]. Progesterone receptor content was determined by the method of Pilchon and Milgrom [15], using [^{3}H]-progesterone as ligand. Both radiochemicals were obtained from Amersham International, Bucks, U.K. Receptor content is expressed as the mean of three determinations (S.E.M. <10%).

Methotrexate transport studies

Approximately 5×10^5 cells were plated onto 5-cm Petri dishes (Sterilin Ltd, Teddington, Middx, U.K.) in routine culture medium with and without 10^{-6}M 17β-oestradiol. After 3–4 days the medium was replaced with medium containing 10^{-7}M [^{3}M]-MTX (3,5',7-[^{3}H]-methotrexate, Amersham International Ltd, Bucks, U.K., sp. act. 20 Ci/mmol) with and without 17β-oestradiol. Influx of MTX was assessed at various times up to 48 hr. Triplicate dishes were used at each time point. At each time point the medium containing the tritiated drug was removed and the monolayer washed four times with 3-ml aliquots of isotonic phosphate-buffered saline at 2°C. The cell monolayer was dissolved in 10.0 ml 0.005 M Tris–HCl buffer, pH 7.4, containing 0.15 M NaCl, 0.01 M EDTA, 0.5% (w/v) sodium dodecyl sulphate and 0.02% (w/v) sodium azide. Radioactivity was determined in an aliquot (500 μl) of the resulting solution using an Intertechnique SL-30 liquid scintillation spectrometer with a counting efficiency of 49%. The remainder of the solution was assayed for protein content [16] using human serum albumin (Behringwerke AG, Mannheim, Germany) to prepare the standard solutions. The results were corrected for counting efficiency, protein content and the specific activity of the label and were expressed as fmol MTX/mg protein.

RESULTS

Table 1 shows that the MDA-MB-436 cell line, when grown in hormone-free medium, has an assayable cytoplasmic oetrogen receptor content of 11.6 fmol/mg cytoplasmic protein. However, the MDA-MB-436 cell line oestrogen receptor content is approximately 10% of that reported for the MCF-7 cell line [17, 18]. We have failed to detect progesterone receptors in the MDA-line (Table 1) and physiological concentrations of oestrogen failed to induce progesterone receptor synthesis. Furthermore, [^{3}H]-UdR or [^{3}H]-TdR incorporation was not significantly influenced by a wide range of concentrations of 17β-oestradiol (Figs 1 and 2), and similar results were obtained when the experiments were repeated in serum-free medium (data not shown).

Table 1. Hormone receptor content of the MDA-MB-436 human breast cancer cell line

	Cytosol protein (fmol/mg)	Dissociation constant K_d
CER	11.65	5×10^{-10}
NER	6	3×10^{-10}
PGR	0	—
PRL	82	6.5×10^{-9}

The cells were grown in L-15 medium (modified) supplemented with 5% 'hormone-free' serum. CER, cytoplasmic oestrogen receptor; NER, nuclear oestrogen receptor; PGR, progesterone receptor; PRL, prolactin receptor.

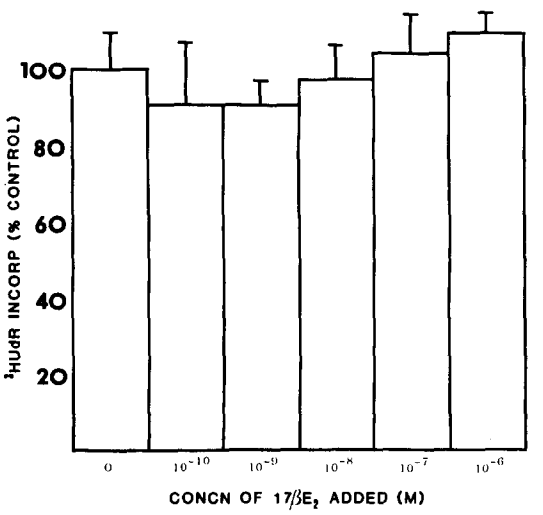

Fig. 1. Effect of 17β-oestradiol on [^{3}H]-UdR incorporation into DNA. The cells were exposed to 17β-oestradiol for 48 hr before determining precursor incorporation into DNA (mean ± S.E. of four determinations).

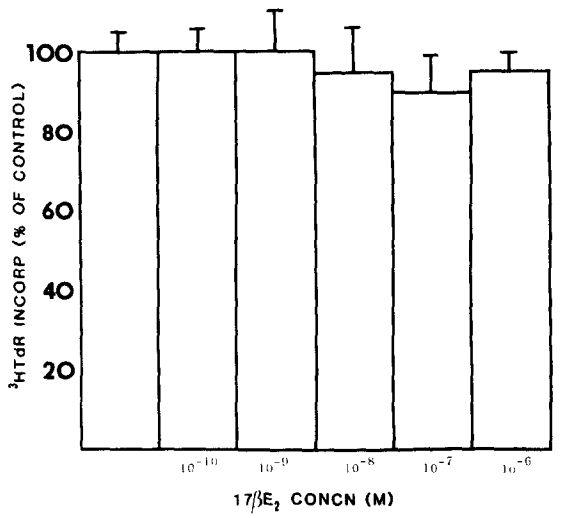

Fig. 2. Effect of 17β-oestradiol on [^{3}H]-TdR incorporation into DNA. The cells were exposed to 17β-oestradiol for 48 hr before determining precursor incorporation into DNA (mean ± S.E. of four determinations).

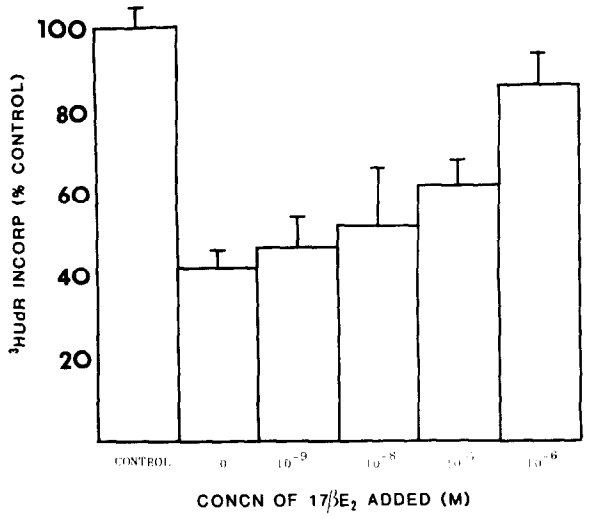

Fig. 3. Effect of 17β-oestradiol and 10^{-8}M MTX on [^{3}H]-UdR incorporation into DNA. Cells were exposed to 17β-oestradiol for 24 hr before exposure to 17β-oestradiol + 10^{-8} MTX for a further 24 hr. Precursor incorporation was determined at the end of the drug exposure period (mean ± S.E. of four determinations).

A 24-hr exposure of MDA-MB-436 cells to 10^{-8}M MTX resulted in a 60% inhibition of DNA synthesis, as determined by [^{3}H]-UdR incorporation (Fig. 3). Co-administration of 17β-oestradiol led to a dose-dependent reversal of the anti-metabolic effect of MTX, which reached significance at 10^{-6}M 17β-oestradiol (Fig. 3).

Figure 4 shows the dose-dependency of the anti-metabolic action of MTX in this cell and the ability of 10^{-6}M 17β-oestradiol to reverse the effect of all concentrations of MTX tested. Figure 5 demonstrates that inhibition of [^{3}H]-UdR incorporation leads to a reduction in the proliferative capacity of the cell population. Furthermore, the ability of 10^{-6}M 17β-oestradiol to reverse MTX-induced inhibition of [^{3}H]-UdR incorporation also leads to a partial reversal of MTX-induced population growth delay. Alone, 10^{-6}M 17β-oestradiol had no effect on the rate of proliferation of MDA-MB-436 cells over the 6-day experimental period.

In an attempt to determine the mechanism of the protective effect of 17β-oestradiol described, its influence on MTX transport kinetics was investigated. Co-administration of 10^{-6}M 17β-oestradiol led to a 20% reduction in the intracellular steady-state level of MTX achieved

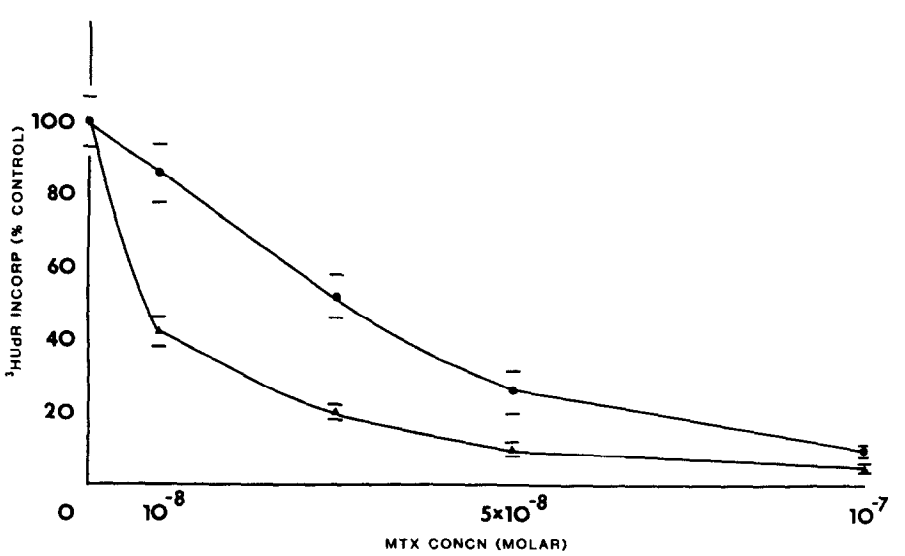

Fig. 4. Effect of MTX ± 10^{-6}M 17β-oestradiol on [^{3}H]-UdR incorporation into DNA. Cells were either exposed to 10^{-6}M 17β-oestradiol for 24 hr before exposure to 10^{-6}M 17β-oestradiol + MTX for a further 24 hr (▲——▲) or were exposed to MTX alone (●——●). Precursor incorporation was determined at the end of the drug exposure period (mean ± S.E. of four determinations).

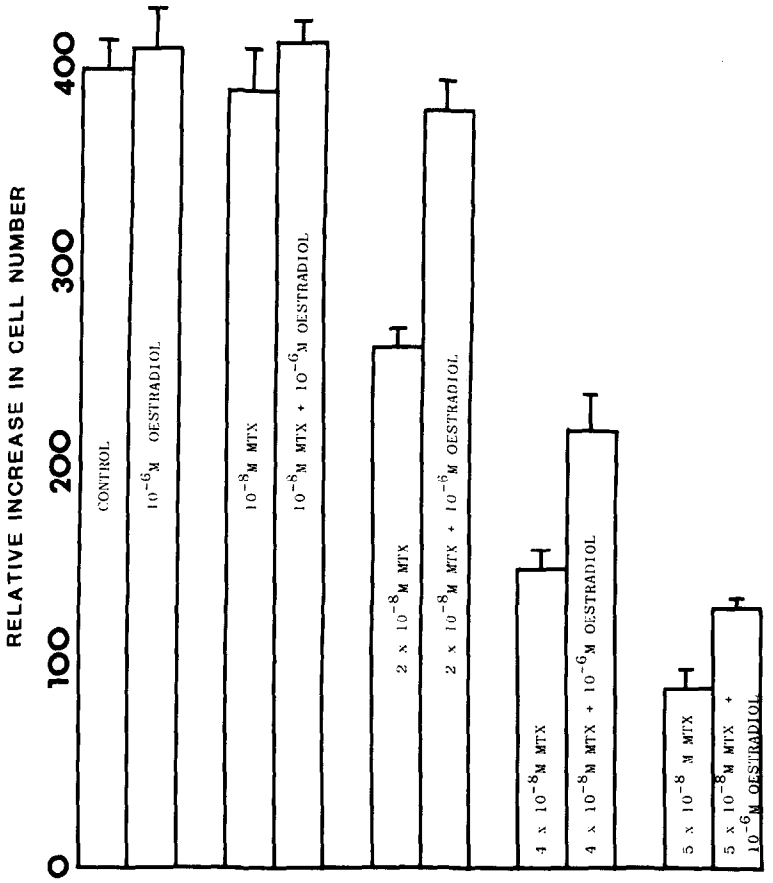

Fig. 5. Effect of MTX ± 10^{-6}M 17β-oestradiol on cell population growth delay. Cells were either exposed to 10^{-6}M 17β-oestradiol for 24 hr before exposure to 10^{-6}M 17β-oestradiol + MTX or were exposed to MTX alone. Cell number was determined at the end of the drug exposure period (day 0) and on day 6, with a medium change on day 3 (mean ± S.E. of four determinations). Relative increase in cell number is expressed as the cell number on day 6 as a percentage of that at the end of the drug exposure period.

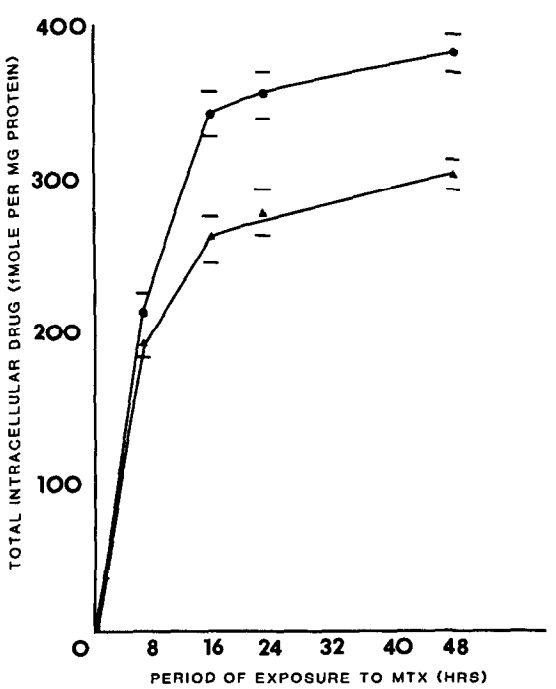

Fig. 6. The influence of 10^{-6}M 17β-oestradiol on the time course of [^{3}H]-MTX influx into MDA-MB-436 cells.

following exposure of MDA-MB-436 cells to 10^{-7}M [^{3}H]-MTX for a 48-hr period (Fig. 6).

DISCUSSION

The MDA-MB-436 cell line was established from a plural effusion in a patient with stage 3 metastatic breast carcinoma [9]. Evidence for its epithelial origin has been presented [9] and it has been distinguished from other breast tumour cell lines in the MDA-MB series by allozyme phenotype analysis [10]. The MDA-MB-436 cell line possesses membrane-associated mammary tumour glycoprotein [11], but fails to produce tumours in nude mice [9].

The modulating influence of 17β-oestradiol on cytotoxic drug-induced perturbations of DNA synthesis and cell proliferation in the MDA-MB-436 cell line contrast markedly with previously reported data using the MCF-7 cell line [7]. Our results demonstrate that, unlike the MCF-7 cell line [17], the MDA-MB-436 line possesses low levels of oestrogen receptor and is progesterone receptor-negative (Table 1). Physiological and

pharmacological concentrations of 17β-oestradiol failed to influence DNA synthesis in the MDA-MB-436 line as assessed by [^{3}H]-TdR or [^{3}H]-UdR incorporation (Figs 1 and 2). Furthermore, 17β-oestradiol was not mitogenic in this cell line. Lippman *et al.* [18] reported that physiological and pharmacological concentrations of 17β-oestradiol stimulated [^{3}H]-TdR incorporation in MCF-7 cells, although the mitogenic effect of the hormone reported by these authors has been questioned [19]. Nevertheless, Weichselbaum *et al.* [7] demonstrated that physiological concentrations of 17β-oestradiol potentiated the cytotoxicity of cytosine arabinoside in MCF-7 cells and concluded that this effect was a reflection of the hormone's mitogenic potential. Physiological concentrations of 17β-oestradiol failed to influence the anti-metabolic effect of methotrexate significantly in the MDA-MB-436 cell line, although a trend to reversal of MTX effect was observed (Fig. 3). 10^{-6}M 17β-oestradiol significantly reversed the anti-metabolic (Fig. 4) and anti-proliferative (Fig. 5) effects of MTX at all drug concentrations tested. In the presence of 10^{-6}M 17β-oestradiol, approximately twice the concentration of MTX was required during a 24-hr exposure period to achieve the same degree of cell population growth rate inhibition that resulted from exposure to MTX alone.

The inability of 17β-oestradiol to induce progesterone receptor synthesis or increase DNA synthetic rate or cell proliferation suggests that the MDA-MB-436 cell line possesses defects distal to the oestrogen receptor. The modulating influence of 17β-oestradiol on MTX cytotoxicity is therefore unlikely to be hormone receptor-mediated or due to cell population kinetic changes. The data shown in Fig. 5 shows that the 17β-oestradiol-induced reversal of MTX anti-metabolic and anti-proliferative effects correlate with a 20% reduction in intracellular steady-state levels of MTX following exposure of the cells to 10^{-6}M [^{3}H]-MTX. The mechanism underlying this effect of 17β-oestradiol on MTX transport is unclear. It has been shown that pharmacological doses of oestradiol significantly reduce both amino acid transport and insulin binding in rat mammary adenocarcinoma *in vitro* [20], and it is possible that oestradiol-induced changes in membrane structure or fluidity may account for these observations. Such changes may also reduce the number or affinity of MTX transport proteins in the cell membrane of MDA-MB-436 cells.

Further insight into the mechanism of 17β-oestradiol modulation of MTX transport will require a more detailed study using a range of extracellular MTX concentrations and incorporating investigations of drug efflux.

Our results suggest that the influence of steroid hormones on the cytotoxicity of drugs used in the treatment of breast cancer will be dependent both on the concentration of the hormone and the receptor status of the target cell. This may have important consequences for combined hormone–drug therapy of human breast cancer consisting of a heterogenous population of tumour cells.

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