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Oestradiol Enhances Doxorubicin Uptake and Cytotoxicity in Human Breast Cancer Cells (MCF-7) *

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Abstract—*The cytotoxic effect of doxorubicin on human breast cancer cells (MCF-7) appeared to be correlated with drug concentration, exposure time and cellular uptake of doxorubicin. The effects of short-term stimulation of the growth of MCF-7 cells with* 30 pM *oestradiol was investigated with respect to the uptake of doxorubicin and cell kill. Culture of MCF-7 cells in steroid hormone-deprived medium resulted in an approx.* 90% *arrest of the cells in the G0G1-phase of the cell cycle. Growth stimulation with* 30 pM *oestradiol caused a* 3–5*-fold increase in the number of cells in S-G2M phase at between* 18 *and* 24 h *after administration of oestradiol to the medium. Incubation of oestradiol-stimulated cells with* 0.37 μM *doxorubicin during both* 1 *and* 6 h *resulted in an augmented inhibition of cell growth compared to unstimulated controls. An enhanced cellular uptake of doxorubicin after administration of oestradiol was observed only after an incubation period of* 6 h *and not of* 1 h. *These observations suggest that both an increased sensitivity to doxorubicin and an augmented cellular uptake of the drug may underlie the cytotoxic effects of doxorubicin after pretreatment with oestradiol.*

INTRODUCTION

IN THE PAST 10–15 years combination chemotherapy as well as treatment with new (anti-)steroidal agents have improved the therapeutic benefit for breast cancer patients [1, 2]. However, for several years a plateau phase has been reached with respect to response rate, duration of response and survival. At its best hormonal treatment or chemotherapy can reach a maximal response rate of 50–75% in (subgroups of) patients with metastatic breast cancer, with a mean duration of response of about 12 or 8 months respectively. Combination therapy with cytostatics and growth inhibitory hormones may cause a modest improvement in treatment results compared to single treatment modalities, especially in postmenopausal patients with steroid receptor positive tumours [3–6]. However, response rates generally do not surpass 75%, and the results of different studies are conflicting. Treatment with growth inhibitory hormones can even decrease the efficacy of chemotherapy in subgroups of patients [4]. Growth inhibitory hormones can interfere with the action of cytostatic drugs indirectly by influencing drug metabolism and immune function, and directly by influencing cellular uptake or cytotsatic drugs or cell kinetics [7]. One of the reasons for treatment failure could be the apparently high proportion of tumour cells in the resting phase of the cell cycle, in which phase cells are generally less sensitive to chemotherapeutic agents [7, 8].

A new approach in the treatment of breast cancer patients involves short-term stimulation of tumour cell growth to improve the therapeutic ratio of cytotoxic agents [7, 9]. Oestradiol and some other hormones and growth factors can induce recruitment of quiescent cells into the proliferative phase of the cell cycle, causing cells to be more vulnerable to subsequent administration of cytotoxic agents [10, 11]. The results of different experimental studies have shown that pretreatment of tumour cells with physiological and low pharmacological dosages of oestrogens can enhance the cytotoxic effect of chemotherapeutic agents *in vitro* [7, 9–11].

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The first clinical studies involving such hormonal manipulation showed an improvement in the percentage of complete remissions and/or survival [12–15], while in only a few small studies no benefit of oestrogen recruitment with subsequent chemotherapy has been reported [16, 17]. However, the points most open to discussion are the following: (a) the importance of cell synchronization using tamoxifen prior to stimulation of tumour cells; (b) the choice of the growth stimulatory agents to be used in addition to oestrogens (if necessary); (c) the optimal dosages of the growth stimuli; and (d) the optimal duration of the stimulation period. Furthermore, at present very little is known on the effects of short-term hormonal stimulation of tumour cells upon the degree of cellular uptake of cytostatic agents.

Therefore, using human breast cancer (MCF-7) cells as an experimental model, we have investigated the effects of pretreatment of these cells with oestradiol on their uptake of doxorubicin and the subsequent tumour cell kill. This study shows the results of experiments in which the effects of various time periods of oestradiol stimulation and doxorubicin incubation were investigated.

MATERIALS AND METHODS

Cell culture

MCF-7 human breast cancer cells were originally obtained from EG&G Mason Research Institute, Worcester, U.S.A. in its 219th passage. Cells were routinely cultured in RPMI 1640 medium containing 5 mg/l phenol red and supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), insulin (10 μg/ml) and 10% heat-inactivated foetal calf serum (i.e. complete growth medium). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air and cells were passaged weekly. For experiments, cells of exponentially growing cells in culture were harvested after incubation for 5 min at 37°C with 2 ml of 0.25% trypsin in Dulbecco's phosphate buffered saline, lacking Ca^{2+} and Mg^{2+} (DPBS). Subsequently, cells were seeded in T25 flasks (ca 10^5 cells/flask) in complete growth medium or in RPMI 1640 medium containing phenol red and supplemented with 10% steroid-depleted male human serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) (i.e. experimental medium). Previous experiments in our laboratory had shown only an approx. 10% stimulation of growth by oestradiol, when cells were cultured in RPMI-1640 medium containing phenol red, supplemented with steroid-depleted foetal calf serum [18]. Replacing the foetal calf serum by 10% steroid-depleted male human serum, as applied by Devleeschouwer *et al.*, revealed a 4–6-fold stimulation of growth with already very low concentrations of oestradiol, 10–100 pM [19–20]. Doxorubicin as doxorubicin hydrochloride/lactosum (in H_2O) and oestradiol (in ethanol) were added as indicated in the legends to the figures. Steroid depletion of male human serum was performed by two 45 min incubations at 50°C with 0.5% charcoal–0.05% dextran T-70 (w/v), with an intermediate 2 h incubation at 37°C with sulphatase (2 U/ml).

Estimation of cell survival following exposure to doxorubicin

Monolayer cultures were incubated for different time periods (1–72 h) in the absence and presence of doxorubicin at concentrations as indicated in the legends to the figures. Immediately after the incubation period the monolayer cells were washed with 0.15 M NaCl and supplied with drug-free complete growth medium. After daily medium refreshment, cells were harvested by trypsinization and cell number was determined with a haemocytometer.

Hormonal manipulation and flow cytometer analysis

Medium containing 30 pM oestradiol or the equivalent volume of the solvent ethanol (final ethanol concentration 0.025%) was added to MCF-7 cell cultures after 6 days of incubation in steroid-deprived experimental medium. At different time points after oestradiol addition cells were harvested by trypsinization and pelleted by centrifugation for 5 min at 100 ***g***. Cells were subsequently resuspended in 150 μl PBS containing 0.01 mg/ml propidium iodide (PI), 1 mg/ml ribonuclease A and 1% Triton X-100. Incubation with PI was performed for 10 min at room temperature. After washing of the cells with 0.15 M NaCl, the PI fluorescence of the nuclei was determined with a Fluorescence Activated Cell Sorter (FACS 440, Becton and Dickinson).

Incubation with doxorubicin and determination of cellular doxorubicin content

Monolayer cells were incubated with different concentrations of doxorubicin, ranging from 0.037 to 37 μM, as mentioned in the legends to the figures. At the indicated time points, cells of triplicate incubations were harvested by trypsinization and washed with 0.15 M NaCl. Cells from three T25-flasks were used for the extraction of cellular anthracycline as previously described by Kokenberg *et al.* [21]. The other T25-flasks of replicate cultures were used for the estimation of cell survival. Doxorubicin contents of cellular extracts were measured by high performance liquid chromatography (HPLC) according to the method adapted from Israel *et al.* [22]. The detection limit after extraction from biological fluid was 10 ng doxorubicin/ml. The metabolite doxorubicinol was detected.

Statistical analysis

The significance of differences between the results

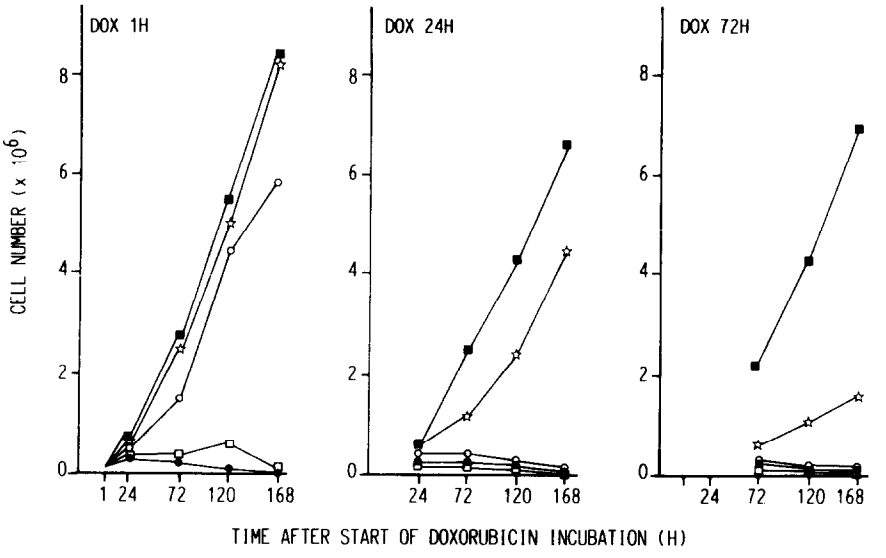

Fig. 1. Survival of MCF-7 cells after incubation with doxorubicin. MCF-7 cells grown for 2 days in complete growth medium were incubated at 0 h in the absence (■) or presence of 0.037 (☆), 0.37 (○), 3.7 (□) and 37 (●) μM doxorubicin. The incubation times with doxorubicin were 1 (left panel), 24 (middle panel) and 72 h (right panel). At the indicated time points the number of cells in the flasks of triplicate cultures was determined.

obtained with the different incubation conditions was calculated using Wilcoxon's test.

RESULTS

The effects of drug concentration and exposure time on cellular content of doxorubicin and cell growth

Exposure of MCF-7 human breast cancer cells *in vitro* to different concentrations of doxorubicin results in a dose-dependent antiproliferative effect of the drug (Fig. 1). Moreover, the antiproliferative effect in the sense of inhibition of cell growth or even decrease in cell number appeared not only to be correlated with the drug concentration of doxorubicin but also with the duration of exposure. In this respect, the cellular uptake of doxorubicin appeared positively correlated with both drug concentration and duration of exposure (Table 1). At the lowest extracellular concentration of doxorubicin (0.037 μM) there was no significant difference between cellular uptake after 1 and 24 h of incubation. The absence of an expected increased uptake after 24 h might be caused by the fact that such low concentrations were at the lower limits of detection.

With respect to growth inhibition, long-term incubation with the lower dosages of doxorubin was as efficient as short-term exposures with 10 times higher dosages (Fig. 1).

Effects of oestradiol on cell cycle

Incubation of resting MCF-7 cell cultures with a low concentration of oestradiol (30 pM) resulted in an increase in the number of actively proliferating cells, as measured by PI-fluorescence with flow cytometry (Fig. 2). The maximal increase in the number of cells in S-G2M phase was found to occur 24 h after the oestradiol pulse (Fig. 3). This fraction increased in this time period from approx. 20 to 50% (Fig. 3), while in the control group only 10% of the cells in the S-G2M phase of the cell cycle were observed at 24 h.

Effect of oestradiol on cellular doxorubicin uptake and tumour cell growth

Pretreatment of MCF-7 tumour cells with 30 pM oestradiol caused a significantly ($P < 0.05$) higher cellular uptake of doxorubicin (259 ± 11 pmol/10^6 cells; mean ± S.D., $n = 3$), when incubated with

Table 1. The effects of drug concentration in medium and drug exposure time on cellular content of doxorubicin (DOX). Monolayer cells were incubated with doxorubicin (0.037, 0.37, 3.7 and 37 μM) *for 1 and 24 h. The cellular uptake of the drug was measured by HPLC*

CELLULAR DOX
(p mol/10^6 cells ± SD)

Extracellular DOX (uM)	DOX (1h)	DOX (24h)
0.037	85 (± 32)	48 (± 9)
0.37	330 (± 103)	565 (± 56)
3.7	3486 (± 2333)	7694 (± 1747)
37	7378 (± 2116)	41066 (± 6108)

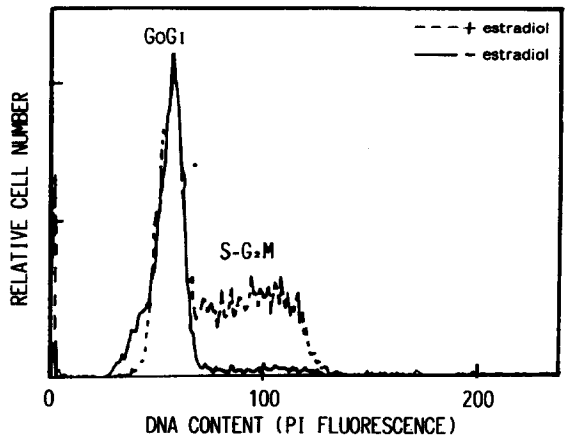

Fig. 2. Effect of oestradiol on cell cycle distribution. MCF-7 cells were cultured for 6 days in steroid hormone-deprived experimental medium. Cell cycle distribution, measured by PI-fluorescence, is plotted for control cultures and cultures stimulated with oestradiol for 24 h. The percentage of cells in the S-G2M-phase was calculated from the areas of the peaks as depicted in this figure.

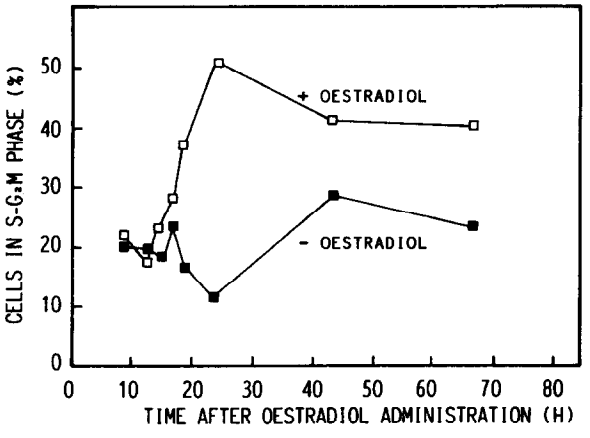

Fig. 3. Kinetics of the oestradiol effect on cell cycle distribution. MCF-7 cells were cultured for 6 days in steroid hormone-deprived experimental medium. At the indicated time points the percentage of cells in S-G2M-phase, determined by flow cytometry, is plotted for the oestradiol (30 pM) stimulated and unstimulated cultures.

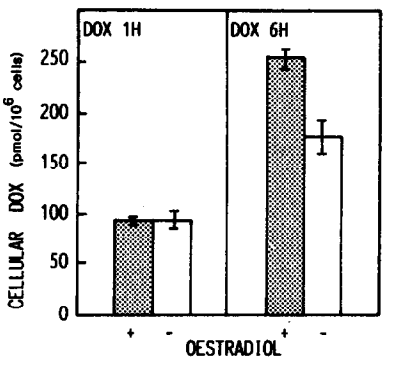

Fig. 4. Effect of oestradiol pretreatment on the cellular uptake of doxorubicin. Cellular doxorubicin content was measured by HPLC after 1 and 6 h incubation with doxorubicin (0.37 μM) in oestradiol (30 pM) stimulated (+) and unstimulated (−) control cultures. The 6 h incubation with doxorubicin started 19 h after the administration of oestradiol. The 1 h incubation started 24 h after oestradiol stimulation.

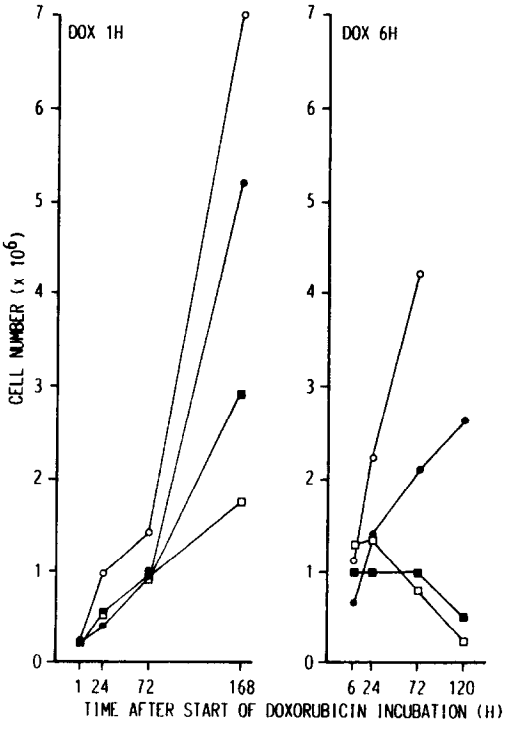

Fig. 5. Effect of oestradiol pretreatment on the cytotoxicity of doxorubicin. After oestradiol (30 pM) stimulation and incubation with 0.37 μM doxorubicin during 1 and 6 h as indicated in Fig. 4, replicate cultures were washed and drug-free complete growth medium was added to the flasks. At the indicated time points the number of cells in the flasks was determined and growth curves plotted. In the left panel it is shown that after 168 h, growth inhibition by a 1-h incubation with 0.37 μM doxorubicin is significantly higher when the cultures are pretreated with oestradiol ($1.7 \pm 0.14 \times 10^6$ cells vs. $2.93 \pm 0.13 \times 10^6$ cells, mean ± S.D., n = 3) ($P < 0.05$). In the right panel the decrease in cell number 120 h after a 6 h incubation with doxorubicin is significantly higher in the cultures pretreated with oestradiol ($0.20 \pm 0.02 \times 10^6$ cells vs. $0.49 \pm 0.04 \times 10^6$ cells; mean ± S.D., n = 3) ($P < 0.05$). [Control plus oestradiol (○), control without oestradiol (●), doxorubicin plus oestradiol stimulation (□), doxorubicin without oestradiol stimulation (■).]

0.37 μM doxorubicin during 6 h (Fig. 4) and compared to unstimulated cells (177 ± 16 pmol/10^6 cells; mean ± S.D., $n = 3$). Such an increased cellular uptake of doxorubicin was not found after an incubation period of 1 h (94 ± 2 vs. 94 ± 8 pmol/10^6 cells; mean ± S.D., $n = 2$). However, with respect to cell growth, pretreatment with oestradiol appeared to enhance the cytotoxic efficacy of doxorubicin (0.37 μM) both during 1- and 6-h incubations (Fig. 5). It has to be noted that a net decrease in cell number caused by doxorubicin was observed not prior than 72 h after an incubation period of 6 hours with the drug (Fig. 5).

DISCUSSION

Chemotherapy of metastatic breast cancer has currently reached a plateau in therapeutic results

with response rates in the range of 50–75% [1, 2]. Moreover, in early breast cancer adjuvant therapy mostly fails to accomplish cure [23]. It seems, therefore, that no further improvement can be expected from the currently used multi-drug therapies alone [23, 24]. Even combined hormono-chemotherapy, which at its best results in an additive effect in subgroups of patients, causes an objective but only temporary response in approx. 75% of the patients [3–6]. An explanation for the lack of cure and the temporary nature of the response in patients with metastatic disease may be found in the observation that, spontaneously or due to growth-inhibitory endocrine therapy, a significant proportion of the tumour cells are in the resting phase of the cell cycle. As a result, tumour cells display cytokinetic drug resistance even to high dosages of chemotherapeutic agents. Most of these agents, including doxorubicin, are primarily effective on proliferating cells. In agreement with these findings are the results of a study of Sulkes *et al.* [25], which show that slowly growing primary breast cancers with a low labelling index (LI) are not or less sensitive to chemotherapy as compared to tumours with a high LI. Recently, it has been shown that growth-stimulatory hormones (such as oestrogens) can increase the cell sensitivity to cytotoxic chemotherapy by increasing the number of actively proliferating cells in these tumours [10, 11]. Several clinical studies, in which the effects of oestrogen 'rescue' or recruitment of tumour cells in the treatment schedule were investigated, show high complete response rates and sometimes an increase in overall survival [12–15]. There are also a few reported studies in which no significant benefit of this treatment modality was found [16, 17]. Nevertheless, the results of these studies are difficult to compare because of a great variation in treatment schedules. These differences involve the dose and choice of chemotherapeutic agents and oestrogens, the use of tamoxifen, the way and duration of treatment, and time scheduling of the drugs used. At present the optimal treatment protocol is unknown.

The results of our study suggest that for optimal cell kill the time interval between administration of oestrogens and doxorubicin should be around 18–24 h, as shown by an increase in the number of proliferating cells from about 10–20% to 50–60% in several experiments. Pretreatment of human breast cancer cells with oestradiol from 19–24 h before administration of doxorubicin results in increased cytotoxic efficacy of the drug. This is the first report showing that pretreatment with a physiological dose of oestradiol can increase the cellular uptake of doxorubicin. Inhibition of cell growth appeared to be correlated with the cellular content of doxorubicin. Moreover, pretreatment with oestradiol increased cytotoxicity also after short-term (1 h) incubation with doxorubicin without enhanced cellular uptake of the drug. These observations suggest that both an increased sensitivity to doxorubicin and an augmented cellular uptake of the drug may underlie the present cytotoxic effects of doxorubicin.

Apart from the time of administration, the dose of oestradiol probably is also important in view of the finding that pharmacological dosages (10^{-6}, 10^{-7} M) caused reduced cellular uptake and antimetabolic effects of methotrexate and nevertheless resulted in reduced cell growth. Thus, short-term pretreatment with a pharmacological dose of oestradiol may indeed enhance drug sensitivity of tumour cells, but it may possibly prevent additional beneficial effects by increased cellular uptake of cytotoxic drugs as observed after pretreatment of cells with physiological dosages of oestradiol. However, the oestradiol-stimulated drug uptake may only affect the uptake of specific cytotoxic drugs, such as doxorubicin. Other drugs may even show a decreased uptake resulting from effects of oestradiol on drug transport via the cell membrane [26].

In conclusion, our study shows that the optimal interval between the start of pretreatment with oestradiol and administration of doxorubicin is 18–24 h. During this period a 3–5-fold increase in the number of proliferating MCF-7 cells was observed. Cells showed a higher uptake of doxorubicin and an increased sensitivity to doxorubicin, which resulted in increased cell kill. Combined treatment with specific hormones and growth factors can improve the cytotoxic ratio of doxorubicin [11]. Therefore, further studies should focus on combinations of hormones and growth factors with respect to recruitment of resting cells prior to chemotherapy.

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