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Prolonged Tamoxifen Exposure Selects a Breast Cancer Cell Clone that is Stable *In vitro* and *In vivo*

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The effects of long-term tamoxifen exposure on cell growth and cell cycle kinetics were compared between oestrogen receptor (ER)-positive (MCF-7) and ER-negative (MDA-MB-231) cell lines. In the MCF-7 cell line, prolonged tamoxifen exposure (0.5 μmol/l for > 100 days) blocked cells in G0–G1 of the cell cycle, and slowed the doubling time of cells from 30 to 59 h. These effects corresponded to an increase in the cellular accumulation of tamoxifen over time [mean area under concentration curve (AUC) = 77.92 μmoles/10^6/cells/day]. In contrast, in the MDA-MB-231 cell line, long-term tamoxifen exposure had no obvious effect on the doubling time, and reduced cellular tamoxifen accumulation (mean AUC = 50.50 μmoles/10^6/cells/day) compared to the MCF-7 cells. Flow cytometric analysis of MDA-MB-231 cells demonstrated that a new tetraploid clone emerged following 56 days of tamoxifen exposure. Inoculation of the MDA-MB-231 tetraploid clone and MDA-MB-231 wildtype cells into the opposite flanks of athymic nude mice resulted in the rapid growth of tetraploid tumours. The tetraploid tumours maintained their ploidy following tamoxifen treatment for nine consecutive serial transplantations. Histological examination of the fifth transplant generation xenografts revealed that the tetraploid tumour had a 25–30 times greater mass, area of haemorrhage and necrosis, a slightly higher mitotic index and was more anaplastic than the control neoplasm. The control wildtype MDA-MB-231 tumours maintained a stable ploidy following tamoxifen treatment until the eighth and ninth transplantation, when a tetraploid population appeared, suggesting that tamoxifen treatment may select for this clone *in vivo*. These studies suggest that prolonged tamoxifen exposure may select for new, stable, fast growing cell clones *in vitro* as well as *in vivo*.
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INTRODUCTION

TAMOXIFEN IS a non-steroidal triphenylethylene anti-oestrogen that has been widely used in the treatment of patients with breast cancer. It is effective in prolonging both disease-free and overall survival of women following primary surgery [1], and it inhibits tumour growth in approximately 50% of women with advanced oestrogen receptor (ER)-positive metastatic breast cancer [2]. It is considered relatively non-toxic when compared with classic chemotherapeutic agents [3]. However, although many patients initially respond to tamoxifen therapy, acquired tamoxifen resistance eventually develops following chronic administration. The mechanism underlying the development of tamoxifen resistance is poorly understood.

It has been shown that tamoxifen can actually stimulate tumour growth, following chronic dosing, in hormone-responsive human breast tumours growing in nude mice [4]. In addition, several metabolites of tamoxifen have been noted to have oestrogenic activity *in vitro* [5, 6]. Metabolism of tamoxifen to oestrogenic metabolites may in part contribute to the stimulation of hormone-responsive tumours, following prolonged exposure to tamoxifen. Interestingly, the presence of monophenol tamoxifen, an oestrogenic metabolite of tamoxifen, has been identified in tamoxifen-resistant human breast cancer tumour tissues [7].

Tamoxifen has also been reported to induce secondary tumours such as endometrial cancers [8–12]. In addition, a more aggressive form of hormone-independent mammary tumour has been reported by Fendl and Zimniski in a dimethylbenzanthracine (DMBA)-induced rat mammary tumour model following tamoxifen treatment [13]. In recent studies performed in our laboratory, tamoxifen and its metabolites were present in secondary endometrial tumours of breast cancer patients treated chronically with tamoxifen (unpublished results). Furthermore, Magriples *et al.* recently published a retrospective study of women who developed endometrial tumours while taking tamoxifen. These authors suggest that tamoxifen-induced endometrial tumours had a poorer prognosis than spontaneously developing endometrial cancer, further suggesting that tamoxifen induces aggressive tumours [14].

These data suggest that tamoxifen may, in fact, stimulate the growth of cells, perhaps through either the induction or selection of a more aggressive cancer clone. In the present study, we examined the *in vitro* and *in vivo* effects of prolonged tamoxifen exposure on the growth characteristics, and cell cycle kinetics in a human breast cancer cell line MDA-MB-231.

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MATERIALS AND METHODS

Cell lines

An ER-negative breast cancer cell line (MDA-MB-231) and an ER-positive (MCF-7) cell line were grown in improved minimum essential medium (IMEM) supplemented with 10% fetal bovine serum (FBS). All cells were grown in quadruplicate Corning T-75 culture flasks and maintained in 5% CO_2 and 95% air at 37°C.

Tamoxifen exposure

Cells were plated at 15 000 cells/ml 24 h prior to the addition of the drug-containing solution. Tamoxifen was dissolved in ethanol and sterile saline (final concentration < 0.1% ethanol) before dilution with media. Sterile saline containing < 0.1% ethanol was added to each control flask. Quadruplicate flasks of cells were exposed to tamoxifen (0.5 μmol/l) for 100+ days. Following exposure, medium was aspirated, and cells were washed with sterile phosphate buffered saline (PBS). Cells were then trypsinised and counted using a Coulter Model ZM Counter. Cells were harvested at various times to assess doubling time and tamoxifen accumulation. Cell cycle measurements were also analysed on every passage using flow cytometric analysis.

Tamoxifen accumulation

During tamoxifen exposure, duplicate flasks of cells were randomly selected for analysis. Cells were washed once with ice-cold saline (4°C), scraped from the flask using a rubber scraper, and removed by adding 4 ml of PBS, and transferred to a glass extraction tube. The exact volume of cell suspension was measured, and a 20–100 μl aliquot (10^6 cells) was removed and counted. Cells were then centrifuged at 200 *g* for 6 min, and the cell pellet analysed for tamoxifen content as described previously [7]. The cellular tamoxifen concentrations were quantified at each time point ($n = 11$), and the area under concentration curve (AUC, μmoles/10^6 cell/day) calculated using the trapezoidal rule.

Flow cytometry

Cells were stained with a modified Krishan technique [15]. Aliquots of 2.0×10^6 cells were centrifuged at 200 *g* for 6 min at 40°C, cell pellets were resuspended in 1.0 ml of Krishan staining solution (50 μg/ml propidium iodide in a hypotonic sodium citrate solution with 0.3% NP-40 and 1.0 mg/ml RNA-Ase-A), vortexed and stained for 30 min at room temperature in the dark. Prior to flow cytometric measurements, samples were filtered through a 37 μ nylon mesh into 12 × 75 mm tubes and stored at 4°C. Flow cytometric measurements were performed on an EPICS 753 instrument (Coulter Cytometry, Hialeah, Florida, U.S.A.), with an argon-ion laser tuned to 488 nm and 400 mW of power. Red fluorescence from the propidium iodide (peak fluorescence at 610 nm) was collected through 515 nm long pass and 610 nm long pass filters. Compartmental analysis of DNA histograms was accomplished with MODFIT software (Verity Software House, Inc., Topsham, Maine, U.S.A.).

In vivo *stability of tamoxifen-induced clone*

The stability and growth rates of both control MDA-MB-231 and tetraploid clones were then examined in nude mice. Six athymic female 4–5-week-old nude mice (BALB-C/nu/nu) were kept in a temperature-controlled room on a 12 h:12 h light:dark schedule with food and water *ad libitum*. Breast cancer cell lines were resuspended in sterile saline at a density of 3 million/200 μl. Both cell lines (3×10^6) were injected subcutaneously (sc) on opposite sides of the same animal in the scapular area. A 20-gauge needle was used for injection. Tumours were allowed to grow for 3 weeks prior to drug administration. Three of the mice were then treated with tamoxifen, 500 μg/day sc. The others served as control animals without any treatment. After 22 days, one tamoxifen-treated and one control mouse were sacrificed. The tumours were collected and the weight recorded. The tumours were analysed by flow cytometry. Two more mice were sacrificed on days 35 and 57. On day 57, we transplanted a small piece of the control tumour and tetraploid tumour into six additional mice. We continued this pattern of transplantations, flow cytometric analysis and tamoxifen treatment for 10 consecutive transplantations.

Histology

Following euthanasia of two representative mice bearing the fifth transplant generation xenografts, tumours were removed for histological examination. The tumour tissues were fixed in 10% neutral buffered formalin, processed conventionally, embedded in paraffin, cut at 5 μ, stained with haematoxylin and eosin, and examined with a light microscope.

In addition to histological examination of these tumours, tumours were cloned *in vitro* and maintained for 27 serial passages without tamoxifen exposure. Flow cytometric measurements were performed on these cells periodically.

RESULTS

Table 1 shows the results of *in vitro* tamoxifen exposure on MDA-MB-231 (ER-negative) and MCF-7 (ER-positive) cell lines. As shown, tamoxifen had a cytostatic effect on the MCF-7 cells, and had a minimal effect on the MDA-MB-231 cell line. These effects are consistent with the anti-oestrogenic properties of tamoxifen at the dose used (0.5 μmol/l). The cellular accumulation of tamoxifen was also examined in both cell lines. Cellular accumulation of tamoxifen in the MCF-7 cell line over the exposure time was also greater than in the MDA-MB-231 cell line (Table 1).

Figures 1 and 2 show the results of flow cytometric analysis on the cell cycles kinetics of MCF-7 and MDA-MB-231 cells following prolonged tamoxifen exposure. Figure 1 shows DNA histograms derived from control (a) and tamoxifen-treated (b) MCF-7 cells. Note the reduction of cells in S-phase and the increase in cells G0–G1 following tamoxifen exposure.

Figure 2 shows DNA histograms derived from quadruplicate control (a) and tamoxifen-treated (b) MDA-MB-231 cells. Compared to controls, tamoxifen caused no accumulation of cells in G0–G1 as would be expected in an ER-negative cell line. However, starting at day 56, an accumulation of cells was noted in the G2 + M phase of the cell cycle, which suggested that tamoxifen was having some effects on the cell cycle. By day 77,

Table 1. Cellular doubling time (DT) and tamoxifen (TAM) accumulation (AUC) following prolonged tamoxifen exposure

Cell line	DT (h) without TAM*	DT (h) with TAM* (0.5 μmol/l)	TAM (AUC)† (μmoles/10^6 cells/day)
MCF-7 (ER+)	29.73 ± 8.29	59.00 ± 10.82	77.92
MDA-MB-231 (ER−)	21.05 ± 2.41	23.17 ± 2.13	50.50

*Values are mean ± standard deviations. †AUC were calculated for 67 days of continuous tamoxifen exposure in both cell lines

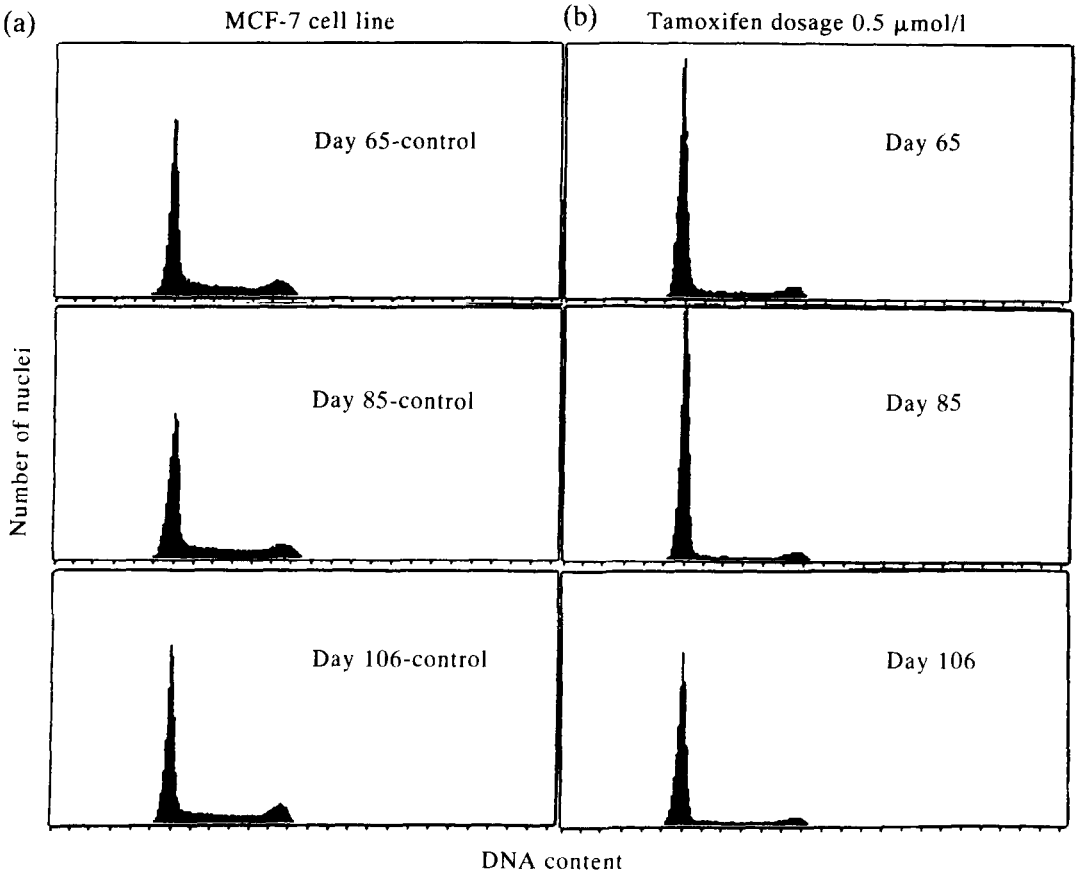

Fig. 1. Flow cytometry histograms of ER-positive MCF-7 cells with and without tamoxifen exposure (0.5 μmol/l) for 100+ days. Duplicate flasks were examined. The percent G0G1 for control cells on days 65, 86 and 106 were 56.7, 55.4 and 52.9%, respectively. The percent G0G1 for treated cells on days 65, 86 and 106 were 72.4, 80.5 and 73.7%, respectively.

this effect was even more pronounced and suggested that tamoxifen had potentially selected a new tetraploid cell clone. By day 102, cells continued to accumulate in G2 + M phase suggesting that the tetraploid condition was stable. The stability of the new tetraploid clone was then examined by growing a portion of the MDA-MB-231 tamoxifen-treated cells (clone) and MDA-MB-231 control cells without tamoxifen for 2 weeks. No further changes in DNA histograms of these cultures were found (Fig. 3).

To further address whether the tetraploid clone was stable, we inoculated into the opposite sides of six athymic nude mice both clone and control MDA-MB-231 cells. The tumours grew rapidly for 3 weeks. Three mice were then treated with tamoxifen (500 μg/day), and three remained as controls. Tumours were harvested at 22, 35 and 57 days from tumour inoculation. Flow cytometric analysis showed no change in the ploidy of cells derived from the individual tumours. Figure 4 shows the DNA histograms of two mice, showing that there was no apparent difference in the histograms with or without *in vivo* tamoxifen treatment.

Figure 5 compares the tumour size of mice inoculated with MDA-MB-231 wild type cells (left scapular) and with tamoxifen-pretreated MDA-MB-231 clones (right scapular). Note the very large tumours induced by inoculation of mice with the MDA-MB-231 clones: these results suggest that prolonged tamoxifen exposure may induce fast growing tumour cell clones in hormone-independent cells.

The results of the second generation transplant of the tetraploid clone show evidence of prolonged *in vivo* stability. The DNA histograms of transplanted tumours evaluated on day 28 are shown in Fig. 6. The tetraploid nature of the transplanted tumour cells compared to wild type tumour cells is clearly distinguishable.

The histological examination of the fifth transplant generation xenografts showed a clear difference between the tetraploid and control tumours (Fig. 7a, b). The tetraploid tumour mass could be first viewed totally in cross section in 12 low power (40×) microscopic fields. The neoplasm was expansive with compression and oedematous change in adjacent peripheral tissues. Moderate pleocellular inflammatory infiltrates were evident in adjacent tissues and within the neoplasm. Large areas of necrosis and haemorrhage were prominent throughout the neoplasm. The anaplastic cells tended to form sheets with occasional clusters. The cells were large, variably sized and shaped but tended to be oval to round with abundant lightly eosinophilic cytoplasms. Nuclei were large, variably sized and shaped, and generally contained multiple distinct nucleoli. Mitotic figures exceeded six per high power field. The control tumour mass could be viewed totally in cross section already in one low power (40×) microscopic field. The neoplasm was minimally expansive, and had only a few inflammatory cells associated with its margins and essentially none in the tumour parenchyma. Only minimal, generally individual, cellular necrosis was seen. No haemorrhage was evident. The anaplastic cells formed no definite architectural pattern. The cells were variably sized but were generally round to oval, with scant to abundant deeply eosinophilic cytoplasms. The nuclei were large, variably sized, with one or more nucleoli. Mitotic figures often exceeded four

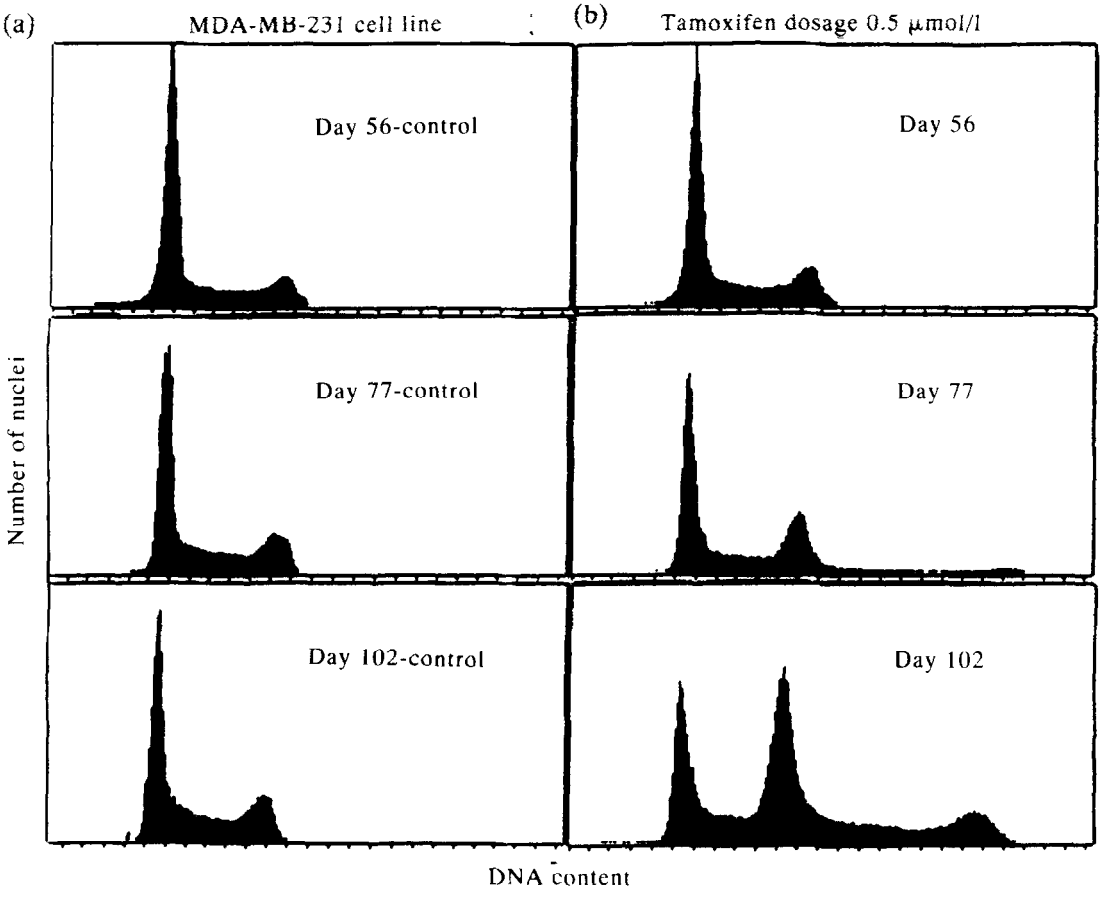

Fig. 2. Flow cytometry histograms of ER-negative MDA-MB-231 cells with and without tamoxifen exposure (0.5 μmol/l) for 100+ days. Tamoxifen selects a new tetraploid cell clone after 56 days, continuing to be stable for 100+ days. Duplicate flasks were examined. The percent G2+M for control cells on days 56, 77 and 102 were 9.8, 14.3 and 16.3%, respectively. The percent G2+M for treated cells on days 65, 86 and 106 were 15.0, 27.6 and 54.0%, respectively.

per high power field. In summary, the tetraploid tumour was much larger, more expansive with marked necrosis and haemorrhage, and had a higher mitotic index compared to the control tumour.

In addition to histological examination of these tumours, ploidy remained stable on the day of sacrifice. Furthermore, flow cytometric analysis of both control and tetraploid tumours, following 27 serial *in vitro* passages (126 days), showed that both tumour-derived cell lines maintain a stable ploidy. These cell lines were maintained in tamoxifen-free media.

Currently, this pattern of *in vivo* tetraploid stability has lasted for nine consecutive transplantations. Interestingly, new data suggest that tamoxifen can select for the tetraploid clone *in vivo*. Following the eighth transplantation in tamoxifen-treated mice, the control MDA-MB-231 tumours became predominantly tetraploid, suggesting that tamoxifen has selected for the tetraploid clone *in vivo*. Interestingly, although the control tumours appeared to be tetraploid, following tamoxifen treatment *in vivo*, the *in vitro* cell lines derived from the fifth transplantation remained diploid without tamoxifen treatment. Therefore, tamoxifen could have selected for this clone *in vivo*. This is important considering that the transplantations were serially performed. The ploidy of the original tetraploid clone remained stable following tamoxifen treatment.

DISCUSSION

Tamoxifen is touted as a safe drug that can be taken for many years with relatively few side-effects. It has been shown that ER-positive breast tumours respond well to tamoxifen treatment; response rates of 48% are reported in ER-positive tumours as compared to 13% in ER-negative tumours [16]. However, tamoxifen has also been associated with the induction of tumours in animals and humans including liver, endometrial and mammary carcinomas [8–12].

During the last few years, there has been a growing number of reports that tamoxifen may actually have a stimulatory effect on cancer cell growth, including results from several animal models [4, 17]. Gottardis *et al.* have shown that tamoxifen can actually stimulate the growth of human endometrial carcinoma following chronic dosing in nude mice [18]. Satyaswaroop *et al.* also observed the emergence of an ER-positive endometrial tumour that will only grow in the presence of tamoxifen [19]. Furthermore, Fendl and Zimniski recently published data using a rat model that suggested that prolonged tamoxifen treatment caused a regression in DMBA-induced mammary tumours but also stimulated the growth of an aggressive form of hormone-independent cancer, appearing during tamoxifen treatment [13]. They also demonstrated that tamoxifen-treated ER-negative rat mammary tumours grow more rapidly than untreated ER-negative or ER-positive tumours. In addition, Anzai *et al.* recently reported that the stimulatory effects of 4-hydroxytamoxifen were greater than oestradiol on the growth of a human endometrial adenocarcinoma cell line [5].

In the present study, we have shown that prolonged tamoxifen treatment (> 100 days) of MDA-MB-231 ER-negative cells may significantly alter cell cycle kinetics and the tumorigenicity of

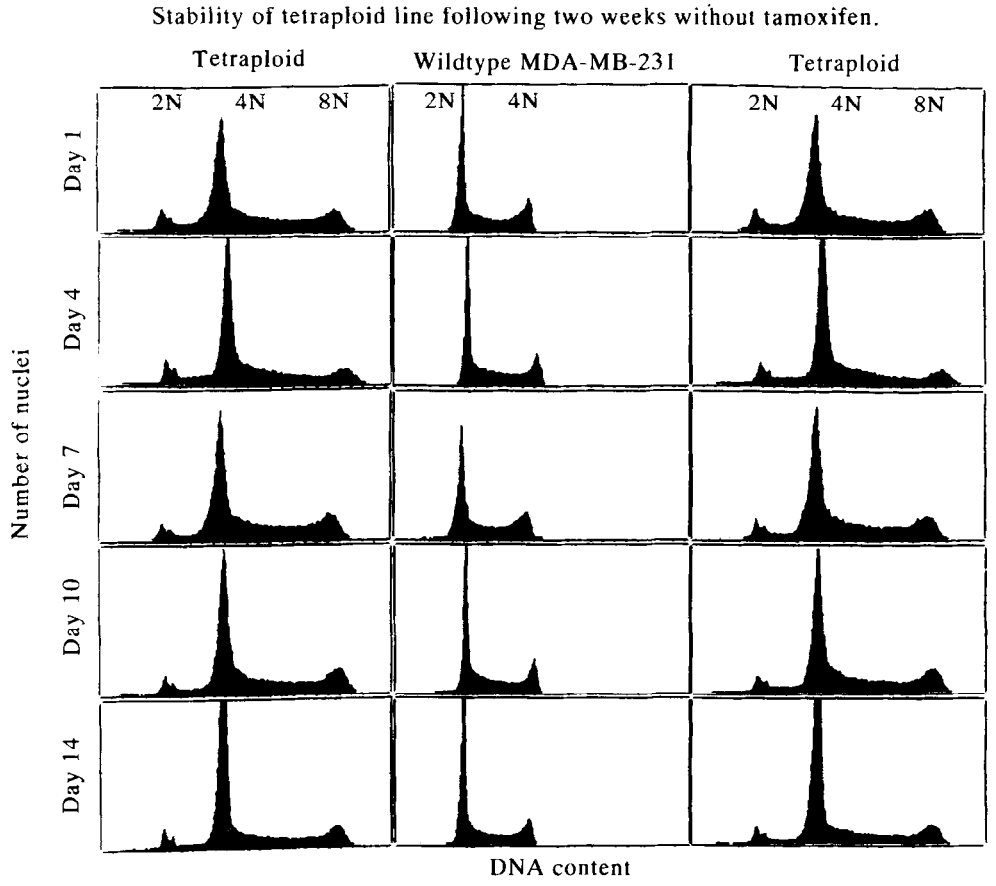

Fig. 3. Flow cytometry histograms of the control cells and the new tetraploid clone induced by tamoxifen exposure (0.5 μmol/l) over a 2-week period without tamoxifen treatment. Duplicate tetraploid (outside panels) and wild type cells were examined.

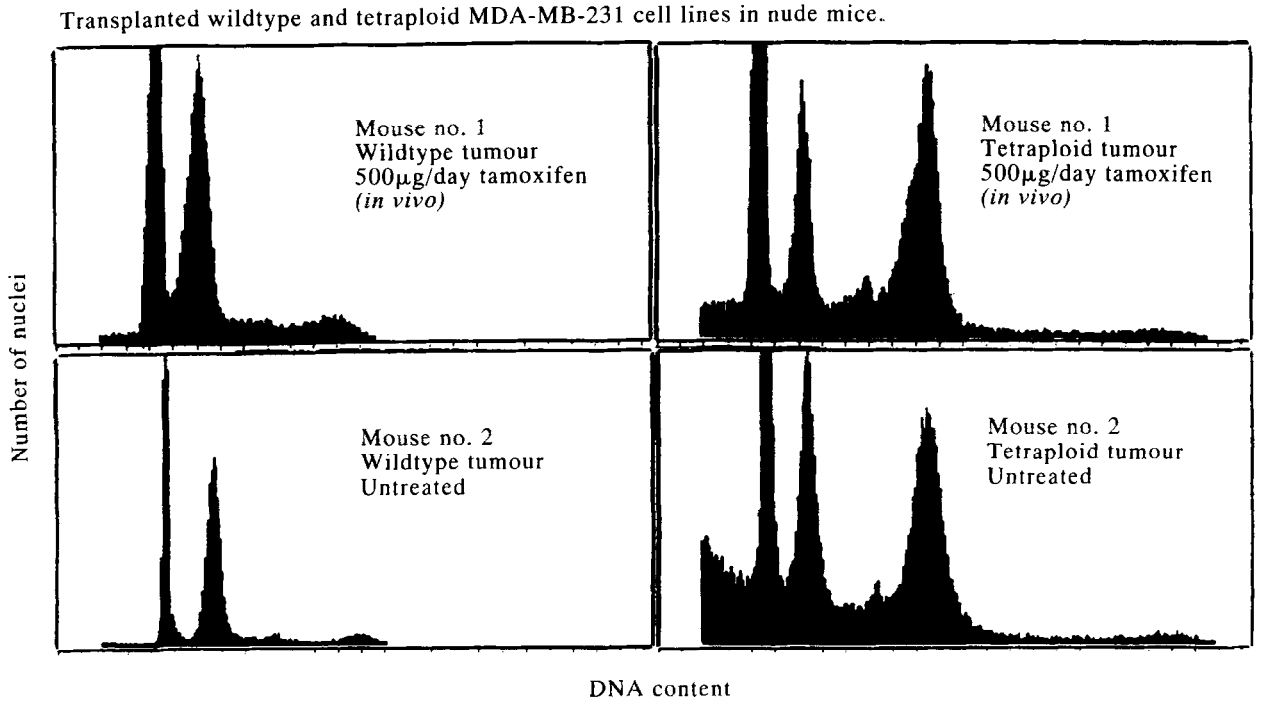

Fig. 4. Flow cytometry histograms of the tumours of two mice 35 days after inoculation with MDA-MB-231 control cells (left), and tetraploid clone cells (right) on opposite sides of the mouse. The peak on the far left of each histogram is mouse DNA.

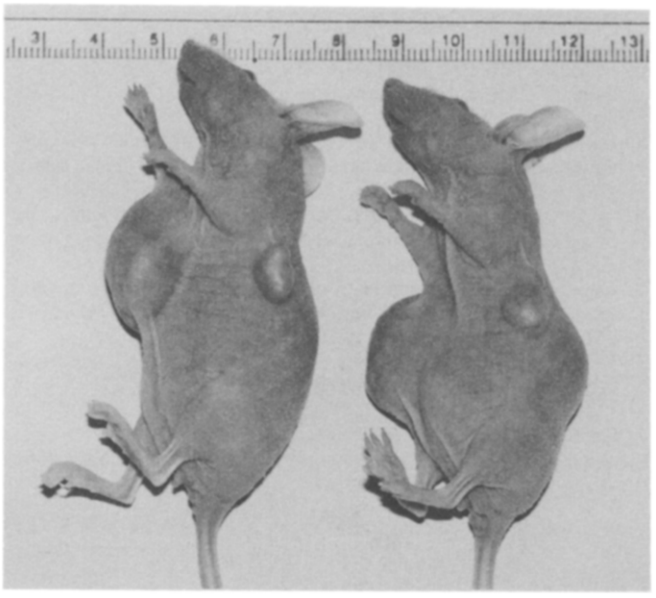

Fig. 5. Tumours on day 28 after the second transplant (Fig. 6). Left: scapular with control MDA-MB-231 cells. Right: scapular with MDA-MD-231 tamoxifen-induced tetraploid clone tumours.

these cells. The genetic instability of these cells has been reported previously with regard to ER status and tumorigenicity [20]. The tamoxifen-selected clone analysed in our study produced aggressive and rapid growing tumours when implanted into nude mice. Furthermore, our studies suggest the tamoxifen-selected clone is stable for long periods of time, and that tamoxifen may select for this clone *in vitro* as well as *in vivo*.

Whether tamoxifen's adverse effects are related to a direct carcinogenic effect on cells, or the selection of a pre-existing cell

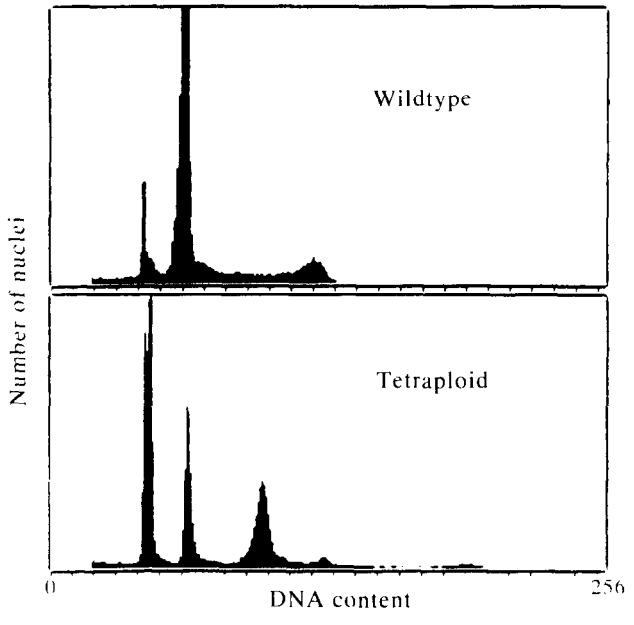

Fig. 6. Flow cytometry histograms of tumours after the second inoculation of MDA-MB-231 control and tamoxifen-induced tetraploid clone transplanted on the opposite sides of a mouse. Tumours were allowed to grow for 28 days without tamoxifen treatment. The peak on the far left of each histogram is mouse DNA.

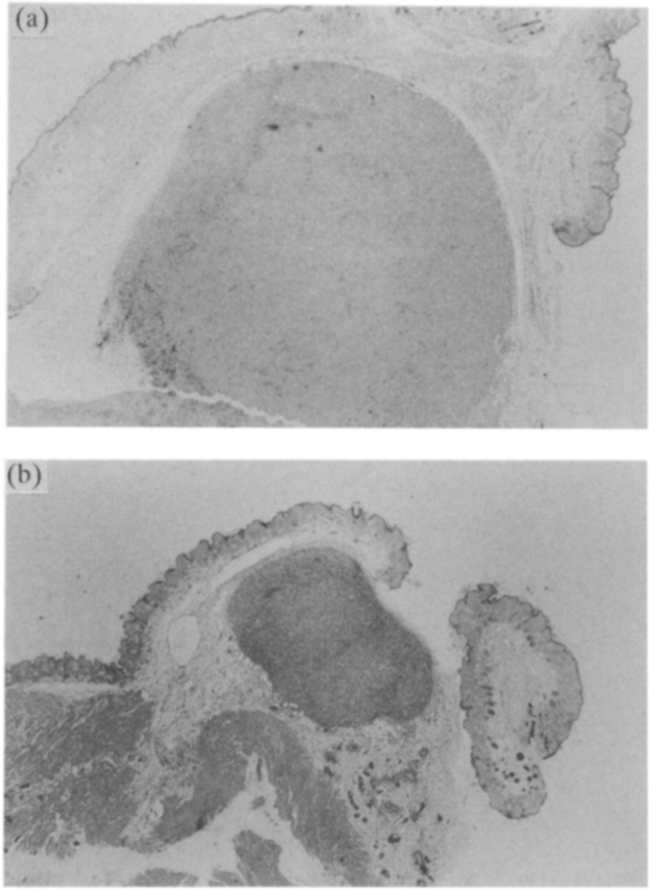

Fig. 7. (a) A low power field of a cross-section of approximately one twelfth of the fifth transplant generation tetraploid tumour shows the expansion compression of adjacent tissue, and extensive area of central necrosis. Haematoxylin and eosin, ×40. (b) A lower power field of a cross-section of the entire fifth transplant generation control tumour illustrates its small relative size, lack of significant expansion and absence of discernible necrosis. Haematoxylin and eosin, ×40.

clone remains unknown. Many authors have questioned the safety of long-term tamoxifen treatment, and especially the use of tamoxifen in women at high-risk for developing breast cancer, particularly in premenopausal women [8, 13, 21]. Further research on the pharmacological properties of tamoxifen are needed.

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Possible Coexistence of Two Independent Mechanisms Contributing to Anthracycline Resistance in Leukaemia P388 Cells

Florentina Soto, Rosa Planells-Cases, Jaume M. Canaves, Antonio V. Ferrer-Montiel, Jordi Aleu, Francisco Gamarro, Santiago Castanys, Jose M. Gonzalez-Ros and Jose A. Ferragut

Murine leukaemia P388 and L1210 cell sublines with varying degrees of resistance to the anthracycline daunomycin (DNM) have been used to monitor (i) intracellular accumulation of DNM, (ii) expression of the drug efflux pump P-glycoprotein (pgp) and (iii) cytoplasmic pH changes. Drug-resistant L1210/65 cells (65-fold resistance), overexpress pgp, and display decreased intracellular accumulation of DNM and identical intracellular pH as compared to the parental drug-sensitive L1210 cell line. On the other hand, moderately drug-resistant P388/20 cells (20-fold resistance), which also exhibit a decreased intracellular drug accumulation with respect to drug-sensitive P388/S cells, display only moderate pgp-encoding *mdr*1 gene transcription without detectable levels of pgp protein, and undergo cytoplasmic alkalinisation (up to ~0.2 pH units). A further increase in the level of drug resistance (P388/100 cells, 100-fold resistance), results in a more pronounced decrease in drug accumulation, significant pgp expression and slightly higher intracellular alkalinisation. Alterations in the degree of protonation of DNM have been shown previously to influence processes such as the rate of uptake and the intracellular accumulation of the drug. On this basis, we propose that the changes in intracellular pH, observed at low levels of drug resistance (P388/20 cells), could constitute an early cellular response aimed at decreasing the intracellular accumulation of ionisable anti-neoplastics. As the level of resistance increases (P388/100), the cells seem to require more efficient mechanisms of defense against the drug, such as that represented by the expression of pgp. Since there is no apparent correlation between the extent of the changes in intracellular pH and the level of pgp expression in DNM-resistant P388 cell sublines, it is suggested that these two cellular responses contributing to drug resistance could operate independently.
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INTRODUCTION

WHEN CONFRONTED with cytotoxic agents, tumour cells having the multidrug resistant (MDR) phenotype frequently exhibit reduced intracellular accumulation of the drugs with respect to the parental drug-sensitive cells [1]. An increased drug efflux has been used to explain this property of resistant cells, based on the overexpression of certain membrane glycoproteins, the P-glycoprotein (pgp) family, responsible for actively pumping the drugs out of the cells [2]. However, recent findings on neuroblastoma cells suggest that the overexpression of pgp does not modify the extent of drug accumulation [3, 4]. Also, there are a number of drug-resistant cell lines and tumours in which overexpression of pgp has not been