Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Steroid Biochemistry & Molecular Biology 88 (2004) 37–51
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

The proliferative effects of 5-androstene-3β,17β-diol and 5α-dihydrotestosterone on cell cycle analysis and cell proliferation in MCF7, T47D and MDAMB231 breast cancer cell lines

S.R. Aspinall*, S. Stamp, A. Davison, B.K. Shenton, T.W.J. Lennard

School of Surgery and Reproductive Sciences, The Medical School, University of Newcastle, Newcastle upon Tyne, Benton NE2 4HH, UK

Received 3 March 2003; accepted 10 October 2003

Abstract

Epidemiological studies suggest that precursor steroids are implicated in the aetiology of breast cancer. However, our understanding of the role of precursor steroids in breast cancer is complicated by fact that there are many precursor steroids, which are metabolically inter-related and have divergent proliferative activities on the growth of breast cancer cell lines. In this study the proliferative affects of 5α-dihydrotestosterone and 5-androstene-3β,17β-diol, which may be considered true metabolites acting at a tissue level, on MCF7, T47D and MDAMB231 breast cancer cell lines have been examined by a flow cytometric technique. DNA cell cycle analysis demonstrates that 5-androstene-3β,17β-diol stimulates the proliferation of hormone-dependent cell lines at physiological levels by an oestrogen receptor mediated mechanism whereas 5α-dihydrotestosterone does not affect the proliferation of MCF7 and T47D cell lines at physiological levels over short (48 h) incubations. Both 5α-dihydrotestosterone and 5-androstene-3β,17β-diol stimulate proliferation of hormone-dependent cell lines at pharmacological levels via and interaction with the oestrogen receptor. In long (6–9 days) incubations both 5α-dihydrotestosterone and 5-androstene-3β,17β-diol inhibit the 17β-oestradiol induced proliferation of MCF7 and T47D cell lines, however, 5α-dihydrotestosterone inhibits while 5-androstene-3β,17β-diol stimulates basal proliferation. These cell line studies suggest a model for the role of precursor steroids in pre- and postmenopausal breast cancer.
© 2004 Elsevier Ltd. All rights reserved.

Keywords: Breast cancer; Androgens; 5-Androstene-3β,17β-diol; 5α-Dihydrotestosterone; MCF7; T47D; MDAMB231 breast cancer cell lines

1. Introduction

Several lines of evidence now implicate precursor steroids in the aetiology of breast cancer. Epidemiological studies have established that serum precursor steroids are elevated in postmenopausal women who develop breast cancer [13] and subnormal in premenopausal women who develop breast cancer [4,5].

Our understanding of the mechanism of action of precursor steroids, however, has been hindered by the findings that precursor steroids are metabolically inter-related and they can inhibit or stimulate the proliferation of hormone-dependent breast cancer through different mechanisms and this activity depends on the nature of the precursor steroids, its concentration, the hormone receptor profile of the breast cancer and the endocrine status of the patient [6].

The metabolism of precursor steroids (Fig. 1) is illustrated below. Precursor steroids are metabolised:

(a) by the sulfatase enzymes to 5-androstene-3β,l7β-diol a metabolite with high affinity for the oestrogen receptor;
(b) via androstenedione and testosterone to 5α-dihydrotestosterone which has high affinity for the androgen receptor;
(c) by the aromatase enzymes to oestrogens.

The ovary and the adrenal cortex both contribute to circulating levels of precursor steroids. However, neither produce significant quantities of either 5α-dihydrotestosterone or 5-androstene-3β,17β-diol. 5α-Dihydrotestosterone and 5-androstene-3β,17β-diol may be considered true metabolites, which act at the tissue level [8]. In the present study, we have therefore examined the actions of 5α-dihydrotestosterone and 5-androstene-3β,17β-diol on breast cancer cell lines.

The oestrogenic actions of androgens are established and are the subject of a review by Rochefort and Garcia [7]. Much attention has centred on 5-androstene-3β,17β-diol, an androgen termed "hermaphrodiol" due to its relatively high affinity for the oestrogen and androgen receptor [6,9]. 5-Androstene-3β,17β-diol induces the secretion of a 46 K oestrogen-dependent protein and stimulates the proliferation

* Corresponding author. Fax: +44-191-222-8514.
E-mail address: sebaspinall@tiscali.co.uk (S.R. Aspinall).

0960-0760/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2003.10.011

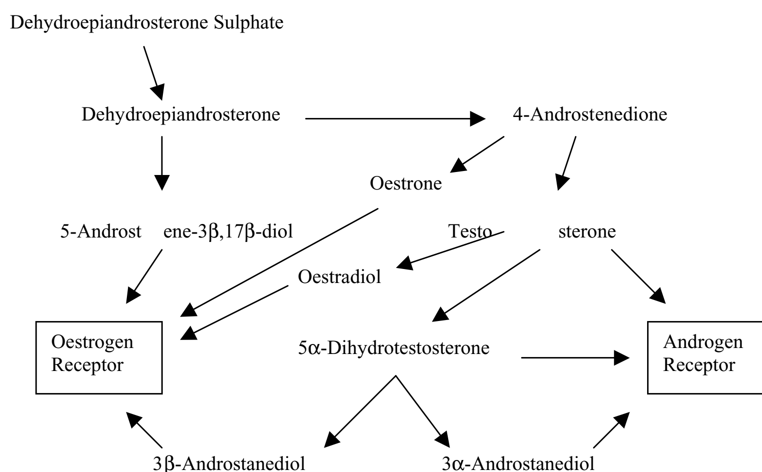


Fig. 1. The metabolism of precursor steroids [7].

of MCF7 [10–12] and ZR751 [13] breast cancer cell lines at physiological concentrations. This action has been demonstrated to occur via a direct interaction with the oestrogen receptor [14].

In addition to its oestrogenic actions, at physiological levels 5-androstene-3β,17β-diol antagonises the oestrogen-induced proliferation of MCF7 cells by an androgen receptor mediated mechanism [11,12].

5-Androstene-3β,17β-diol is formed by peripheral conversion of DHEA, which is in equilibrium with DHEAS thus providing a buffer to maintain levels of its metabolites [8]. In contrast to 5-androstene-3β,17β-diol, DHEA has a very low and DHEAS a negligible affinity for the oestrogen receptor [7]. However, DHEAS and DHEA have both been shown to stimulate the proliferation of hormone-dependent cancer cell lines, the former at physiological levels [10,15] and to be elevated in the serum of postmenopausal women who develop breast cancer [1–3].

The mechanism of action of DHEAS was unknown. However, it has been shown that sulfatase inhibitors block the DHEAS-stimulated growth of MCF7 cells [16]. This suggests that the mitogenic affect of DHEAS on breast cancer cell lines is mediated in part via its metabolism to 5-androstene-3β,17β-diol. This pathway is of clinical significance as it enables the ongoing stimulation of hormone-dependent breast cancers by adrenal androgens in patients on aromatase inhibitors.

The interim findings of the Arimidex, Tamoxifen, alone or in combination (ATAC) trial [17] demonstrate that aromatase inhibitors are superior to tamoxifen as the adjuvant therapy postmenopausal women with breast cancer. In light of these findings the evidence-base for the routine use of aromatase inhibitors in clinical practice is currently being established [18]. The properties of 5-androstene-3β,17β-diol are therefore of particular significance and it is timely therefore to examine the effects 5-androstene-3β,17β-diol on breast cancer growth.

5α-Dihydrotestosterone, an androgen with high affinity for the androgen receptor and low affinity for the oestrogen receptor [7] has also been shown to have anti-oestrogenic activity at physiological levels in the MCF7 cell line [19]. In steroid free medium physiological levels of 5α-dihydrotestosterone inhibit the proliferation of T47D, ZR751 and MFM223 breast cancer cell lines via an interaction with the androgen receptor [20–22]. Although 5α-dihydrotestosterone stimulates the proliferation of MCF7 and MDAMB453 cell lines at physiological levels this may be due to the activation of androgen receptor-independent pathways by active metabolites with oestrogenic activity [21,23]. At pharmacological levels, 5α-dihydrotestosterone has been shown to stimulate the proliferation of MCF7 cells via the oestrogen receptor [24,25].

In general it appears that androgen receptor mediated mechanisms in breast cancer inhibit growth. The mechanism of this action is unknown. However, recently it has been suggested that this effect may he mediated by the production of one or more proteins, which prevent entry of the target cell into the cell cycle. These proteins have been termed androcyclone-II [26]. The aim of the present study was therefore to examine the affects of 5-androstene-3β,17β-diol and 5α-dihydrotestosterone on cell cycle and proliferation using a flow cytometric technique. The hormone-dependent cell lines MCF7 and T47D have been examined as well as the hormone-dependent cell line MDA231.

2. Materials and methods

2.1. Cell line studies

Cell lines were used as an in vitro model to assess the effects of 5α-dihydrotestosterone (Sigma–Aldrich, Poole, UK), 5-androstene-3β,17β-diol (Sigma–Aldrich, Poole, UK) and l7β-oestradiol (Sigma–Aldrich, Poole, UK) on

cell cycle and cell proliferation. The study of synchronous populations of cells allows steroid induced changes in the cell cycle to be quantified. The breast cancer cell lines used are all epithelial cells, which grow as a monolayer. They were the MCF7 cell line (European Collection of Animal Cell Cultures, ECACC, Salisbury, Wiltshire, UK), which was established from a pleural effusion obtained from a 69-year-old female [27]: the T47D cell line (ECACC) which was established from a 54-year-old female with an infiltrating ductal carcinoma of the breast [28] and the MDA-MB-231 cell line CACC) which was established from the pleural effusion of a 51-year-old female with breast carcinoma [29].

To achieve synchronous cell populations, cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 1000 mg/l glucose and without sodium pyruvate (GIBCO Life Technologies, Paisley, UK) supplemented with 10% heat-inactivated foetal calf serum (Sigma, Poole, UK), 1% L-glutamine (GIBCO) and 0.5% gentamycin (GIBCO) in 75 cm^3 flasks (Corning Ltd, High Wycombe, UK) at 37 °C in a humidified atmosphere containing 5% CO_2 in air. Cells were passaged every 3–4 days when they had grown to 80% confluence, when they were harvested 1 × trypsin/EDTA (GIBCO Life Technologies, Paisley, UK).

The cells were then pelleted by centrifugation and re-suspended in stripped medium containing DMEM with 1000 mg/l glucose and without phenol red or sodium pyruvate (GIBCO) supplemented with 10% charcoal/dextran treated foetal calf serum (HyClone, Perbio Science UK Ltd, Cheshire, UK), 1% L-glutamine (GIBCO) and 0.5% gentamycin (GIBCO). Cells were seeded in 12-well culture plates (Corning) for cell cycle studies and 6-well plates (Corning) for proliferation studies. The cells were allowed to adhere to the wells for 24 h. At this point, DNA cell cycle analysis or cell proliferation was determined, as outlined below. The stripped medium was aspirated from the remaining wells and replaced with stripped medium supplemented with steroids containing 0.01% ethanol (BDH Chemicals, Poole, UK). Wells were aspirated and fresh steroid medium was added every 48 h.

2.2. Staining and analysis of DNA

The DNA content of a cell cycle determined by flow cytometry provides information on aneuploidy (i.e. an abnormal amount of DNA) and gives a static estimate of the percentage of cells present in each phase of the cell cycle (i.e. G0/1, S and G2/M).

The following method was used to prepare and stain cell lines for DNA analysis. Cultured cells were harvested with 1 × trypsin/EDTA and pelleted by centrifugation at 2500 rpm for 5 min. The cell pellet was resuspended in 350 μl of full medium and incubated for 5 min at 37 °C. One hundred microlitres of 0.25 mg/ml propidium iodide (PI, Sigma, Poole, UK) containing 5% Triton X-100 (BDH Chemicals, Poole, UK) and 50 μl of 1 mg/ml RNAse (Sigma, Poole, UK) was

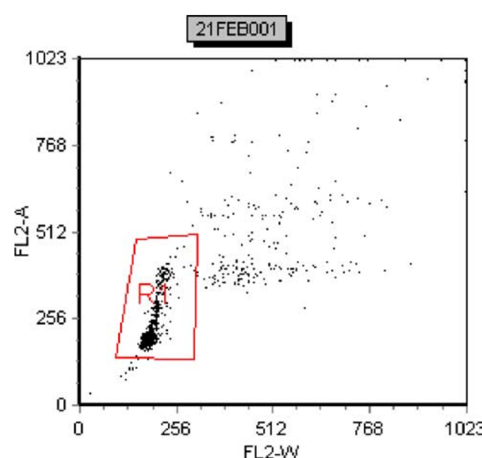


Fig. 2. Dot plot of fluorescence-2 area vs. width with gate to exclude doublet, triplet, etc. molecules.

added to the cell suspension. Analysis was performed on a Becton Dickinson FACScan using LYSYS II software with pre-stored settings.

Permeabilisation of the cell membrane with the detergent Triton X-100 was required to allow the dye propidium iodide access to the DNA. RNAse was required to remove RNA to which the propidium iodide would otherwise bind. Propidium iodide is a nucleic acid dye, which intercalates between the bases in double stranded nucleic acids. When excited by a 488 nm laser, propidium iodide fluoresces at 620 nm, which can be detected by fluorcscence-2.

Data files were converted from HP to PC format using HP disk (Applied Cytometry Systems, South Yorkshire, UK). DNA data analysis was then performed using Multicycle software (Phoenix Flow Systems, San Diego, USA). This software uses algorithms to decouvolute the DNA histogram and fit a cell cycle model. The DNA is identified on a dot plot of fluorescence-2 area against fluorescence-2 width and gated to exclude doublet, triplet molecules (Fig. 2).

A fluorescence-2 histogram of these gated events displays the DNA profile, and the Multicycle was then used to fit the three phases of the cell cycle and calculate the percentage of cells within each phase (Fig. 3). A DNA histogram exhibiting more or less than one G0/1 peak is classified as aneuploid [30].

2.3. Cell proliferation studies

To assess the effect of steroids on the proliferation of cultured cells, the following method was followed. Cultured cells were harvested from 6-well plates by 1 × trypsin/EDTA and pelleted by centrifugation at 2500 rpm for 5 min in falcon tubes. The cells were then resuspended in 350 μl of strip medium and incubated for 5 min at 37 °C. Forty microlitres of 1 mg/ml propidium iodide (Sigma, Poole, UK) and 10 μl of flow count fluorospheres (Coulter, Bedfordshire, UK) were added. Analysis was performed on a Becton

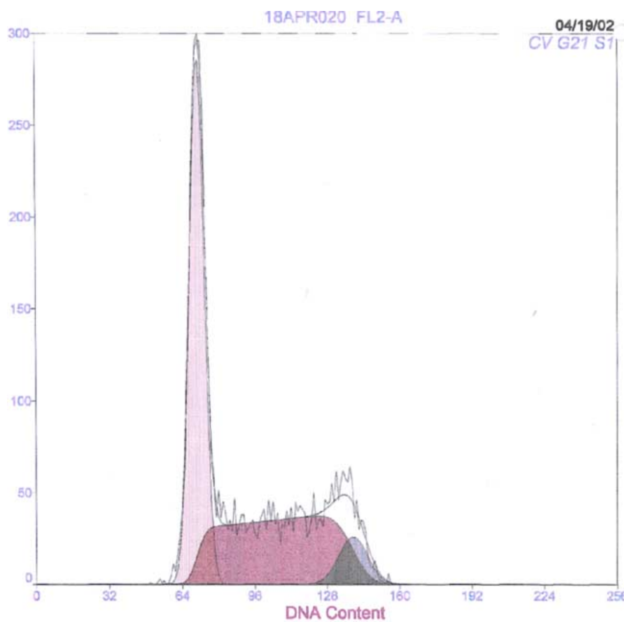


Fig. 3. DNA histogram showing percentage of cells in each phase of the cell cycle.

Dickinson FACScan using LYSYS II software on pre-stored settings.

The incubation step prior to adding propidium iodide was necessary, as harvesting with 1 × trypsin/EDTA damages the cell membrane rendering the cell leaky to small molecules such as propidium iodide. The incubation step enabled the cells to repair the cell membrane allowing all viable cells to remain impenetrable to propidium iodide. Therefore, propidium iodide was excluded from viable cells with an intact cell membrane, but intercalated with DNA from dead cells with a leaky cell membrane. Flow count fluorospheres consist of 10 μm polystyrene fluorescent particles in an aqueous solution at a known concentration per microlitre. They are excited by laser light at 488 nm and have an emission spectrum of 525–700 nm.

Cells were analysed on a dot plot of FSC against SSC and a R1 gate set around cells to exclude debris and an R2 gate set on the fluorospheres. A dot plot of fluorescence-1 against fluorescence-2 was constructed for all events within the R1 and R2 regions (Fig. 4). Dead cells took up propidium iodide staining with higher fluorescence-2 intensity, and therefore could be easily identified. Quadrants were set so that the upper left area contained the dead cells, the lower left area the live cells and the upper right area the fluorospheres.

Using the number of events in each quadrant the number of live cells was calculated using the following formula:

$$\frac{\text{number of events in lower left}}{\text{number of events in upper right}} \times \text{fluorospheres per } \mu\text{l} \times \text{volume of fluorospheres added to sample } (\mu\text{l})$$

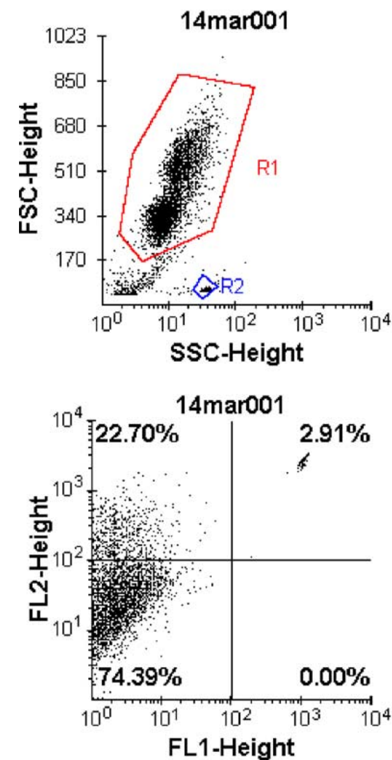


Fig. 4. Dot plot of forward scatter (FSC) against side scatter (SSC) with cells gated R1 and fluorospheres gated R2. Fluorescence-2 against fluorescence-1 dot plot of events within R1 and R2 gates, quadrants have been set to distinguish live from dead cells and fluorospheres.

3. Results

3.1. Analysis of steroid hormone receptor expression of breast cancer cell lines

The cell lines used in this study, namely MCF7, T47D and MDAMB231 express different levels of steroid receptors [31]. MCF7 and T47D cells have been reported to express oestrogen and androgen receptors, though the reported level of steroid receptor expression differs between studies

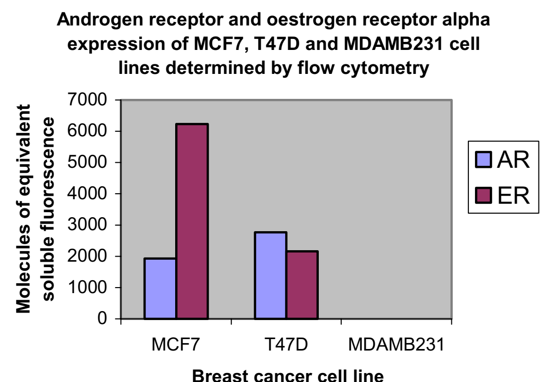


Fig. 5. Androgen and oestrogen receptor alpha expression of MCF7, T47D and MDAMB231 cell lines.

[21,23,28,32] MDAMB231 cells do not express steroid receptors [31].

The steroid receptor expression of the cell lines used in the present study has been determined using a flow cytometric technique. Flow cytometry is an established technique for

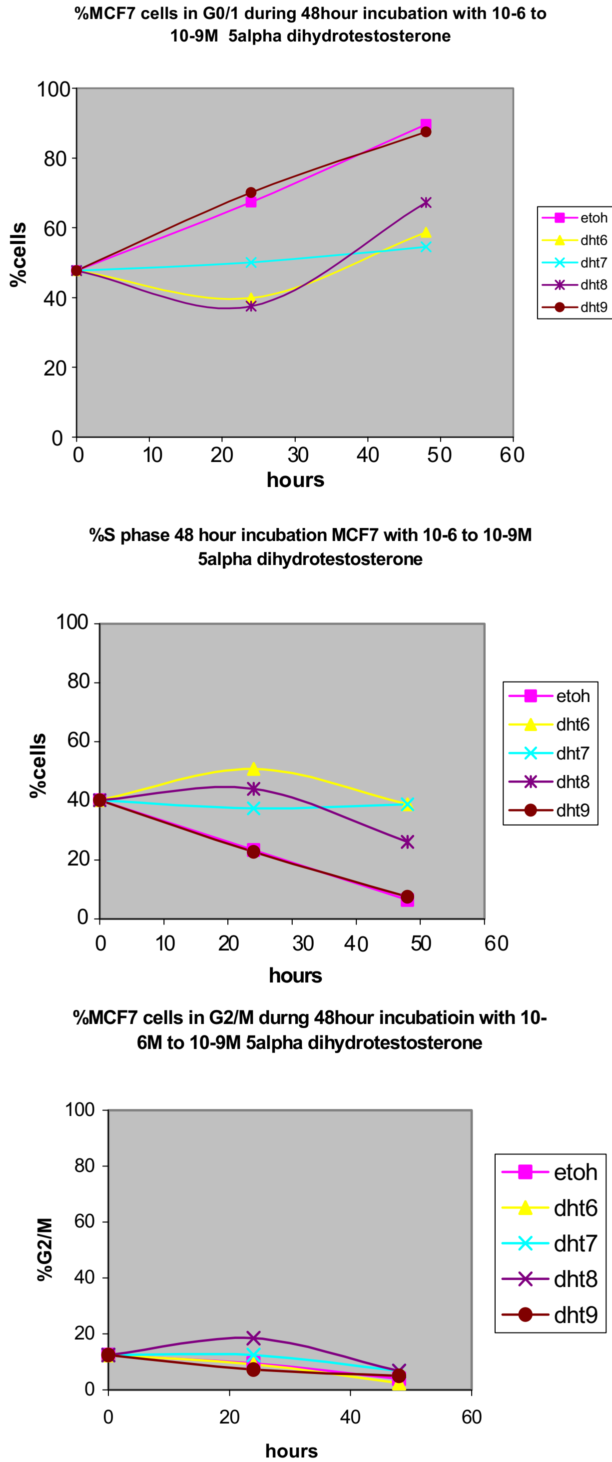


Fig. 6. Percentage of MCF7 cells in G0/1, S and G2/M on incubation with 10^{-9} to 10^{-4} M 5α-dihydrotestosterone (nDHT = 10^{-n} M 5α-dihydrotestosterone, ETOH = 0.01% ethanol).

the determination of cytoplasmic and nuclear antigens [33]. Methods for the determination of oestrogen receptor alpha and androgen receptor expression in breast cancer [34] have been established in our laboratory. Details of the method for determination of oestrogen receptor alpha expression are included in the above publication. The method for androgen receptor follows a similar method using an androgen receptor antibody AR 441 (DAKO, Cambridge, UK) The steroid receptor profile is illustrated in Fig. 5.

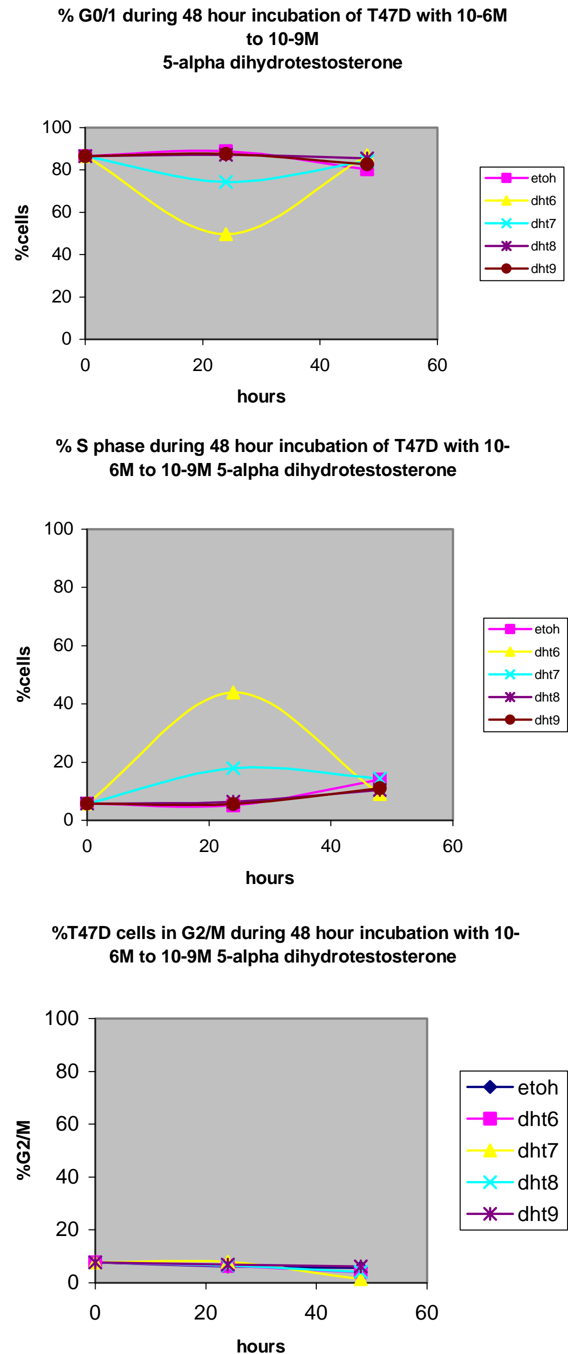


Fig. 7. Percentage of T47D cells in G0/1, S and G2/M on incubation with 10^{-9} to 10^{-6} M 5α-dihydrotestosterone (nDHT = 10^{-n} M 5α-dihydrotestosterone, ETOH = 0.01% ethanol).

3.2. Analysis of DNA cell cycle during 48-h incubation of MCF7, T47D and MDA231 with 5α-dihydrotestosterone +/− faslodex

MCF7, T47D and MDA231 breast cancer cell lines were incubated over 48 h with a range of concentrations of 5α-dihydrotestosterone. The percentage of cells in each phase of the cell cycle G0/1, S and G2/M was determined at 0, 24 and 48 h as outlined in Section 2.

MCF7 Cells were incubated with a range of concentrations of 5α-dihydrotestosterone from physiological (10^{-9} to 10^{-8} M concentrations) to phamacological levels (10^{-7} to 10^{-6} M). Fig. 6 illustrates that pharmacological levels of 5α-dihydrotestosterone resulted in a fall in the percentage of cells in G0/1 arid a rise in the percentage of cells in S phase within 24 h. These findings would be interpreted as a stimulation of proliferation. Likewise, 10^{-8} M 5α-dihydrotestosterone stimulated the proliferation

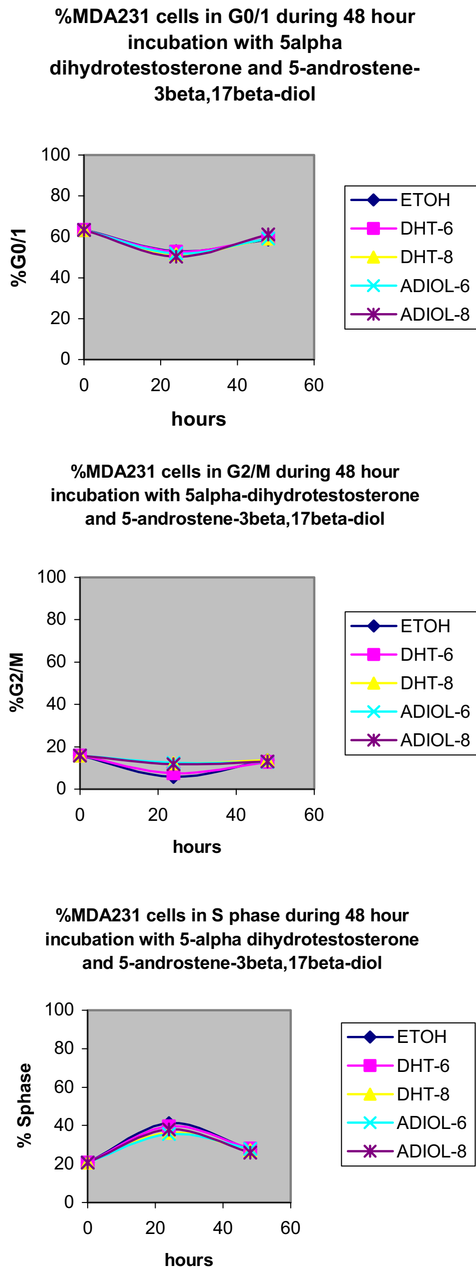
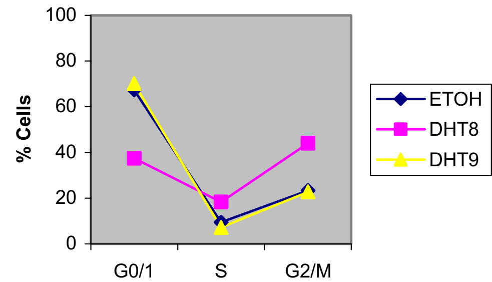
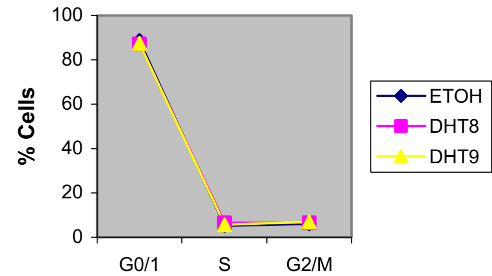


Fig. 8. Percentage of MDA231 cell lines on incubation with 10^{-8} and 10^{-6} M 5α-dihydrotestosterone and 5-androstene-3β,17β-diol (nDHT = 10^{-n} M 5α-dihydrotestosterone, ETOH = 0.01% ethanol).





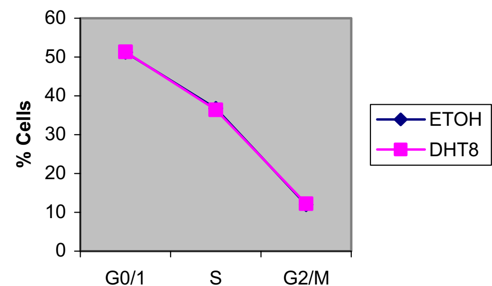


Fig. 9. Percentage of MCF7, T47D and MDA231 cells in G0/1, S and G2/M on incubation with 10^{-9} and 10^{-8} M 5α-dihydrotestosterone (nDHT = 10^{-n} M 5α-dihydrotestosterone, ETOH = 0.01% ethanol).

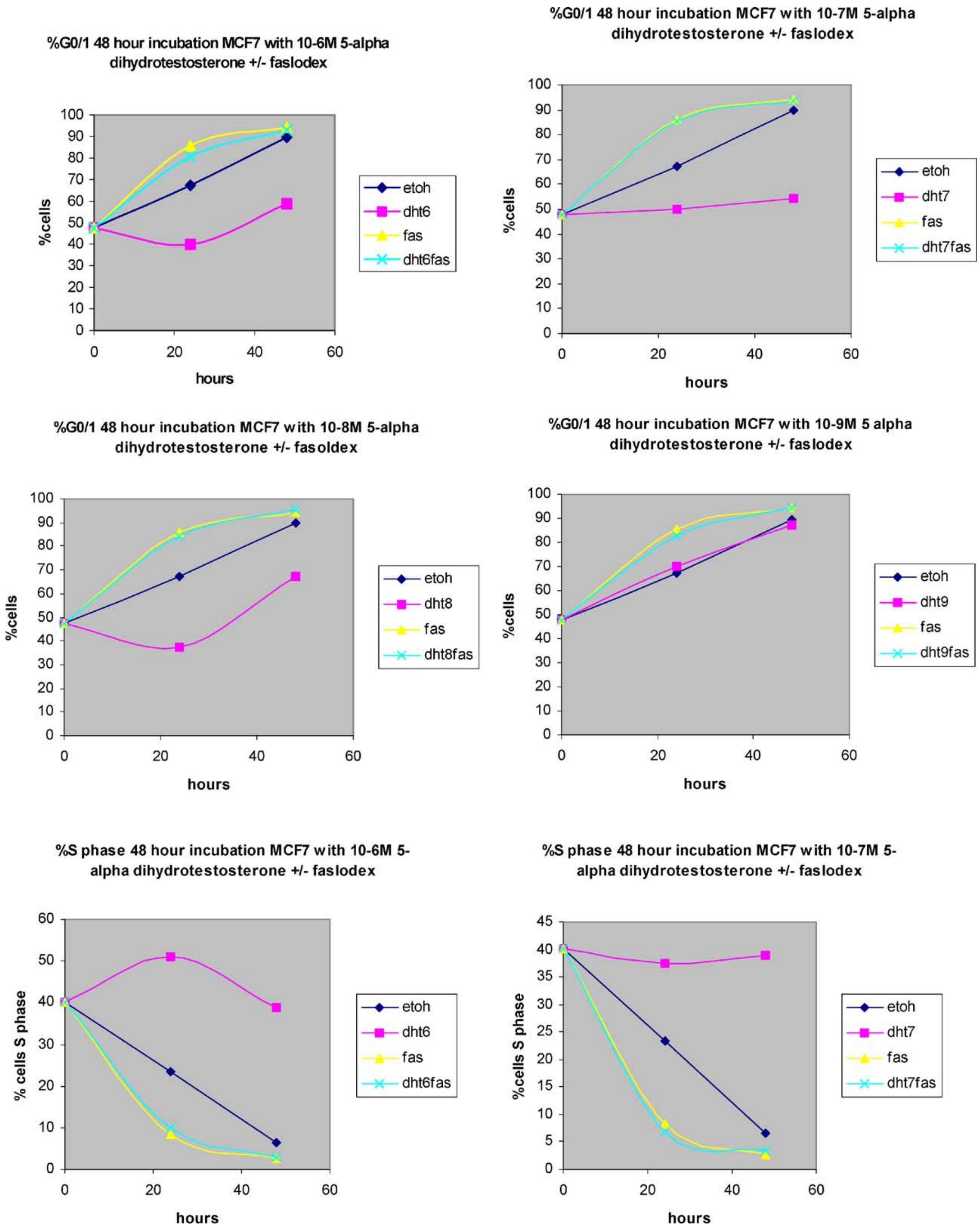


Fig. 10. Percentage of MCF, T47D cells in G0/1, S phase on incubation with 10^{-9} to 10^{-6} M 5α-dihydrotestosterone and 10^{-6} M faslodex (nDHT = 10^{-n} M 5α-dihydrotestosterone, ETOH = 0.01% ethanol, fas = 10^{-6} M faslodex).

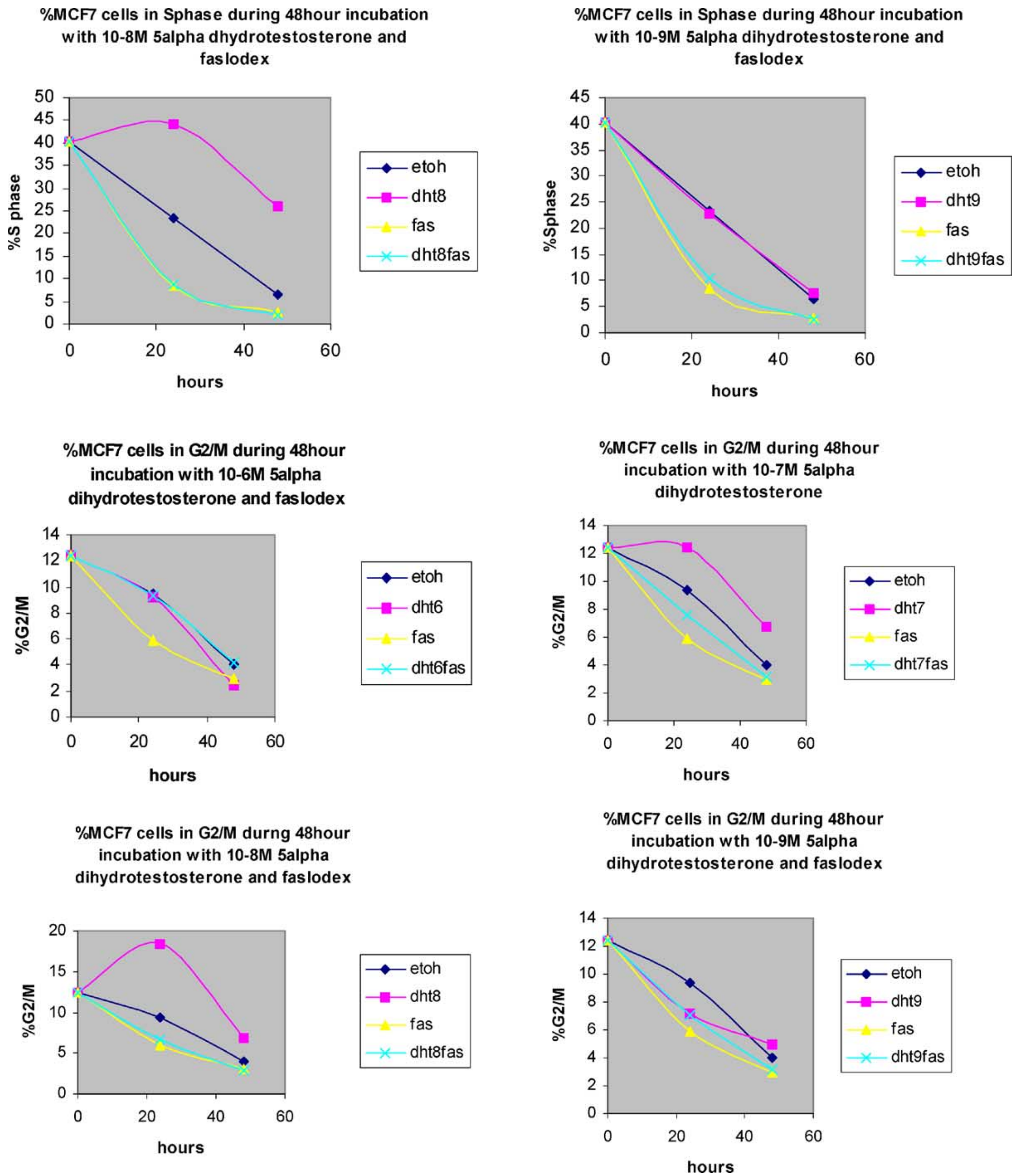


Fig. 10. (*Continued*)

of MCF7 cells, whereas 10^{-9} M 5α-dihydrotestosterone did not affect proliferation over a 48-h period. The effect on the progression of all breast cancer cells to G2/M was less consistent.

Similar results were obtained with T47D cells. Pharmacological doses of 5α-dihydrotestosterone stimulated the proliferation of T47D cells over 48 h, however, physiological doses did not affect the proliferation of this cell line. Again the effect on progression of cells to G2/M was inconsistent. On incubating the MDA231 cell lines with 10^{-9} to 10^{-6} M 5α-dihydrotestosterone and 5-androstene-3β,17β-diol which express low or zero amounts of oestrogen receptors, no significant effect on proliferation was observed. These results are illustrated in Figs. 7 and 8.

In order to look at the action of physiological levels of 5α-dihydrotestosterone in more detail the following graphs are presented in Fig. 9. These illustrate the percentage of cells in each phase of the cell cycle following 24-h incubation. These graphs clearly illustrate that apart from the action of 10^{-8} M 5α-dihydrotestosterone, which stimulates the MCF7 cell line, physiological levels of androgens do not

affect the proliferation of breast cancer cell lines over this time period.

MCF7 and T47D cell lines were incubated with 5α-dihydrotestosterone and the anti-oestrogen 10^{-6} M faslodex. The percentage of cells in each phase of the cell cycle G0/1, S and G2/M was determined at 0, 24 and 48 h, as previously. Fig. 10 demonstrates that the proliferation of MCF7 cells by 5α-dihydrotestosterone was inhibited by faslodex confirming that 5α-dihydrotestosterone stimulates proliferation in the MCF7 cell line via interaction with the oestrogen receptor. However, faslodex failed to inhibit the proliferation of T47D cells, suggesting that 5α-dihydrotestosterone stimulates proliferation in T47D cell line via a pathway other than the oestrogen receptor.

3.3. Cell proliferation of MCF7 and T47D cell lines during 9-day incubation with 5α-dihydrotestosterone

MCF7 and T47D cells were incubated for 9 days with physiological levels of 5α-dihydrotestosterone and 17β-oestradiol (10^{-9} M). Fig. 11 illustrates that physiological levels of 5α-dihydrotestosterone inhibit the baseline and oestrogen-induced proliferation of MCF7 and T47D breast cancer cell lines.

3.4. Analysis of DNA cell cycle during 48-h incubation of MCF7 and MDA231 cell lines with 5-androstene-3β,17β-diol +/− faslodex

MCF7 and MDA231 breast cancer cell lines were incubated over a 48-h period with physiological levels of 5-androstene-3β,17β-diol (10^{-9} to 10^{-6} M). The percentage of cells in each phase of the cell cycle G0/1, S and G2/M was determined at 0, 24 and 48 h as outlined in Section 2.

Figs. 12 and 13 illustrate that both physiological and pharmacological levels of 5-androstene-3β,17β-diol resulted in a fall in the percentage of cells in G0/1 and a rise in the percentage of cells in S phase at 24 h. Again the progression of cells to G2/M was less consistent. It can be interpreted from these results that physiological levels

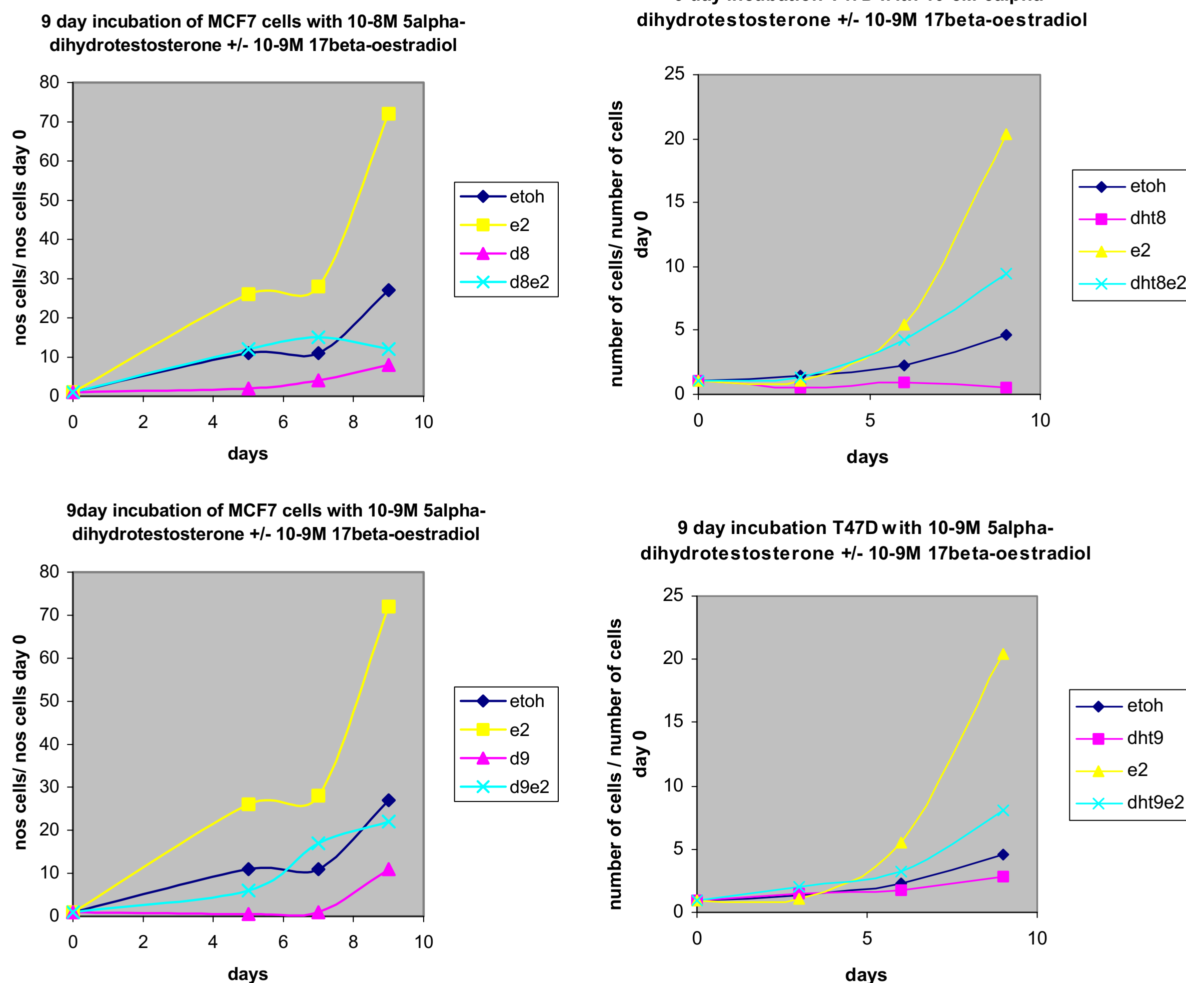


Fig. 11. Proliferation of MCF7 and T47D breast cancer cell lines during 9-day incubation with physiological levels of 5α-dihydrotestosterone and 17β-oestradiol (nDHT = 10^{-n} M 5α dihydrotestosterone, e2 = 17β-oestradiol, ETOH = 0.01% ethanol).

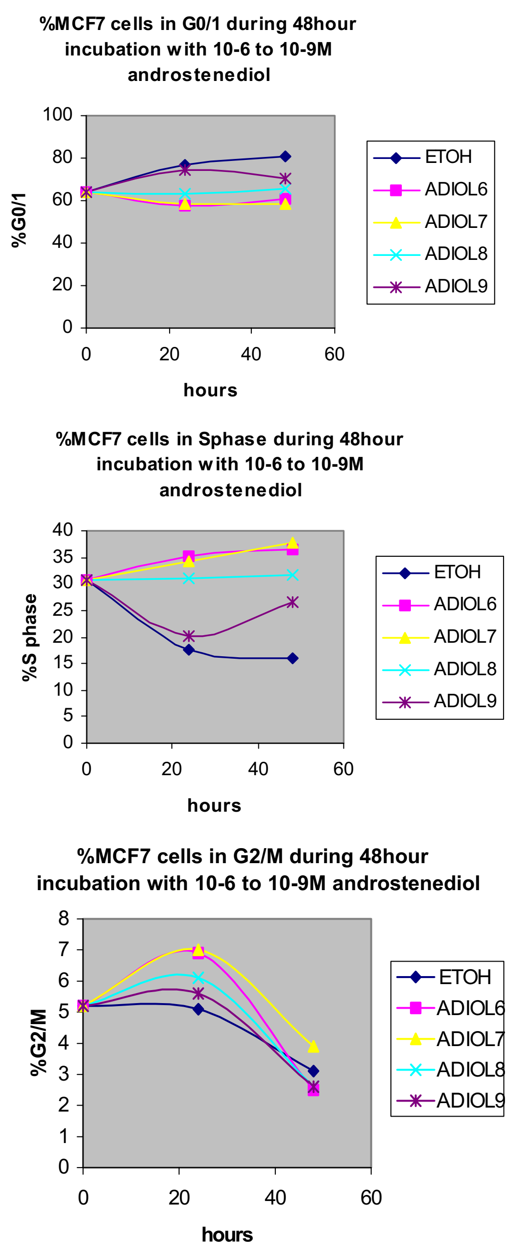


Fig. 12. Percentage of MCF7 cells in G0/1, S and G2/M during 48-h incubation with 10^{-6} to 10^{-9} M 5-androstene-3β,17β-diol (ADIOLn = 10^{-n} M 5-androstene-3β,17β-diol, ETOH = 0.01% ethanol).

of 5-androstene-3β,17β-diol stimulate the proliferation of MCF7 cell line. In addition, stimulation increased up to a concentration of 100 nm 5-androstene-3β,17β-diol, but no additional stimulation was observed at greater concentrations. No significant affect on proliferation of the MDA231 cell line was observed (see Fig. 3).

On co-incubating MCF7 cells with 5-androstene-3β, 17β-diol and faslodex it can be seen from Figs. 12 and 13 that faslodex completely inhibits the stimulatory affect of physiological doses of 5-androstene-3β,17β-diol and partially inhibits the stimulation of pharmacological doses of 5-androstene-3β,17β-diol.

3.5. Cell proliferation of MCF7 and T47D cell lines after 6-day incubation with physiological levels of 5 androstene-3β,17β-diol and 17β-oestradiol

MCF7 and T47D cell lines were incubated for 8 days with physiological levels of oestradiol and 5-androstene-3β, 17β-diol. Cell numbers were determined after 6 and 8 days by the methods outlined above. As illustrated in Fig. 14, physiological levels of 5-androstene-3β,17β-diol stimulated baseline and inhibited oestrogen-induced proliferation of MCF7 and T47D cells.

4. Discussion

4.1. Actions of 5α-dihydrotestosterone on proliferation of breast cancer cell lines

This study has observed that over 8–9-day incubations physiological concentrations of 5α-dihydrotestosterone inhibit the proliferation of MCF7 and T47D cell lines. Physiological levels of 5α-dihydrotestosterone have previously been reported to either stimulate [21,35] or not affect [24] the proliferation of MCF7 cells and inhibit the proliferation of T47D breast cancer cells via an androgen receptor mediated mechanism [21].

MCF7 cells have been shown to metabolise 5α-dihydrotestosterone to androstene-3β,l7β-diol, an androgen with oestrogenic properties [36]. This may account for the stimulatory affect of 5α-dihydrotestosterone observed in MCF7 cells, in contrast to the inhibitory action of 5α-dihydrotestosterone observed in other breast cancer cell lines [21,36]. The MCF7 cell line used in this study has been passaged several hundred times. It is not known how this affects glucuronyltransferase activity. It may be that repeated passages result in downregulation or loss of glucuronyltransferase expression in MCF7 cells, which may account for the inhibitory affect of physiological concentrations of 5α-dihydrotestosterone observed in this study.

In addition to the inhibition of MCF7 and T47D cell lines demonstrated in this study, 5α-dihydrotestosterone has been observed to inhibit the oestrogen-induced proliferation of MCF7 and T47D cell lines. These findings are consistent with previous studies on MCF7 [19] and ZR751 cell lines [20] by a mechanism, which was mediated via the androgen receptor.

Over 48 h 10^{-9} M 5α-dihydrotestosterone does not affect the progression of MCF7 or T47D cells through the cell cycle. Likewise, 10^{-8} M 5α-dihydrotestosterone does not affect the proliferation of T47D cells during over 48-h incubation, however, 10^{-8} M 5α-dihydrotestosterone stimulates the proliferation of MCF7 cells via an oestrogen receptor mediated mechanism.

Cell cycle progression in breast cancer is regulated by a series of cyclin-dependent kinases and cyclin-dependent kinase inhibitors [37,38]. These proteins control the

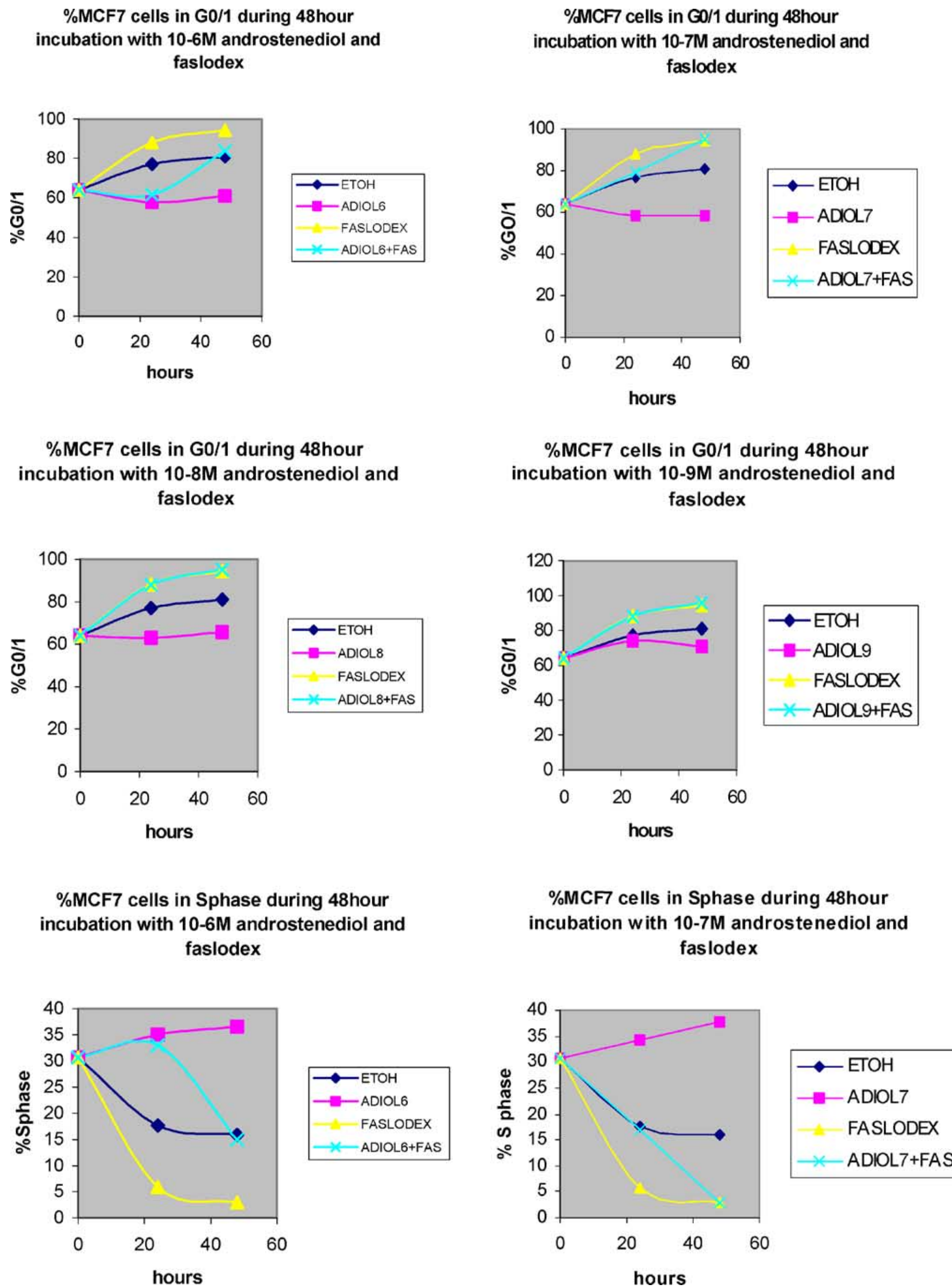


Fig. 13. Percentage of MCF7 cells in G0/1, S and G2/M during 48-h incubation with 5-androstene-3β,17β-diol and faslodex (ADIOLn = 10^{-n} M 5-androstene-3β,17β-diol, fas = 10^{-6} M faslodex, ETOH = 0.01% ethanol).

transition through checkpoints between different cell cycle states occurring, for example, at the initiation of DNA replication (S phase) and cell division (mitosis). Androgens have been shown to upregulate expression of the cyclin-dependent kinases CDK2 and CDK4 and down-regulate expression of the cyclin-dependent kinase inhibitor CKIp16 in the androgen-dependent prostate cancer cell line LNCaP. Thereby stimulating the cell to enter S phase of the cell cycle and enhancing proliferation [39]. Androgen regulation of another cyclin-dependent kinase inhibitor p27 is also of importance in prostate cancer [40].

How androgens regulate cellular proliferation in breast cancer remains poorly understood. Szelei et al. using a synthetic non-metabolised androgen R1881 and MCF7 cells transfected with androgen receptor observed these MCF7-AR1 cells were arrested in G0/1 phase of the cell cycle after 24-h incubation with androgen. It was postulated that this effect was due to androgens inducing the synthesis of a gene product, termed androcyclone-II,

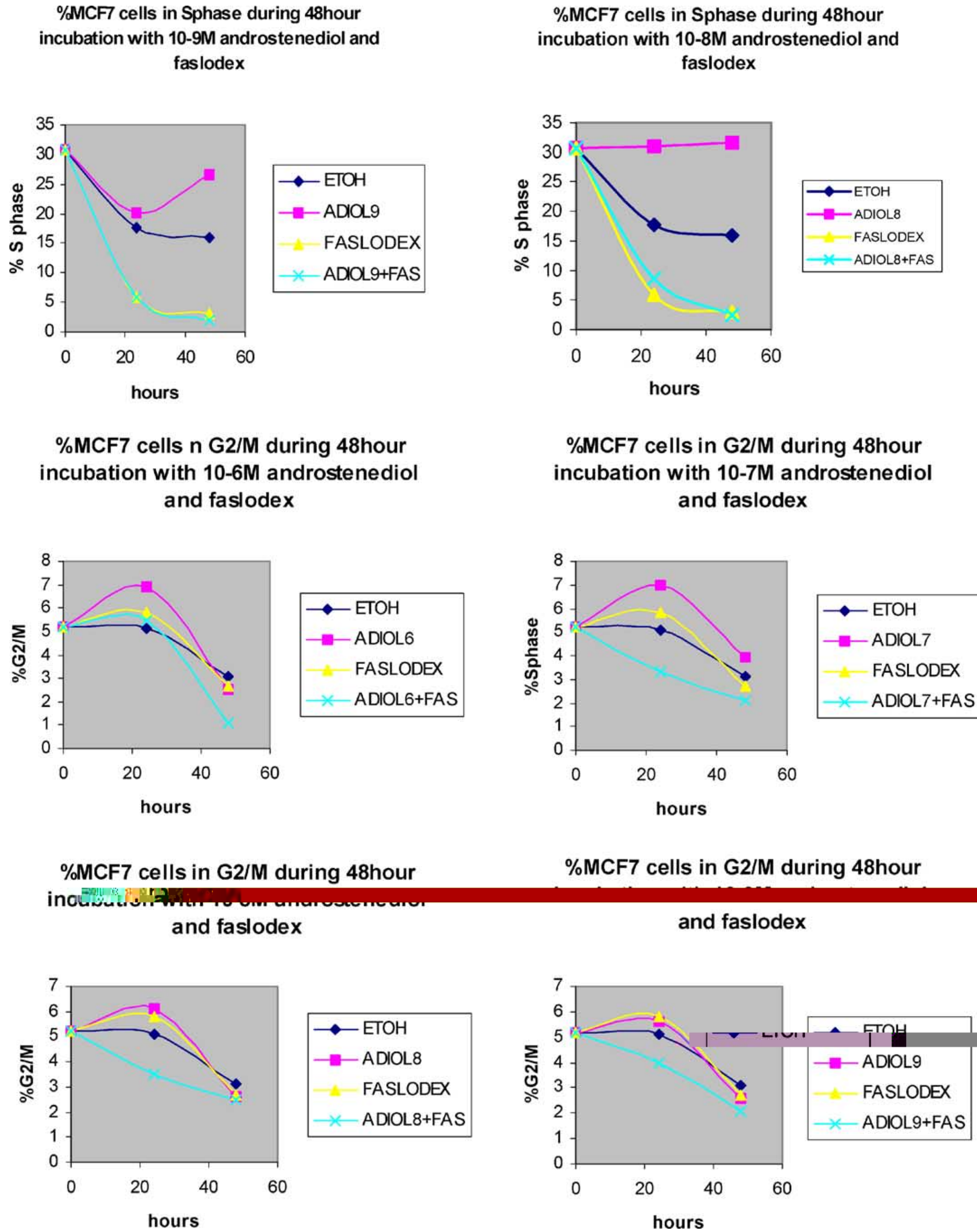


Fig. 13. (*Continued*)

which prevented entry of target cells into the cell cycle [26].

In the present study, despite 5α-dihydrotestosterone inhibiting the proliferation of MCF7 and T47D breast cancer cell lines over longterm incubations no affect on progression of cells through the cell cycle was observed over 48 h. These findings are consistent with those of Poulin et al. [20] and Birrell et al. [21] who observed that the inhibitory actions of 5α-dihydrotestosterone on breast cancer cell lines were not observed until 6 days incubation with androgen. Further studies are necessary to determine how androgens affect the growth of breast cancer via the androgen receptor, and in particular whether they affect the expression of cyclin-dependent kinases and cyclin-dependent kinase inhibitors in breast cancer cell lines.

The present study demonstrates that pharmacological levels of 5α-dihydrotestosterone stimulate MCF7 and T47D cells to enter the S phase and enhance proliferation. In MCF7 cells this action is blocked by co-incubation with faslodex implying an oestrogen receptor mediated mechanism, consistent with previous observations [24,25]. Faslodex does not block this action in T47D cells, suggesting an alternative pathway is involved in mediating the proliferation of T47D cells by pharmacological levels of 5α-dihydrotestosterone.

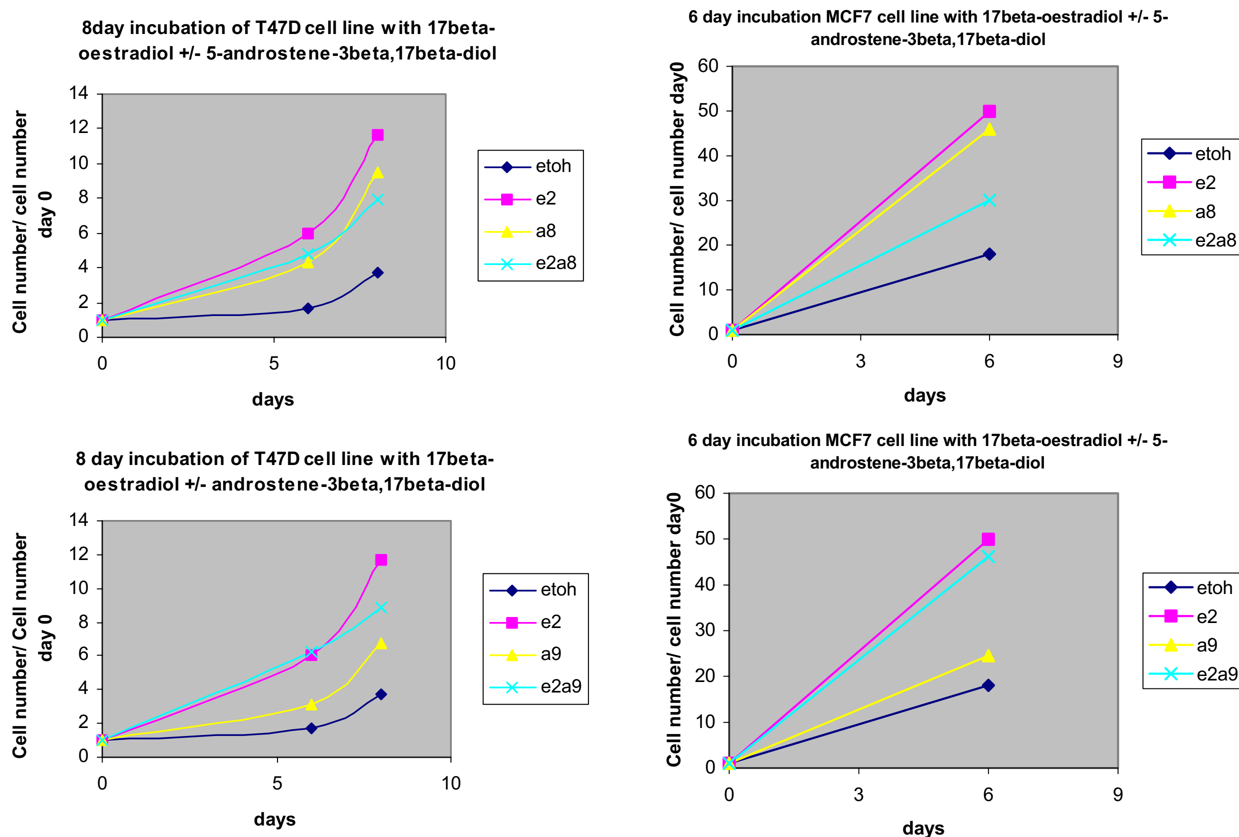


Fig. 14. Proliferation of MCF7 and T47D cell lines after 6-day incubation with physiological levels of 17β-oestradiol and 5-androstene-3β,17β-diol (ADIOL$n = 10^{-n}$ M, 5-androstene-3β,17β-diol, e2 = 10^{-6} M 17β-oestradio1, ETOH = 0.01% ethanol).

4.2. The actions of 5-androstene-3β,17β-diol on the proliferation of breast cancer cell lines

Several previous studies have reported that 5-androstene-3β,17β-diol stimulates the proliferation of hormone-responsive breast cancer cell lines via interaction with the oestrogen receptor at physiological levels [10–15]. The present study demonstrates that within 24 h 5-androstene-3β,17β diol stimulates MCF7 cells to enter S phase of the cell cycle thereby enhancing cell proliferation. Furthermore, this affect was blocked by co-incubation with faslodex, confirming that this action is mediated via the oestrogen receptor.

5-Androstene-3β,17β-diol is a steroid, which has affinity for both the oestrogen and androgen receptor and has therefore been termed “hermaphradiol” [6,9]. Consistent with its affinity for the androgen receptor the present study confirms previous findings demonstrating that 5-androstene-3β,17β-diol like 5α-dihydrotestosterone inhibits the oestrogen-induced proliferation of hormone-dependent breast cancer cell lines [11,12,41].

4.3. Implications for the role of androgens in breast cancer

Precursor steroids are metabolised to substrates, which inhibit (5α-dihydrotestosterone) or stimulate (5-androstene-3β,17β-diol, oestrogens) the proliferation of hormone-dependent breast cancer cells at physiological levels. The net affect of precursor steroids in vivo seems likely to be a balance between the stimulatory and inhibitory affects of their metabolites. The findings by several groups that serum precursor steroids are elevated in postmenopausal women who develop breast cancer suggest that the net activity in vivo is stimulatory.

In premenopausal women, the hormonal milieu is rich in ovarian oestrogens. In the present study, both 5α-dihydrotestosterone and 5-androstene-3β,17β-diol antagonise the oestrogen-induced proliferation of breast cancer cells. Elevated levels of precursor steroids in premenopausal women by antagonising the oestrogen-induced proliferation of hormone-dependent breast cancer may therefore protect against the development of breast cancer. This mechanism may underlie the observations of Bulbrook and Hayward [4] and others that premenopausal women who develop breast cancer have subnormal androgen levels.

The observation that 5-androstene-3β,17β-diol stimulates the proliferation of hormone-dependent breast cancer cell lines at physiological levels has implications for patients on aromatase inhibitors, as this pathway provides a mechanism for the ongoing stimulation of hormone-dependent breast cancer. Evidence that this pathway may have importance in vivo is suggested by a recent study, which has found that elevated levels of serum dehydroepiandrosterone sulphate

predict breast cancer progression in postmenopausal women on aromatase inhibitors [42].

References

[1] J.A. Cauley, F.L. Lucas, L.H. Kuller, K. Stone, W. Browner, S.R. Cummings, Elevated serum estradiol and testosterone concentrations are associated with a high risk for breast cancer, Ann. Intern. Med. 130 (1999) 270–277.

[2] J.F. Dorgan, F.Z. Stanczyk, C. Longcope, H.E. Stephenson, L. Chang, R. Miller, C. Franz, R.T. Falk, L. Kahle, Relationship of serum dehydroepiandrosterone (DHEA), DHEA sulfate, and 5-androstene-3beta,17beta-diol to risk of breast cancer in postmenopansal women, Cancer Epidemiol. Biomarkers Prev. 6 (3) (1997) 177–181.

[3] G.B. Gordon, T.L. Bush, K.L. Helzlsouer, S.R. Miller, G.W. Comstock, Relationship of serum levels of dehydroepiandrosterone and dehydroepiandrosterone sulfate to risk of developing postmenopausal breast cancer, Cancer Res. 50 (1990) 3859–3862.

[4] R.D. Bulbrook, J.L. Hayward, Relation between urinary androgen and corticosteroid excretion and subsequent breast cancer, Lancet 2 (1971) 395–397.

[5] K.L. Helzlsouer, G.B. Gordon, A.J. Alberg, T.L. Bush, G.W. Comstock, Relationship of prediagnostic serum levels of dehydroepiandrosterone and dehydroepiandrosterone sulfate to the risk of developing premenopausal breast cancer, Cancer Res. 52 (1992) 1–4.

[6] J.B. Adams, Adrenal androgens and human breast cancer: a new appraisal, Breast Cancer Res. Treat. 51 (1998) 183–188.

[7] H. Rochefort, M. Garcia, The estrogenic and antiestrogenic activities of androgens in female target tissues, Pharmacol. Ther. 23 (1984) 193–216.

[8] C. Longcope, Adrenal and gonadal androgen secretion in normal females, Clin. Endocrinol. Metab. 15 (2) (1986) 213–228.

[9] J. Poortman, J.A. Prenen, F. Shwartz, J.H.H. Thussen, Interaction of 5-androstene-3beta,17beta-diol with oestradiol and dihydrotestosterone receptors in human myometrical and mammary cancer tissue, J. Clin. Endocrinol. Metab. 40 (1975) 137–143.

[10] J. Adams, M. Garcia, H. Rochefort, Estrogenic effects of physiological concentrations of 5-androstene-3beta,17beta-diol and its metabolism in MCF7 human breast cancer cells, Cancer Res. 41 (1981) 4720–4726.

[11] R. Hackenberg, I. Turgetto, A. Filmer, K.-D. Schulz, Estrogen and androgen receptor mediated stimulation and inhibition of proliferation by androst-5-en-3beta,17beta-diol in human mammary cancer cells, J. Steroid Biochem. Mol. Biol. 46 (1993) 597–603.

[12] G. Boccuzzi, E. Brignardello, M. DiMonaco, C. Forte, L. Leonardi, A. Pizzini, Influence of dehydroepiandrosterone and 5-en-androstene-3beta,17beta-diol on the growth of MCF7 human breast cancer cells induced by 17beta-oestradiol, Anticancer Res. 12 (1992) 799–804.

[13] R. Poulin, F. Labrie, Stimulation of cell proliferation and estrogenic response by adrenal C19-steroids in the ZR751 human breast cancer cell line, Cancer Res. 46 (1986) 4933–4937.

[14] M. Maggiolini, G. Donze, F. Jeannin, S. Ando, D. Picard, Adrenal androgens stimulate the proliferation of breast cancer cells as direct activators of estrogen receptor alpha, Cancer Res. 59 (1999) 4864–4869.

[15] J.C. LeBail, F. Marre-Fournier, J.C. Nicolas, G. Habrioux, C19 steroids estrogenic activity in human breast cancer cell lines: importance of dehydroepiandrosterone sulfate at physiological plasma levels, Steroids 63 (1998) 673–683.

[16] A. Billich, P. Nussbaumer, P. Lehr, Stimulation of MCF7 breast cancer cell proliferation by estrone sulfate and dehydroepiandrosterone sulfate: inhibition by novel non-steroidal steroid sulfatase inhibitors, J. Steroid Biochem. Mol. Biol. 73 (2000) 225–235.

[17] M. Baum, The ATAC (Arimidex, Tamoxifen, Alone or in Combination) adjuvant trial in postmenopausal women, Breast Cancer Res. Treat. 69 (2001) 210.

[18] E.P. Winer, C. Hudis, H.J. Burstein, R.T. Chlebowski, J.N. Ingle, S.B. Edge, E.P. Mamounas, J. Gralow, L.J. Goldstein, K.I. Pritchard, S. Braun, M.A. Cobleigh, A.S. Langer, J. Perotti, T.J. Powles, T.J. Whelan, G.P. Browman, American Society of Clinical Oncology Technology Assessment on the Use of Aromatase Inhibitors as Adjuvant Therapy for Women with Hormone Receptor-Positive Breast Cancer: Status Report, J. Clin. Oncol. 20 (15) (2002) 3317–3327.

[19] J.H. MacIndoe, L.A. Etre, An antioestrogeic action of androgens in human breast cancer cells, J. Clin. Endocrinol. Metab. 53 (1981) 836–841.

[20] R. Poulin, D. Baker, F. Labrie, Androgens inhibit basal and estrogen-induced cell proliferation in ZR751 human breast cancer cell line, Breast Cancer Res. Treat. 12 (1988) 213–225.

[21] S.N. Birrell, J.M. Bentel, T.E. Hickey, C. Ricciardelli, M.A. Weger, D.J. Horsfall, W.D. Tilley, Androgens induce divergent proliferative responses in human breast cancer cell lines, J. Steroid Biochem. Mol. Biol. 52 (5) (1995) 459–467.

[22] R. Hackenberg, S. Luttchens, J. Hofmann, R. Kunzmann, F. Hotzel, K.-D. Schulz, Androgen sensitivity of the new human breast cancer cell line MFM-223, Cancer Res. 51 (1991) 5722–5727.

[23] R.E. Hall, S.N. Birrell, W.D. Tilley, R.L. Sutherland, MDA-MB-453 an androgen-responsive human breast carcinoma cell line with high level androgen receptor expression, Eur. J. Cancer 30A (4) (1994) 484–490.

[24] D.T. Zava, W.L. McGuire, Androgen action through estrogen receptor in human breast cancer cell line, Endocrinology 103 (1978) 624–631.

[25] M. Lippman, G. Bolan, K. Huff, The effects of androgens and antiandrogens on hormone-responsive human breast cancer in long-term tissue culture, Cancer Res. 36 (1976) 4610–4618.

[26] J. Szelei, J. Jimenez, A.M. Soto, M.F. Luizzi, C. Sonnenschein, Androgen-induced inhibition of proliferation in human breast cancer MCF7 cells transfected with androgen receptor, Endocrinology 138 (1997) 1406–1412.

[27] H.D. Soule, J. Vasquez, A. Long, S. Albert, M.B.A. Brennan, Human cell line from a pleural effusion derived from a breast carcinoma, J. Natl. Cancer Inst. 51 (1973) 1049–1416.

[28] I. Keydar, L. Chen, S. Karby, F.R. Weiss, J. Delerea, M. Radu, S. Chaitcik, H.J. Brenner, Establishment and characterisation of a cell line of human breast cancer origin, Eur. J. Cancer 15 (1979) 659–670.

[29] R. Caillean, R. Young, M. Olive, W.J. Reeves, Breast tumor cell lines from pleural effusions, J. Natl. Cancer Inst. 53 (1974) 661–674.

[30] M.G. Ormerod, Analysis of DNA-general methods, in: M.G. Ormerod (Ed.), Flow Cytometry, Oxford University Press, Oxford, 2000, pp. 83–98.

[31] K.B. Horwitz, D.T. Zava, A.K. Thilager, E.M. Jenson, W.L. McGuire, Steroid ceptor analyses of nine breast cancer cell lines, Cancer Res. 38 (1978) 2434–2437.

[32] K.B. Horwitz, M.E. Costlow, W.L. McGuire, A human breast cancer cell line with estrogen, androgen, progesterone and glucocorticoid receptors, Steroids 26 (1975) 785–795.

[33] J.K. Larsen, Measurement of cytoplasmic and nuclear antigens, in: M.G. Ormerod (Ed.), Flow Cytometry, Oxford University Press, Oxford, 2000, pp. 133–158.

[34] I. Brotherick, T.W.J. Lennard, S. Cook, R. Johnstone, B. Angus, M.P. Winthereik, B.K. Shenton, Use of the biotinylated antibody DAKO-ER 1D5 to measure oestrogen receptor on cytokertin positive cells obtained from primary breast cancer cells, Cytometry 20 (1995) 74–80.

[35] R. Hackenberg, J. Hofmann, F. Lolzel, K.-D. Schulz, Stimulatory effects of androgen and antiandrogen on the in vitro proliferation of human mammary carcinoma cells, J. Cancer Res. Clin. Oncol. 114 (1988) 593–601.

[36] R. Roy, S. Dauvois, F. Labrie, A. Belanger, Estrogen-stimulated glucuronidation of dihydrotestosterone in MCF-7 human breast cancer cells, J. Steroid Biochem. Mol. Biol. 41 (3–8) (1992) 579–582.
[37] X. Grana, E.P. Reddy, Cell cycle control in mammalian cells: role of cyclins, cyclin-dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors, Oncogene 11 (1995) 211–219.
[38] T. Hunter, J. Pines, Cyclins and cancer II: cyclin D and CDK inhibitors come of age, Cell 79 (1994) 573–582.
[39] S. Lu, S.Y. Tsai, M.-J. Tsai, Regulation of androgen-dependent prostatic cancer cell growth: androgen regulation of CDK2, CDK4, and CKI p16 genes, Cancer Res. 57 (1997) 4511–4516.
[40] E. Macri, M. Loda, Role of p27 in prostate carcinogenesis, Cancer Metastasis Rev. 17 (1999) 337–344.
[41] G. Boccuzzi, E. Brignardello, M. DiMonaco, V. Gatto, L. Leonardi, A. Pizzini, M. Gallo, 5-En-androstene-3beta,17beta-diol inhibits the growth of MCF-7 breast cancer cells when oestrogen receptors are blocked by oestradiol, Br. J. Cancer 70 (1994) 1035–1039.
[42] K.T. Morris, S. Toth-Fejel, J. Schmidt, W.S. Fletcher, R.F. Pommier, High dehydroepiandrosterone-sulfate predicts breast cancer progression during new aromatase inhibitor therapy and stimulates breast cancer growth in tissue culture: a renewed role for adrenelectomy, Surgery 130 (2001) 947–953.