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Inhibitory Action of Androstenedione on the Proliferation and Cell Cycle Kinetics of Aromatase-free MXT and MCF-7 Mammary Tumour Cell Lines

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Abstract—*The effects of androstenedione (AD) on cell proliferation and kinetics have been measured in MXT mouse and MCF-7 human mammary cancer cell lines using SAMBA 200 cell image analysis of Feulgen-stained nuclei. At a concentration of* 0.01 μM *AD inhibited the proliferation of both cell lines whereas a higher dose* (1 μM) *was inhibitory on MCF-7 cell proliferation but stimulatory in MXT cells. It is unlikely that these effects are due to aromization of AD into oestrogen since (a) both cell lines were devoid of aromatase and (b) both cell lines were similarly affected by oestradiol (E2), being stimulated at low concentrations and inhibited at high doses. Furthermore, inhibition by AD seems to occur, at least in part, by blockade of the cell cycle whereas that by E2 appears to be cell cycle independent.*

INTRODUCTION

IT HAS BEEN shown from clinical [1–4] and experimental [5] observations that oestrogens and androgens exert direct antagonistic effects on the growth and development of normal and malignant human breast tissue. Androgens and oestrogens also exert antagonistic actions in established cell lines as, for example, the inhibition of oestradiol-induced progesterone receptor synthesis by androgens [6, 7] and the oestrogen down-regulation of androgen receptor [2]. However, only a few reports are available on the influence of androgens on the cell kinetic parameter of human breast cancers [1, 4, 8].

The present study is an attempt to characterize the *in vitro* influence of androstenedione (AD) or oestradiol (E2, control of the methodology) on the growth and the cell cycle kinetic parameters (percentage of cells in the G0–G1, S and G2 + M phases) of the mouse MXT and the well-documented MCF-7 hormone-sensitive human breast cancer cell lines (for review see [9]). The MXT mammary tumour of the B6D2F1 strain is a subcutaneously transplantable model [10], whose cell proliferation is influenced *in vivo* by oestrogens [11, 12], progesterone [13, 14], prolactin [15] and the luteinizing-hormone releasing-hormone [16]. We established this MXT tumour growing *in vitro* as monolayers [17]. Our experiments were performed by analysing Feulgen-stained nuclei through a cell image processor, i.e. the System for Analytical Microscopy in Biomedical Applications (SAMBA 200, see [18]). We also assayed the aromatase activity within these MXT and MCF-7 cell lines.

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Abbreviations used: AD: androstenedione; E2: oestradiol; SAMBA 200: System for Analytical Microscopy in Biomedical Applications; S.E.M.: standard error of the mean.

MATERIALS AND METHODS

1. *Chemicals, medium and cells*

17-Beta-oestradiol and androstenedione were purchased from Sigma Chemicals Co (St Louis, MO). Hormones in ethanolic solutions were added to the culture medium with a final ethanol concentration of less than 0.2% (vv). As found in our own preliminary experiments and reported in earlier literature [9], this concentration had no effect on the proliferation of MXT or MCF-7 cells under the experimental conditions used.

The MCF-7 cells were kindly provided by Dr. M. Rich (Michigan Cancer Foundation) and the MXT cell line that we used was the 190th passage of the MXT tumour adapted to grow *in vitro* in our laboratory, as previously described [17]. The cells were maintained as monolayers cultured at 37°C in closed Falcon plastic dishes (Becton Dickinson, Oxnard, CA) containing Eagle's minimal essential medium (MEM) (Gibco Europe, Paisley, U.K.) supplemented with 10% foetal calf serum (FCS) (Gibco), 0.6 mg/ml glutamine (Gibco) and a mixture of 100 IU/ml penicillin and 10 μg/ml streptomycin (Difco, Detroit, MI).

In our experiments FCS was heat-inactivated for 1 h at 56°C and subsequently treated with dextran coated charcoal for 30 min at room temperature.

2. *Experimental schedule*

The MXT or MCF-7 cells growing in the log phase were plated at a density of 10,000 cells/ml medium in 35 × 10 mm Petri dishes (Becton Dickinson, U.S.A.), with a 18 × 18 mm coverslip on the bottom, and containing 3 ml of MEM + 5% FCS (v/v). After the cells had become firmly attached to the coverslip (24 h after plating), the medium was replaced by experimental MEM containing 5% FCS (v/v) and supplemented either with AD (1, 0.01 or 0.0001 μM) or with E2 (1, 0.01 or 0.0001 μM).

Three coverslips for each experimental condition were fixed in EFA (ethanol 95°: 75 vol, neutral formol 40%: 20 vol, acetic acid: 5 vol) for 10 min respectively 12, 24, 36, 48 or 72 h after the addition of the experimental medium to the Petri dishes. Control cells, cultured in an experimental medium without any hormones, were fixed at the same time. At the end of the fixation procedure, the coverslips (cell back-up) were mounted on histological slides with DPX (BDH Chemicals) and stored in the dark at 4°C until staining.

3. *Preparation of slides*

The slides supporting the coverslips were stained by the Feulgen reaction [19] according to the procedure described by Moustafa and Brugal [20]. All the slides of a given experimental condition were stained together for 1 h in Feulgen reagent (Fluka AG, CH) after hydrolysis in 6 N HCl for 1 h at 20°C. The cell preparations were then stored in the dark and cold (4°C) before analysis. The Feulgen reaction was used because it allows selective and quantitative (stoichiometric) staining of the DNA.

4. *Description of the SAMBA 200 system analysis*

About 250–300 cell nuclei from each slide were automatically analysed using a SAMBA 200 (TITN, France) microscopic image processor. The hardware and software of this system have been described elsewhere [18]. The DNA histograms were assessed on the basis of their integrated optical density (IOD) which measures the amount of absorbant material, i.e. the nuclear DNA content, in arbitrary units. This parameter is based on the fundamental notion of spectrophotometry, which defines optical density (OD) as a function of transmission values.

For each coverslip analysed, we also assessed the 'mean number of cells per arbitrary unit of scanned area' (MNC/AU) by counting the number of cells recorded on a 16 mm^2 coverslip area.

5. *Aromatase activity*

Aromatase levels were determined in MXT and MCF-7 cell lines cultured in 125 cm^2 Falcon dishes until 0.4 mg of cells were obtained. This was carried out according to the method previously described in detail by Vermeulen *et al.* [21], in which tritium liberated during the aromatization of 1-beta-3H-androstenedione was used as a parameter of aromatization. The number of moles of oestrogen formed was calculated from the corrected disintegrations per minute using the specific activity of 1-beta-3H-androstenedione in the incubate. Results are expressed as fmol E1-mg protein/h.

6. *Statistical analyses*

Results are given as a mean ± standard error of the mean (S.E.M.); statistical comparisons of data were performed by mean of the Fisher *F*-test (one-way variance analysis). Normal parameter distribution fitting was assessed by the chi-square test and homogeneity of variance was verified by the Hartley test.

RESULTS

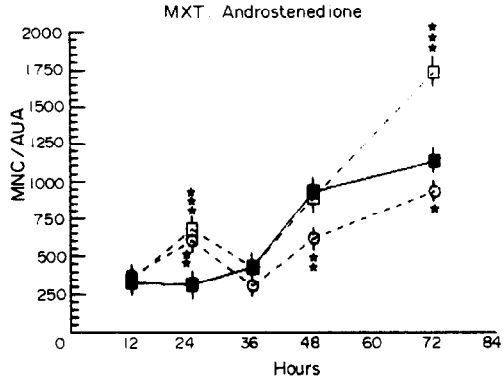
All the data reported both in the Results section and in the complete set of figures relate to the mean values (± S.E.M.) recorded during one of four experiments, i.e. the third one was performed on triplicates. We present here the results that only relate to this third experiment because the data from the four experiments were identical.

1. *Aromatase activity*

Aromatase activity was totally absent in both MXT and MCF-7 cultured cells.

2. *Effects of AD or E2 on the cell count*

a. *MXT cell line.* Androstenedione significantly slowed down MXT proliferation assessed by the 'mean number of cells/arbitrary unit of scanned area' (MNC/AUA) between 48 and 72 h after administration of 0.01 μM in the incubation medium (Fig. 1). In contrast, this concentration significantly increased the MXT MNC/AUA at the



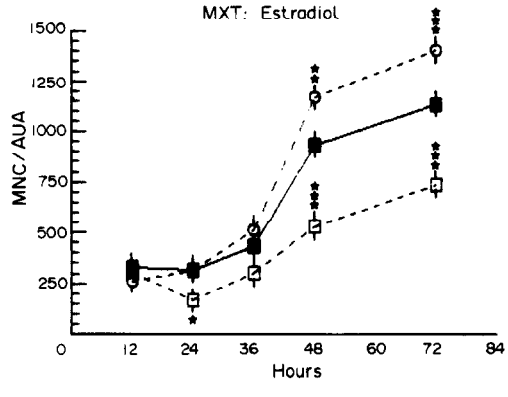
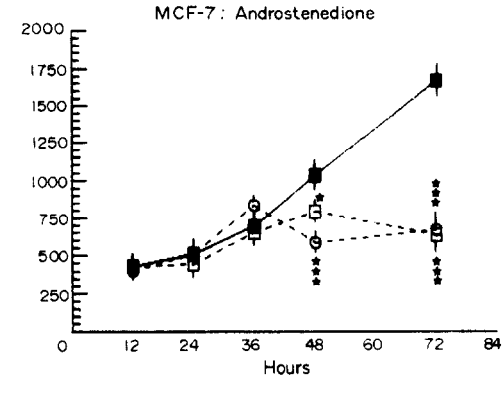


Fig. 1. Effect of androstenedione or oestradiol on the proliferation of MXT cells over time. Cells were plated for 24 h *into* 35 × 10 mm *Petri dishes, whose bottom supported a* 18 × 18 mm *glass coverslip, containing minimal essential medium with* 5% *DCC-treated foetal calf serum. At hour* 0 (24 h *after the plating*), 0.01 (○) *or* 1 (□) μM *of AD or E2 was added to the medium. Control cells remained in the medium without hormone* (■). *Triplicate coverslips were analysed per experimental condition by using a cell image processor, the SAMBA* 200 *system, that recorded the number of Feulgen-stained nuclei on a* 16 mm^2 *arbitrary area scanned/coverslip* (*MNC/AUA*). *The mean number of nuclei* (± *S.E.M.*) *recorded in each hormone-treated condition was statistically compared* (*Fisher* F-*test*) *to the control value recorded at the same time* (*P < 0.05; **P < 0.01; ***P < 0.001).



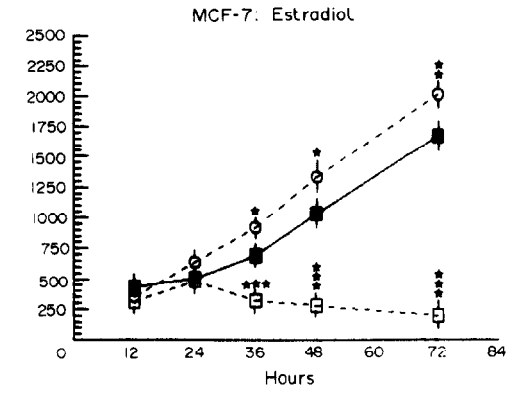


Fig. 2. Effect of androstenedione or oestradiol on MCF-7 cell proliferation over time. All the experimental and analytical procedures used are identical to those described in caption of Fig. 1. ■ = *Control*; ○ = 0.01 μM *of AD or E2*; □ = 1 μM *of AD or E2. The mean number of nuclei* (± *S.E.M.*) *recorded in each hormone-treated condition was statistically compared* (*Fisher* F-*test*) *to the control value recorded at the same time* (*P < 0.05; **P < 0.01; ***P < 0.001).

24th hour. The 1 μM dose induced two significant increases in cell counts, i.e. at the 24th and the 72nd hour (Fig. 1). We observed a lower number of cells at the 36th and the 48th hours, a feature that was apparently related to a significant AD-induced inhibition of MXT cell proliferation at these times. We did not investigate the AD- (or E2-) induced effects after the 72nd hour of culture because, by that time, the hormones had induced the appearance of clusters of tumour cells, and this precluded cell counts since there were many superimposed cells.

Oestradiol exerted a significant stimulatory effect on the MXT cell count when administered at 0.01 μM, between the 48th and the 72nd hour. In contrast, it significantly slowed down this cell count when administered at 1 μM, at the 24th hour and between the 48th and the 72nd hour (Fig. 1).

b. *MCF-7 cell count.* Androstenedione at both 0.01 and 1 μM significantly slowed down MCF-7 cell proliferation 48 to 72 hours after its administration in the incubation medium (Fig. 2).

Oestradiol slightly but nevertheless significantly increased the MNC/AUA of MCF-7 cell line when administered at 0.01 μM in the medium; this effect occurred between the 36th and the 72nd hour (Fig. 2). At 1 μM, it dramatically decreased MCF-7 cell proliferation between the 36th and the 72nd hour after its administration (Fig. 2).

3. *Effects of AD or E2 on the cell cycle kinetic parameters of MXT and MCF-7 cell lines*

a. *Effects of AD.* As illustrated in Fig. 3A, androstenedione exerted an inhibitory effect (concomitant increase in the G0–G1 value and a decrease in the S value) on the MXT cells at the 12th, 48th and 72nd hours when administered at 1 μM, and at the 12th and 48th hours when administered at 0.01 μM; at the 36th hour, 0.01 μM AD induced a decrease in the G0–G1

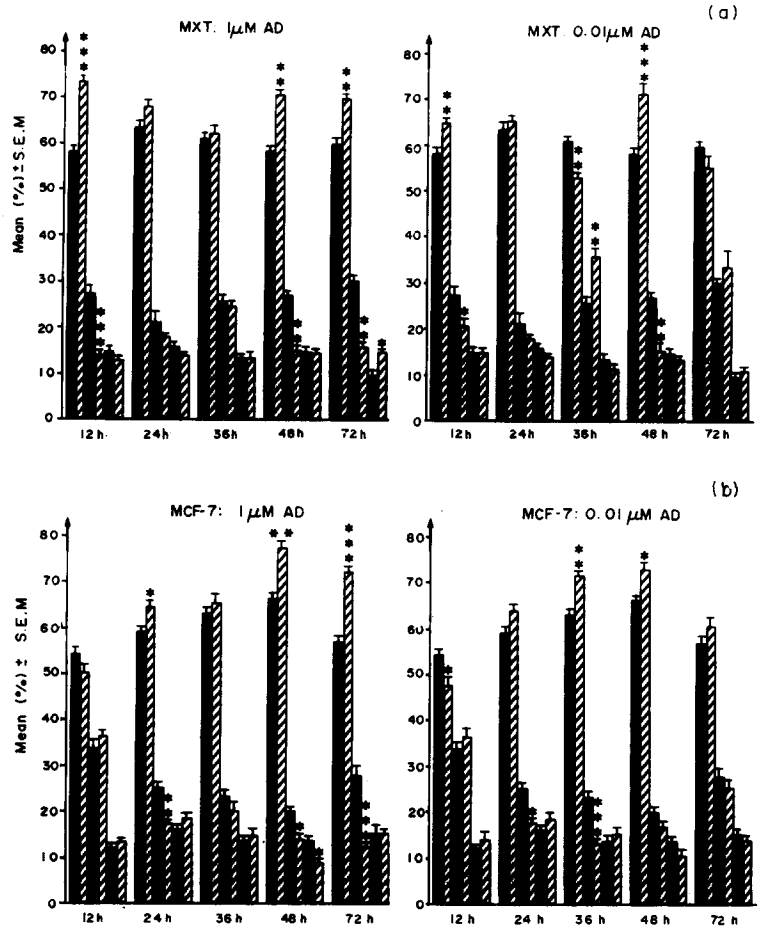


Fig. 3. Repartition as a percentage of the MXT (A) or MCF-7 (B) cells into the G0–G1 (first paired histograms), S (second paired histograms) and G2 + M (third paired histograms) fractions. The cells were cultured for various times (12–72 h) *on glass coverslips put in Petri dishes containing control media* (■) *or media containing* 1 *or* 0.01 μM *androstenedione* (□). *The cells grown on the coverslip were thereafter fixed for histology and stained by the Feulgen reaction, allowing a quantitative and selective DNA determination by using a cell image processor, i.e. the SAMBA* 200. *The mean percentage value* (± *S.E.M.*) *of the AD-treated condition was compared* (*Fisher* F-*test*) *to the corresponding control value* (*P < 0.05; **P < 0.01; ***P < 0.001). *Three coverslips were analysed per experimental condition and* 250–300 *nuclei were analysed per coverslip. The whole methodology described here is identical for the caption of Fig.* 4.

value concomitantly with an increase in the S value. This phenomenon appears as a synchronization of the MXT cells at the S phase rather than a true mitogenic effect: no significant increase of the G2 + M value was observed 12 h later, i.e. 48 h after steroid administration (for comparison see Fig. 4B showing the effect of 0.01 μM E2 on the MCF-7 cells). Androstenedione induced no effect on the MXT cells at 0.0001 μM (data not shown for the sake of clarity in the figure).

The results obtained on the MCF-7 cells (Fig. 3B) are comparable to those described above for MXT: AD increased the G0–G1 fraction and concomitantly decreased the S fraction at the 24th, 48th and 72nd hours when administered at 1 μM, and at the 36th hour when administered at 0.01 μM; no effect was observable with 0.0001 μM AD (data not shown).

b. *Effect of E2.* Figure 4A shows that oestradiol exerted no effect except on the G0–G1 phase at the 12th hour at 0.01 μM and on the MXT cell cycle kinetic parameters whatever the level used, i.e. at concentrations of 1, 0.01 or 0.0001 (data not shown) μM. The results obtained on the MCF-7 cells (Fig. 4B) are quite different: 1 μM E2 decreased the G0–G1 fraction at the 24th and 48th hour and concomitantly increased the S and G2 + M fractions. At 0.01 μM this steroid seemed to induce two mitogenic waves 24 and 48 h after its administration: the G0–G1 value decreased concomitantly with an increase of the S value, and 12 h later (at the 36th hour) an increase in the G2+ M values appeared. No effect was detectable at 0.0001 μM (data not shown).

DISCUSSION

The present study deals with the characterization of the effect of an androgen, i.e. androstenedione, which occupies a central position in steroidogenesis, on the cell kinetic parameters (breakdown of the cells into the G0–G1, S and G2 + M phases) and

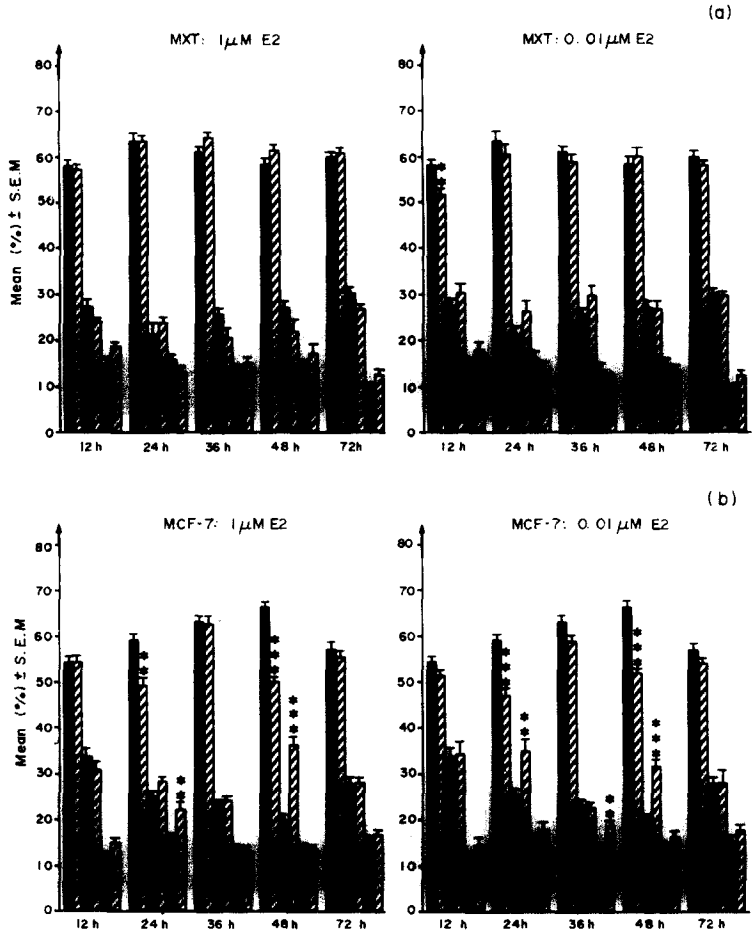


Fig. 4. Repartition as a percentage of the MXT (A) or MCF-7 (B) cells into the G0–G1 (first paired histograms), S (second paired histograms) and G2 + M (third paired histograms) fractions. The cells were cultured for 12–72 h *in control media* (■) *or media containing* 1 *or* 0.01 μM *E2* (▨). *The mean percentage value* (± *S.E.M.*) *of each fraction, assessed by the SAMBA* 200 *cell image processor through* 250–300 *Feulgen-stained nuclei, recorded in the E2-treated condition, was compared (Fisher* F*-test) to the control value recorded at the same time* (*P < 0.05; **P < 0.01; ***P < 0.001).

the proliferation of MXT mouse [17] and MCF-7 human [22] mammary cancer cell lines. Oestradiol was also studied as a positive control of the methodology. The 'non-oestrogenic' influence of AD was ascertained thanks to the total absence of any aromatase activity in both the MXT and MCF-7 cell lines. However, the possibility remains that the AD-induced effects observed might be due to other androgens relative to androstenedione metabolism inside MXT or MCF-7 cells.

The influence of androstenedione and oestradiol on the cell cycle kinetics and the proliferation of MXT and MCF-7 cells were approached by means of the SAMBA 200 cell image processor [18] which permits the determination of the DNA content of each analysed nucleus by computing the densitometry of Feulgen-stained preparations. Using cell image analysis, Boilly *et al.* [23] have shown that the percentage of cells, including non-cycling cells, in all of the cell-cycle phases can be obtained very rapidly and in one step, whereas traditional methods do not enable changes in the proportion of G1 cells to be determined directly. Furthermore, as Moustafa and Brugal [20] argue, the method has the advantage of permitting cell kinetics analysis from a single sample of a cell population and does not entail any *a priori* knowledge about the validity of the growing mode. Lastly, using cell image analysis, we can ascertain that our results are devoid of any 'false positive' results that might occur with an *in vitro* evaluation of cell proliferation under hormone influence using methodologies such as tritiated thymidine autoradiography [24] or tritiated thymidine incorporation [25].

Our results indicate that high-dose (1 μM) AD administration exerts an inhibitory influence on MCF-7 proliferation and a stimulatory effect on that of MXT cells. At 0.01 μM, AD significantly slows down both MXT and MCF-7 cell proliferation. With regard to cell cycle kinetics, this hormone partly blocks both cell types at the G0–G1 phase at the two doses studied, i.e. 0.01 and 1 μM.

This apparent conflict of AD-induced effects might be because the significant increase in cell numbers corresponds to a decrease in cell death induced by the significant inhibition of the cells

at the G0-G1 phase. Such differential hormone-induced effects on cell growth and cell loss have already been reported [26, 27]. As previously mentioned, the possibility also remains that these effects might be induced by androstenedione metabolites.

Administered into the incubation medium at a 0.01 μM dose, oestradiol exerted a significant and well-known stimulating influence on MCF-7 (see [9, 28]) as well as on MXT cell proliferation. In contrast, at 1 μM, it dramatically decreased both MCF-7 and MXT proliferation. These results are corroborated by much previous work showing that the stimulation and the inhibition of breast cancer cell proliferation can be obtained with low- and high-dose oestrogens (see [29]) respectively. However, our cell count data provide a poor match with the cell kinetics. Indeed, 0.01 μM stimulated MXT proliferation without significantly affecting cell kinetics. We used calf serum stripped of endogenous hormones but phenol red, which possesses weak oestrogenic activity [30, 31], was present in MEM as a pH indicator. Nonetheless, on the basis of cell proliferation data and of the results obtained on the MCF-7 cells in such a schedule, we do not believe that the absence of sensitivity of MXT cell kinetics to oestradiol was due to phenol red. It should be pointed out that cell counts represent the net result between proliferation and death whereas cell kinetics only concern a selection of still living cells, a fact that might explain this poor match between cell count data and cell kinetics. The 1 μM E2-induced stimulation of MCF-7 cell cycle kinetics was accompanied by a dramatic reduction of the cell count to below the control value. The cell count indicates that 1 μM E2 exerts a cytocidal effect on MXT as well as on MCF-7 cells although it does not influence MXT and activated MCF-7 cell cycle kinetics. It thus appears from these two different analyses and from the experimental conditions used that high-dose oestradiol exerts its cytocidal effect in a cell cycle-independent manner.

In conclusion, our results show that high-dose (1 μM) androstenedione exerts an inhibitory influence on MCF-7 human mammary cancer cell proliferation and a stimulatory effect on that of the MXT mouse model, both cell lines being devoid in the present case of any detectable aromatase activity. At 0.01 μM, AD inhibits both MCF-7 and MXT proliferation. Oestradiol at a high-dose (1 μM) dramatically slows down both MXT and MCF-7 proliferation but stimulates them at lower dose (0.01 μM). The inhibitory influence of AD seems partly to occur through a blockade of the cells at the G0–G1 phase whereas the E2-induced cytocidal effect appears as a cell cycle-independent effect.

A better understanding of androgen action on breast cancer cells would improve the androgen treatment of breast cancer used for several decades to treat or prevent oestrogenic responsive mammary cancers (see [4]). We are presently conducting further *in vivo* and *in vitro* experiments on MXT and MCF-7 cell lines using aromatizable and non-aromatizable androgens and various androgen metabolites in order to study their influence on the cell proliferation in such models.

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