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Type I IGF Receptor and Acquired Tamoxifen Resistance in Oestrogen-responsive Human Breast Cancer Cells

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Tamoxifen inhibited the oestrogen-stimulated proliferation of MCF-7 cells but had little effect on the oestrogen-stimulated proliferation of two tamoxifen-resistant variants (RL-3 and AL-1). The lack of oestrogen antagonist activity in the resistant cells was largely a result of an increased oestrogen agonist activity of tamoxifen on cell proliferation. Proliferation of the tamoxifen-resistant cells was also stimulated by 4-hydroxytamoxifen but not by ICI 164,384, a structurally distinct pure anti-oestrogen. Tamoxifen does not have increased oestrogen agonist activity for the induction of a series of oestrogen-regulated RNAs, and this suggests that the increased agonist activity may be restricted to key components involved in the proliferative response. Tamoxifen-stimulated cell proliferation was dependent on insulin-like growth factor I (IGF-1) in the resistant cells, suggesting that tamoxifen stimulates cell proliferation by sensitising cells to the proliferative effects of IGF-1. This may involve induction of the type-I IGF receptor.
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INTRODUCTION

THE ANTI-OESTROGEN tamoxifen is used widely in the treatment of breast cancer. It provides effective low toxicity palliation for women with advanced breast cancer, is used extensively as adjuvant therapy and, in the future, may be used for the prevention of breast cancer in high-risk women [1, 2]. Although tamoxifen is of benefit to approximately one third of all patients and to at least one half of patients with oestrogen receptor-positive tumours, response to tamoxifen is frequently of limited duration and disease ultimately progresses in the majority of patients. Tamoxifen resistance is, therefore, an important clinical problem and influences the survival of large numbers of women. Even though an understanding of the mechanisms involved in anti-oestrogen resistance might suggest strategies for extending the period of effective palliation, these have received little attention.

Two types of hormone resistance can be defined. A significant proportion of patients fail to respond when they are first exposed to hormone therapy on relapse and are, therefore, resistant from the outset (innate resistance). The remainder respond but subsequently relapse, and these patients acquire hormone resistance during therapy (acquired resistance).

Potentially, there are many mechanisms involved in the acquisition of hormone resistance [3]. Disease recurrence may involve selection of cells that lack the oestrogen receptor or of cells that have recruited other signal transduction pathways and thus become independent of the effects of oestradiol. Alternatively, tumour cells could retain their hormonal responsiveness but their responsiveness changes so that proliferation is stimulated rather than inhibited by the therapeutic agent.

Oestrogen-responsive breast cancer cell lines, such as the MCF-7 cell line [4] provide tractable experimental systems for studying the effects of oestrogens and anti-oestrogens on cell proliferation and gene expression in breast cancer cells. Selection of tamoxifen-resistant variants of oestrogen-responsive breast cancer cells by prolonged culture in tamoxifen has in most [4–8], but not all [9], cases given rise to cells which retain oestrogen receptors. In addition, nude mice implanted with xenografts of oestrogen receptor-positive breast cancer cells and treated with tamoxifen gave rise to oestrogen receptor-positive tumours whose growth was stimulated by tamoxifen [10, 11].

We have recently described culture conditions for obtaining substantial stimulation of MCF-7 cell growth by oestradiol [12, 13]. Under these conditions, cells do not proliferate significantly in the absence of oestrogen and it is, therefore, possible to measure the oestrogen agonist activity of tamoxifen. In this study we characterise a new tamoxifen-resistant MCF-7 variant (RL-3), and measure the effects of oestradiol, tamoxifen and the steroidal pure anti-oestrogen ICI 164,384 [14] on proliferation and on the expression of oestrogen-responsive genes in MCF-7 and RL-3 cells, as well as a previously isolated tamoxifen-resistant variant (AL-1)[6]. Our data suggest that the resistance of RL-3 and AL-1 cells to the antiproliferative effects of tamoxifen results from an enhanced oestrogen agonist activity for cell proliferation. This enhanced oestrogen agonist activity for cell proliferation may result from an acquired ability of tamoxifen to sensitise cells to the proliferative effects of insulin-like growth factors (IGFs), possibly by inducing the type I IGF receptor.

MATERIALS AND METHODS

Media

Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with various concentrations of fetal calf

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serum, insulin and tamoxifen. MCF-7 cells were cultured in medium containing serum (0.5%) and insulin (6 ng/ml). AL-1 cells were cultured in the same medium but supplemented with tamoxifen (10^{-6} mol/l). RL-3 cells were cultured in medium containing serum (1.5%), tamoxifen (10^{-6} mol/l) and insulin (1 μg/ml).

Conditioned medium was prepared by culturing near-confluent MCF-7 cells in growth medium for 3 days. The medium was collected, filtered and stored frozen. When used, it was mixed with an equal volume of fresh growth medium.

Selection of RL-3 cells

MCF-7 cells, adapted to grow in low concentrations of serum [15], were plated out and cultured in normal growth medium supplemented with 10^{-6} mol/l tamoxifen. Initially, the medium was changed every 2 days but as the number of viable cells decreased, it was changed less frequently. After approximately 4 weeks, few cells remained attached to the bottom of the flask, and the medium was replaced with conditioned medium containing no tamoxifen and left for 2 weeks, during which time the remaining cells grew into small colonies. The colonies were then transferred to 8-mm diameter wells and cultured initially in conditioned medium and subsequently in growth medium. When sufficient numbers of cells had grown from each of the colonies, selection for tamoxifen resistance was resumed by including 10^{-6} mol/l tamoxifen in the growth medium.

A preliminary screening experiment (data not shown) with six tamoxifen-resistant clones showed that all retained oestrogen-responsive proliferation and all showed greater tamoxifen-stimulated proliferation than the parent MCF-7 cell line. The RL-3 cell line was typical of the variant lines and was characterised in more detail.

Growth experiments

Cells were plated into 16-mm wells at approximately 10% confluence and allowed to attach overnight. They were withdrawn from endogenous steroids present in fetal calf serum by culturing the cells in withdrawal medium [phenol red-free minimal essential medium containing 0.06% sodium bicarbonate supplemented with 20 mmol/l HEPES, charcoal-stripped newborn calf serum (10%) and insulin (1 μg/ml)] for 3 days and changing the medium twice daily. Thereafter, the medium was changed daily and treatment was started after 5 days of withdrawal. After 6 and 9 days of treatment, cells were lysed in a solution of Triton X-100 and ammonia and the DNA measured using bisbenzimidazole (Hoechst 33258, Germany) on a Kontron SFM 25 spectrofluorometer as described previously [12]. The DNA content of one of the wells treated with oestradiol for 9 days was taken as the 100% value for each experiment. Triplicate wells were measured for each time point and in the Results, the bars represent the standard errors.

RNA extraction and hybridisation

Cells were withdrawn and then treated with various combinations of oestradiol and tamoxifen as described previously [16]. Total RNA was extracted and the levels of specific oestrogen-regulated mRNAs measured after northern transfer [17] by hybridisation with cDNA probes labelled with [^{32}P]dCTP (cytidine triphosphate) as described elsewhere [18]. Duplicate measurements were made for each concentration of oestradiol and tamoxifen.

Measurement of oestrogen receptor

Cells were grown to confluence in T175 flasks and then cultured for 2 days in phenol red-free medium containing charcoal-treated serum and insulin (1 μg/ml) to remove oestradiol. Cells were then washed twice with phosphate buffered saline (PBS), scraped off with a rubber policeman and homogenised in a low ionic strength buffer (10 mmol/l Tris pH 7.3). Cytosol was prepared by centrifiguration at 100 000 *g* for 1 h, incubated with varying concentrations of [^{3}H]oestradiol for 2 h at 4°C in the presence and absence of an excess of unlabelled oestradiol, and bound oestradiol measured by chromatography on LH20 sephadex [19]. Binding affinity and number of sites were determined by Scatchard analysis and the figures shown are the mean of two measurements.

Measurement of type-1 IGF receptor

Cells (200 000) were plated into 16-mm diameter wells, withdrawn from oestrogen present in routine culture medium and then treated with various combinations of oestrogens and anti-oestrogens as described elsewhere [13]. Cell monolayers were incubated with various concentrations of [^{125}I]IGF-1 in the presence of 30 μmol/l unlabelled insulin in PBS containing 1 mg/ml bovine serum albumin (BSA) for 3 h at 4°C. the monolayers were washed three times with 1 ml ice-cold PBS and the cell monolayer dissolved in 400 μl 0.4 mol/l NaOH. Radioactivity was counted in a gamma counter and the amount of [^{125}I]IGF-1 specifically bound to the type I IGF receptor calculated by subtracting the amount of [^{125}I]IGF-1 bound in the presence of insulin (non-specific binding) from the amount bound in its absence (total binding). Cross-linking experiments have shown that insulin suppresses binding to the type I IGF receptor but not to other lower molecular weight IGF-I binding proteins present on MCF-7 cells, and this value, therefore, represents binding to the type I IGF receptor. The binding constant of IGF-I was 3×10^{10} mol/l, consistent with binding to the type I IGF receptor rather than to the insulin or type II IGF receptors, and this has been validated by competition experiments (data not shown).

RESULTS

Selection of tamoxifen-resistant MCF-7 cells

Tamoxifen-resistant MCF-7 cells were selected as described in the Materials and Methods. Cells derived from individual colonies were maintained routinely in growth medium supplemented with tamoxifen. Oestrogen stimulated the proliferation of all sublines tested. One of these sublines, RL-3, and another tamoxifen-resistant cell line, AL-1 [6], were chosen for comparison with the parent MCF-7 cell line. The concentrations and affinities of the oestrogen receptors in the three cell lines were measured and all three expressed similar levels (MCF-7, 83; AL-1, 81 and RL-3, 76 fmol/mg protein) of a high affinity (Kd 0.16 nmol/l) oestrogen receptor.

Decreased oestrogen-antagonist effects of tamoxifen in RL-3 and AL-1 tamoxifen-resistant cells

The oestrogen antagonist activity of tamoxifen was measured in MCF-7, RL-3 and AL-1 cells. Cells were treated with oestradiol alone (2×10^{-10} mol/l) or in the presence of tamoxifen (10^{-7}, 10^{-6} or 3×10^{-6} mol/l). Oestradiol stimulated the proliferation of all three cell lines so that there were approximately five times more cells in wells treated with oestradiol than in control wells after 9 days of treatment (Fig. 1). The effect of tamoxifen was dose dependent in the MCF-7 cells; there was

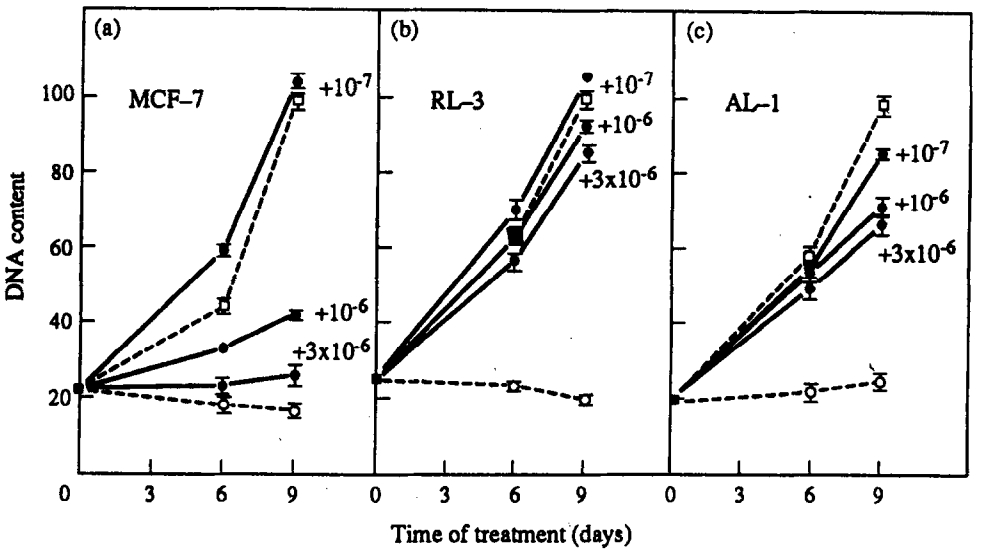

Fig. 1. Oestrogen antagonist effects of tamoxifen on the proliferation of MCF-7 (a), RL-3 (b) and AL-1 (c) cells. Cells were withdrawn for 4 days, and then cultured in withdrawal medium alone (○), or medium containing oestradiol (2×10^{-10} mol/l ■) or oestrodiol (2×10^{-10} mol/l □) together with the indicated concentration of tamoxifen (●).

little effect of 10^{-7} mol/l tamoxifen, 10^{-6} mol/l tamoxifen greatly reduced oestradiol-induced growth and 3×10^{-6} mol/l tamoxifen almost completely abolished it. In RL-3 cells, neither 10^{-7} nor 10^{-6} mol/l tamoxifen significantly reduced the number of cells below that in wells treated with oestradiol alone, and 3×10^{-6} mol/l tamoxifen reduced cell numbers by only 15%. A similar pattern was seen in AL-1 cells; proliferation was not reduced after 6 days but was reduced by approximately 15% after 9 days in medium containing 10^{-7} and 30% in medium containing 10^{-6} mol/l tamoxifen and 3×10^{-6} mol/l. These experiments established that the two tamoxifen-resistant sublines have retained oestrogen responsiveness and that tamoxifen has less marked oestrogen-antagonist activity in these sublines than in the parent cell line.

The stability of the tamoxifen-resistant phenotype was then determined by measuring the oestrogen-antagonist activity of tamoxifen in RL-3 cells that had been cultured in the absence of tamoxifen for 2 months. Figure 2 shows that while oestrogen-stimulated proliferation of MCF-7 cells was inhibited by concentrations of tamoxifen above 10^{-7} mol/l, RL-3 cells showed little inhibition by tamoxifen irrespective of whether they had been grown continuously in tamoxifen or not. The tamoxifen-resistant phenotype of RL-3 cells is, therefore, stable.

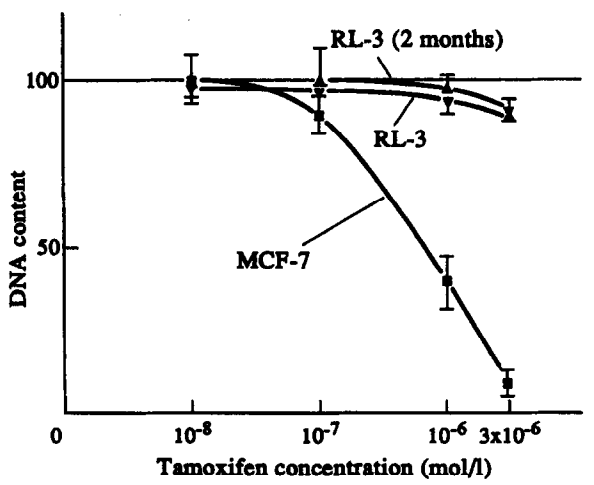

Fig. 2. Stability of the tamoxifen-resistant phenotype in RL-3 cells. RL-3 cells, maintained in medium containing (▼) or lacking (▲) tamoxifen for 2 months and MCF-7 cells (■) were withdrawn for 4 days and then cultured in the presence of 2×10^{-10} mol/l oestradiol and the indicated concentration of tamoxifen.

Increased oestrogen agonist effects of tamoxifen in RL-3 and AL-1 cells

To investigate whether the decreased oestrogen antagonism of tamoxifen was associated with increased oestrogen agonism in tamoxifen-resistant cells, the growth of MCF-7, AL-1 and RL-3 cells cultured in tamoxifen (10^{-11}–10^{-5} mol/l) alone or oestradiol (10^{-8} mol/l) alone was measured (Fig. 3). Tamoxifen alone (10^{-7} and 10^{-6} mol/l) significantly ($P < 0.05$ Student's *t*-test) increased the growth of MCF-7 cells (Fig. 3a) so that after 9 days there were 1.2 (10^{-7} mol/l) and 1.4 (10^{-6} mol/l)-fold more cells present in tamoxifen-treated wells than in control wells. Other concentrations of tamoxifen had no effect. In this experiment, oestrogen treatment resulted in an 8-fold increase in cell numbers over the same period. In both RL-3 and AL-1 cells, the same concentrations of tamoxifen had markedly more pronounced oestrogen agonist activity. Both concentrations of tamoxifen (10^{-7} and 10^{-6} mol/l) increased cell numbers to about 75% of those obtained with oestradiol in RL-3 cells. Tamoxifen had slightly less agonist activity in AL-1 than in RL-3 cells.

Resistance of RL-3 cells to 4-hydroxytamoxifen and ICI 164,384

To determine if tamoxifen-resistant cells are resistant to other anti-oestrogens, the oestrogen antagonist activity of 4-hydroxytamoxifen (an active tamoxifen metabolite) and ICI 164,384 (a structurally unrelated pure anti-oestrogen) were compared in the MCF-7 and RL-3 cell lines. In both the parent MCF-7 cells and the resistant RL-3 cells, ICI 164,384 showed dose-dependent inhibition of oestradiol-stimulated growth (Fig. 4); although somewhat higher concentrations of ICI 164,384 were required to inhibit the effects of oestradiol in RL-3 cells than in MCF-7 cells.

In contrast, 4-hydroxytamoxifen had little effect on oestradiol-stimulated proliferation of RL-3 cells at concentrations up to 10^{-6} mol/l (Fig. 4b). This concentration completely abolished

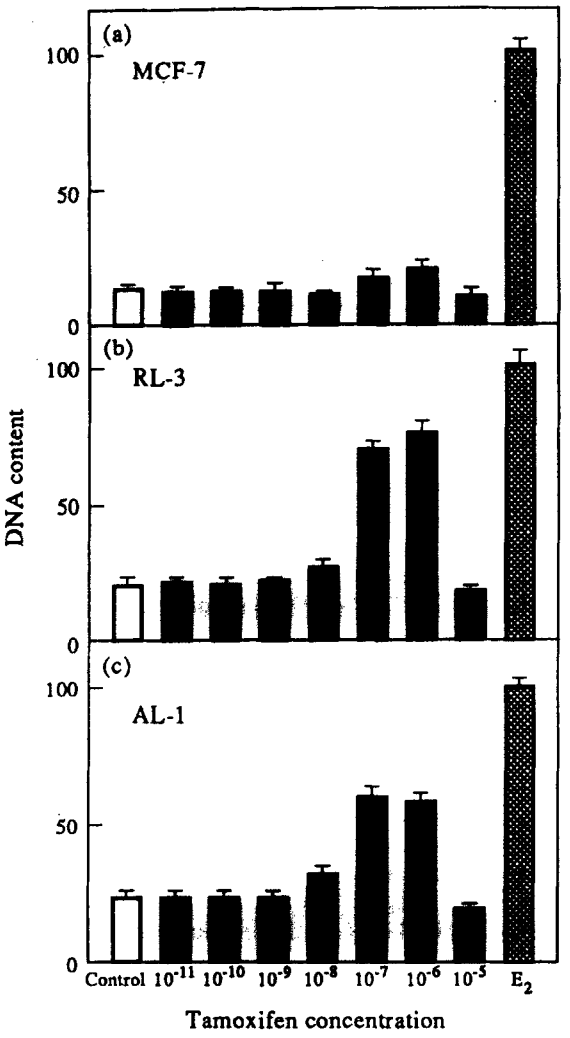

Fig. 3. Oestrogen agonist effects of tamoxifen on the proliferation of MCF-7 (a), RL-3 (b) and AL-1 (c) cells. Cells were withdrawn for 4 days and then cultured for 9 days in withdrawal medium alone (control), or containing oestradiol (10^{-8}, E_2), or tamoxifen at the indicated concentration.

oestrogen-stimulated proliferation in the parent MCF-7 cells (Fig. 4a).

As the lack of an oestrogen antagonist effect appeared to result from an increased oestrogen agonist effect of tamoxifen in RL-3 and AL-1 cells, the oestrogen agonist activities of 4-hydroxytamoxifen and ICI 164,384 were compared in MCF-7 and RL-3 cells. Figure 5 shows that 10^{-8} and 10^{-7} mol/l 4-hydroxytamoxifen increased the growth of MCF-7 cells, while 10^{-9} and 10^{-8} mol/l 4-hydroxytamoxifen increased the growth of RL-3 cells. This increase was much more pronounced in RL-3 cells. In contrast, ICI 164,384 had little effect in either cell line at any concentration tested.

The ability of ICI 164,384 to inhibit the oestrogen agonist effects of tamoxifen on RL-3 cell proliferation was then measured. Figure 6 shows that the oestrogen agonist activity of tamoxifen (10^{-6} mol/l) was inhibited by ICI 164,384. This activity was partially inhibited at concentrations of ICI 164,384 as low as 10^{-9}mol/l and completely inhibited by 10^{-7}mol/l and above.

These experiments, therefore, showed that cross resistance is conferred on a closely related anti-oestrogen but not on a structurally unrelated anti-oestrogen.

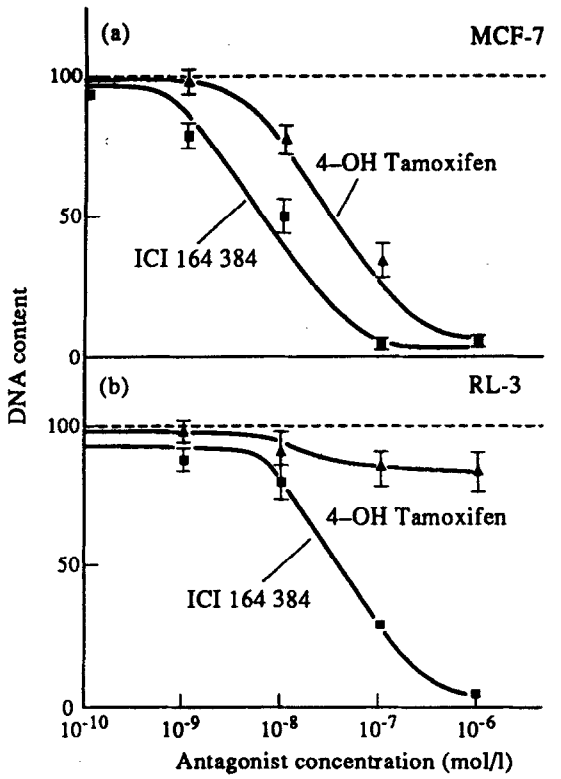

Fig. 4. Oestrogen antagonist effects of ICI 164,384 and 4-hydroxytamoxifen on the proliferation of MCF-7 (a) and RL-3 (b). Cells were withdrawn for 4 days and then cultured for 9 days in withdrawal medium containing oestradiol (2×10^{-10} mol/l) together with the indicated concentration of ICI 164,384 or 4-hydroxytamoxifen. The dotted line indicates the 100% value for wells treated with oestradiol alone.

Regulation of oestrogen-responsive mRNAs by oestradiol and tamoxifen in MCF-7, AL-1 and RL-3 cells

Oestrogen regulates the expression of a number of oestrogen-responsive genes in breast cancer cells [17, 18]. Figure 7 shows the regulation of the pNR-1, pNR-2, pNR-25 and cathepsin D oestrogen-regulated mRNAs by oestradiol in MCF-7, RL-3 and AL-1 cells. The four mRNAs were present and induced by the same concentrations of oestradiol in the three cell lines. The oestrogen-induced level of the pNR-1 RNA was 1.5-fold higher in AL-1 cells than in MCF-7 and RL-3 cells. The pNR-2 mRNA was induced to a similar extent in all three cell lines. The pNR-25 RNA was induced by oestradiol in MCF-7 cells and the two resistant cell lines. The induction was much more pronounced in MCF-7 cells than in the resistant cell lines, and consequently oestrogen-treated MCF-7 cells had considerably more pNR-25 RNA than oestrogen-treated AL-1 and RL-3 cells. Cathepsin D was less induced in the two tamoxifen-resistant cell lines than in MCF-7 cells.

The oestrogen agonist activity of tamoxifen on the induction of the four RNAs was then measured in the three cell lines (Fig. 8). Overall, the oestrogen agonist activity of tamoxifen was similar for the pNR-1, pNR-2 and pNR-25 mRNAs in the three cell lines. As described previously [12], tamoxifen had greater oestrogen agonist activity than oestradiol for the induction of the pNR-1 RNA and the induction was similar in MCF-7 and AL-1 cells and slightly less in RL-3 cells. Tamoxifen was a very weak oestrogen agonist for the induction of the pNR-2 and pNR-25 RNAs with maximal induction occurring at 10^{-7}mol/l tamoxifen for the pNR-2 RNA and 10^{-6}–10^{-5} mol/l for the pNR-25 RNA.

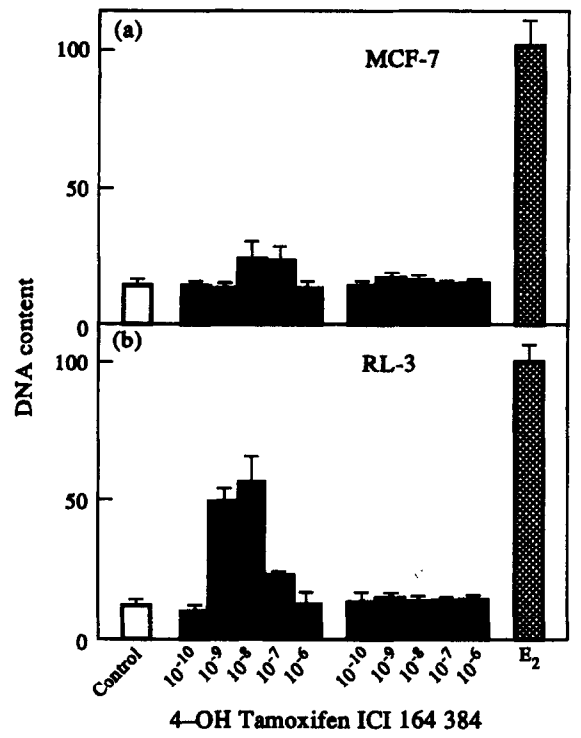

Fig. 5. Oestrogen agonist effects of 4-hydroxytamoxifen and ICI 164,384 on the proliferation of MCF-7 (a) and RL-3 (b) cells. Cells were withdrawn for 4 days and then cultured for 9 days in withdrawal medium alone (control), or containing oestradiol (10^{-8} mol/l, E_2) or 4-hydroxytamoxifen or ICI 164,384 at the indicated concentration.

Tamoxifen was a full oestrogen agonist for the induction of cathepsin D in MCF-7 cells but was a partial oestrogen agonist in RL-3 and AL-1 cells.

These experiments, therefore, established that although tamoxifen showed increased oestrogen agonist activity for cell proliferation in the tamoxifen-resistant cell lines, it did not show significantly greater oestrogen agonist activity for the induction of four oestrogen-regulated RNAs. The proliferative response is, therefore, selectively enhanced by tamoxifen in tamoxifen-resistant cells.

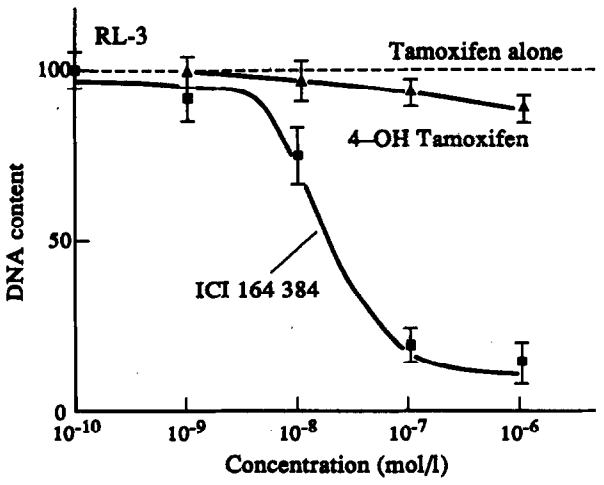

Fig. 6. Tamoxifen antagonist effects of ICI 164,384 in tamoxifen-resistant RL-3 cells. Cells were withdrawn for 4 days and then cultured in medium containing tamoxifen (10^{-6}mol/l) together with the indicated concentration of 4-hydroxytamoxifen or ICI 164,384. The dotted line indicates the 100% value for wells treated with tamoxifen alone.

Effects of tamoxifen on the proliferative response to IGF-1, and on the type I IGF receptor concentration in RL-3 cells

We have suggested previously that oestrogens increase cell proliferation by sensitising MCF-7 breast cancer cells to the proliferative effects of IGF-I [13]. To determine whether the oestrogen agonist effect of tamoxifen may involve a similar mechanism, the agonist effect of tamoxifen on cell proliferation was measured in the presence and absence of IGF-I (Fig. 9). In this experiment, oestradiol treatment together with IGF-I resulted in a 6-fold increase in cell numbers over 9 days. Tamoxifen alone did not increase RL-3 cell proliferation. In the presence of IGF-I, tamoxifen stimulated a large increase in cell proliferation to approximately 65% of the level obtained with oestradiol and IGF-I. Similar results were obtained with AL-1 cells (data not shown).

The enhanced sensitivity to IGF-I may result from an induction of the type I IGF receptor by oestradiol [13]. We, therefore, measured the levels of the type I IGF receptor in MCF-7 and RL-3 cells following oestrogen and anti-oestrogen treatment (Fig. 10).

Whereas oestrogen increased IGF-I binding to the type I IGF receptor 5-fold in MCF-7 cells, tamoxifen and ICI 164,384 had no effect. In marked contrast, tamoxifen but not ICI 164,384 increased IGF-I binding to the type I IGF receptor in RL-3 cells to about 50% of the oestrogen-induced level. This suggests that tamoxifen may increase the proliferation of tamoxifen-resistant cells by inducing higher type I IGF receptor concentrations, thereby sensitising the cells to the proliferative effects of IGF-I.

DISCUSSIONS

In this study, we have used oestrogen-responsive breast cancer cell lines to study the mechanisms involved in the acquisition of tamoxifen resistance by breast cancer cells. Both the RL-3 and AL-1 tamoxifen-resistant variants expressed oestrogen receptors and their proliferation was oestrogen responsive. Although the selection of tamoxifen-resistant variants in culture may not accurately mirror the selection pressures operating in women with breast cancer, cell culture systems can generate models of tamoxifen resistance which may be applicable *in vivo*. The data presented in this paper are consistent with a growing body of experimental and clinical evidence suggesting that acquired tamoxifen resistance does not necessarily involve a transition to hormone unresponsiveness.

The ability of both RL-3 and AL-1 cells to grow in the presence of tamoxifen resulted from an altered response rather than an acquired insensitivity to tamoxifen. Tamoxifen had no (RL-3) or very limited (AL-1) oestrogen antagonist activity, largely because of the dramatically enhanced oestrogen agonist effect of tamoxifen on cell proliferation. This is the first demonstration of an increased agonist effect of tamoxifen on cell proliferation of tamoxifen-resistant cells in culture. We believe these effects of tamoxifen are mediated by the oestrogen receptor because the required concentration of tamoxifen is consistent with the affinity of tamoxifen for the oestrogen receptor, and the increased oestrogen agonist effect of tamoxifen is inhibited by the pure anti-oestrogen ICI 164,384. Other studies [5, 7] with cultured cells are consistent with our findings, although agonist effects of tamoxifen were not observed, probably because they were masked by the oestrogenic effect of phenol red.

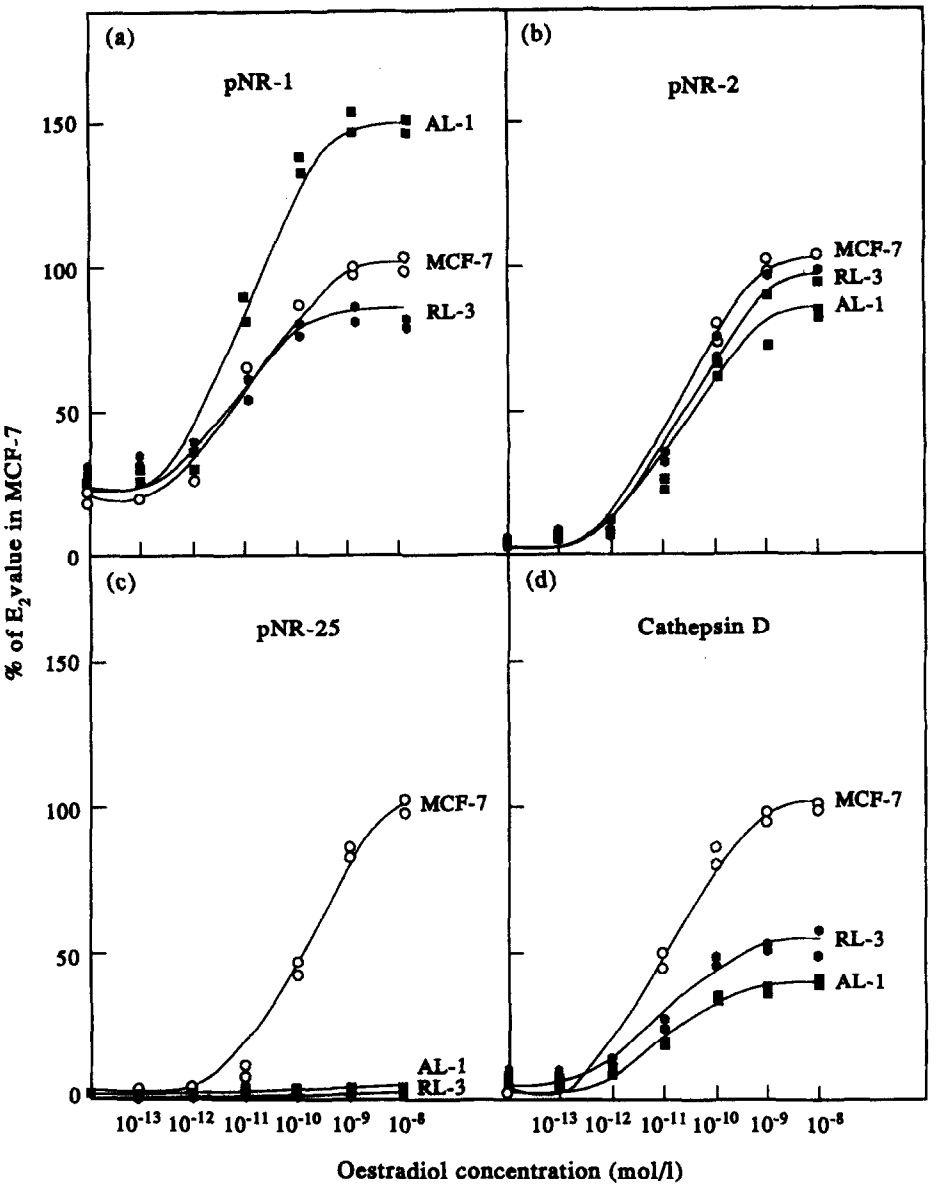

Fig. 7. Induction of pNR-1, pNR-2, pNR-25 and cathepsin D mRNAs by oestradiol in RL-3, AL-1 and MCF-7 cells. Cells were withdrawn and then treated with various concentrations of oestradiol. Total RNA was extracted and the levels of individual RNAs measured by northern transfer analysis. The values shown represent the percentage of the amount of the RNA present in MCF-7 cells treated with 10^{-8} mol/l oestradiol.

Studies on breast cancer cells growing as xenografts in athymic mice have also suggested that tamoxifen resistance is not associated with loss of hormone responsiveness. Three groups have shown that long-term treatment of such mice with tamoxifen results in the selection of tumours whose growth is dependent on tamoxifen [8, 10,11].

Breast cancer cells from patients who have relapsed while receiving tamoxifen can also retain oestrogen responsiveness. In early studies, oestrogen receptor assays were compromised by the interference of tamoxifen [20]. More recently, oestrogen receptor expression has been detected unequivocally in tumour cells following relapse on endocrine therapy [20–24].

These results may help to understand some of the different types of response to endocrine therapy in breast cancer patients. Clinical data, including the documentation of positive objective clinical responses in patients following withdrawal from tamoxifen therapy, and responses to second-line endocrine therapy, also suggest that tamoxifen resistance is not necessarily associated with hormone insensitivity but rather is the result of an altered response to tamoxifen (reviewed in [25]). Withdrawal responses would be predicted if cells in which tamoxifen has increased oestrogen agonist effects had been selected during tamoxifen therapy.

Of particular clinical interest was the observation that the tamoxifen-resistant RL-3 cells were resistant to 4-hydroxytamoxifen but not to ICI 164,384. Our data are not consistent with another study [5] which suggests that acquisition of resistance to one anti-oestrogen can confer resistance to another, structurally unrelated anti-oestrogen but are consistent with the data of Gottardis *et al.* [26], who showed that tamoxifen-resistant MCF-7 cells growing as xenografts were inhibited by ICI 164,384. Thus, resistance to tamoxifen appears to differ from certain forms of resistance to cytotoxic drugs, where overexpression of the P-glycoprotein results in resistance to a variety of structurally unrelated compounds. Overall, our data suggest that anti-oestrogens, which are structurally related to ICI 164,384, may

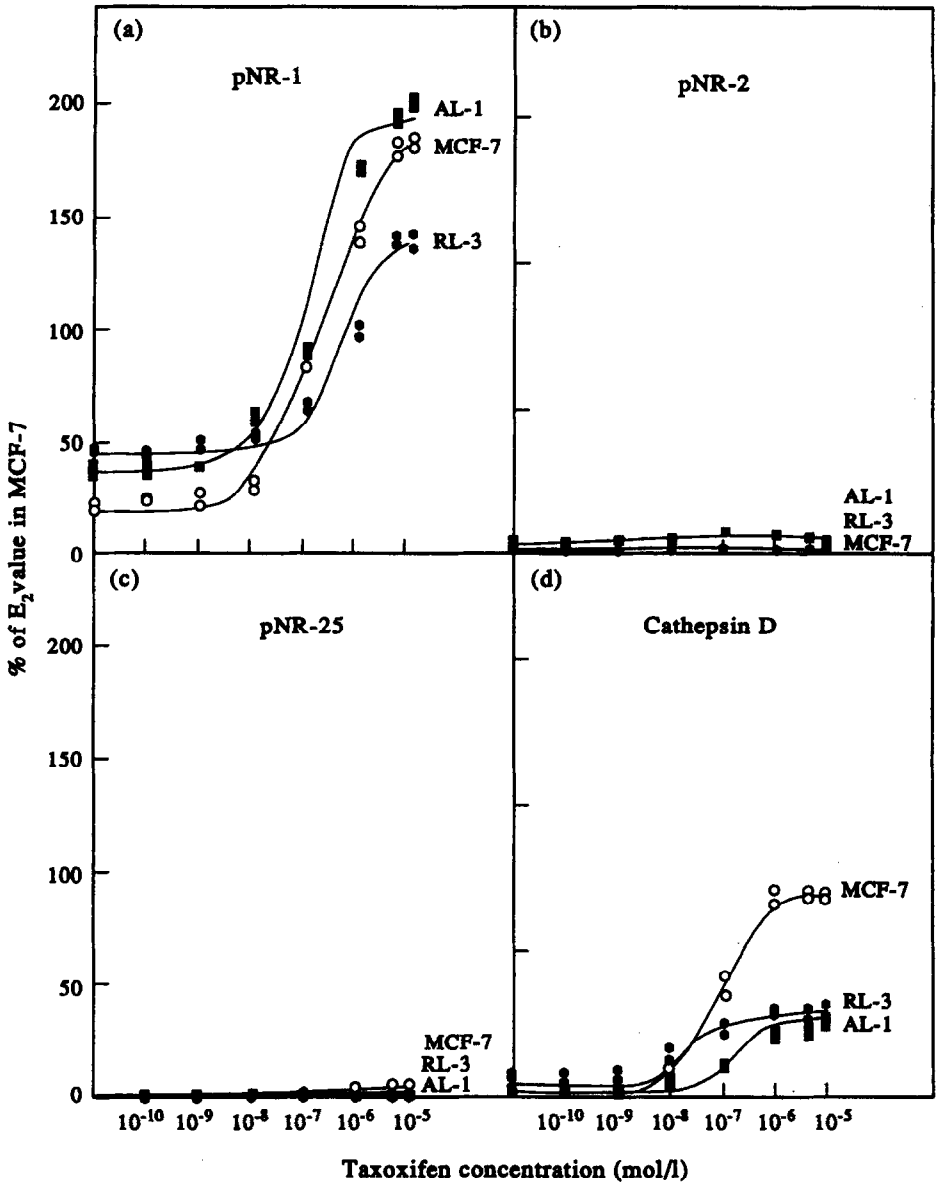

Fig. 8. Induction of pNR-1, pNR-2, pNR-25 and cathepsin D mRNAs by tamoxifen in RL-3, AL-1 and MCF-7 cells. Cells were withdrawn and then treated with various concentrations of tamoxifen. Total RNA was extracted and the levels of individual RNAs measured by northern transfer analysis. The values shown represent the percentage of the amount of the RNA present in MCF-7 cells treated with 10^{-8} oestradiol.

be useful for treating patients who have relapsed on tamoxifen therapy, and one such compound (ICI 182,780) has recently entered clinical trials.

The mechanisms responsible for alterations to tamoxifen responsiveness remain speculative. It is already recognised that tamoxifen can range from being a full oestrogen agonist to a pure oestrogen antagonist, depending on the species and response being studied. We have shown that even in a single cell type, two oestrogen-responsive genes [17, 18] can be regulated very differently by tamoxifen [16] and have suggested that these differences could result from the way their response elements interpret a receptor complexed with an anti-oestrogen. Given that oestrogens act by controlling the interaction of their receptors with oestrogen-response elements [27] adjacent to oestrogen-regulated genes, an altered response could result from changes to the receptor of the response element. In addition, other transcription factors may modulate the interaction of the receptor with the response elements, and could conceivably alter the way in which a response element interprets a tamoxifen–oestrogen receptor complex.

A number of oestrogen-regulated genes have been implicated in mediating the effects of oestrogens on the proliferation of breast cancer cells. For instance, cathepsin D may act as an oestrogen-regulated autocrine mitogen for MCF-7 cells [28], and the oestrogen-regulated pNR-2/pS2 protein may have hormonal activity [29]. RL-3 and AL-1 cells could provide a system for screening candidate genes thought to be implicated in oestrogen-regulated growth control as such genes would be expected to be induced by tamoxifen to a greater extent in tamoxifen-resistant cells than in the parent MCF-7 cells.

In fact, tamoxifen had no more agonist activity for the induction of four oestrogen-responsive mRNAs in tamoxifen-resistant cells than in tamoxifen-sensitive cells, showing that there is not a general increase in tamoxifen agonist activity for all oestrogen responses. This observation is not predicted by other models of tamoxifen resistance [30] which involve reduced

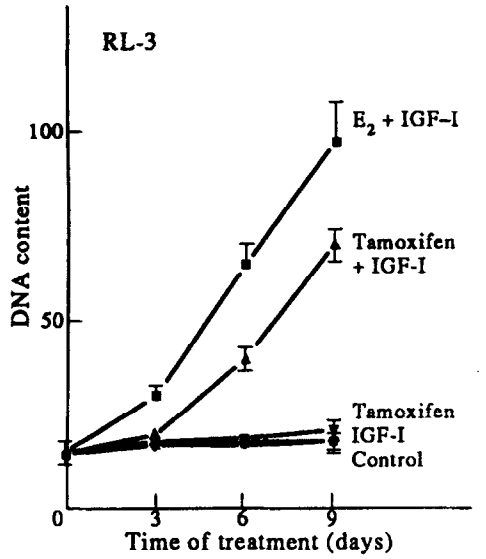

Fig. 9. The effects of IGF-I, oestradiol and tamoxifen on the growth of RL-3 cells. Cells were withdrawn and then treated for 3, 6 or 9 days with withdrawal medium alone (control), or containing 50 ng/ml IGF-I (IGF-I), 10^{-6} mol/l tamoxifen, 10^{-6} mol/l tamoxifen and IGF-I or 10^{-9} mol/l oestradiol and IGF-I (E_2 + IGF-I).

intratumoral accumulation of tamoxifen and increased metabolism to oestrogenic metabolites. This does suggest, however, that the regulation of a limited repertoire of genes involved in mediating the proliferative response may indeed be altered in tamoxifen-resistant cells.

We have shown previously that oestrogen-induced proliferation is dependent on the presence of ligands which interact with the type I IGF receptor, suggesting that oestrogen sensitises cells to the proliferative effect of IGFs [13]. In this study, we have extended this observation and shown that the partial agonist activity of tamoxifen in tamoxifen-resistant cells is also dependent on tamoxifen. The observation that tamoxifen-stimulated proliferation also appears to involve sensitisation to IGFs suggests that one or more of the components of the IGF signal transduction pathway may be increased by tamoxifen in tamoxifen-resistant cells. The type I IGF receptor is the first component, and as we have suggested that oestrogens increase the sensitivity to IGFs by increasing type I IGF receptor, we measured the effect of tamoxifen on binding of IGF-I to this receptor. Binding to the type I receptor was increased by oestrogen and tamoxifen in RL-3 cells but only by oestradiol in the parent MCF-7 cells. Taken together, these observations suggest that the acquired induction of the type I IGF receptor by tamoxifen, and consequent sensitisation to the proliferative effects of IGF-1, may determine the ability of these breast cells to proliferate in the presence of tamoxifen.

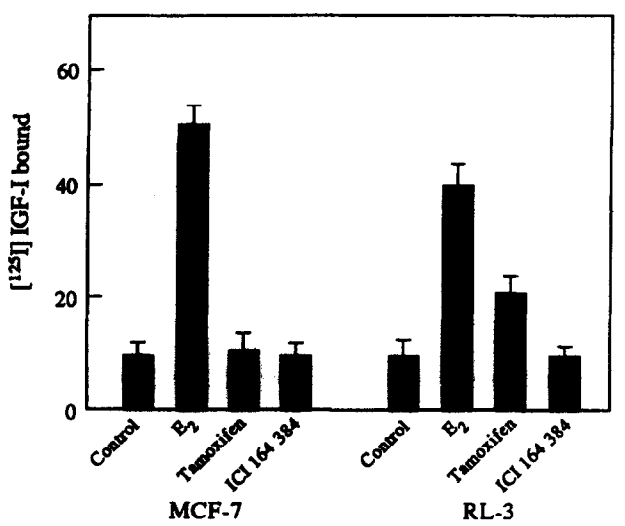

Fig. 10. Effects of oestradiol, tamoxifen and ICI 164,384 on the type I IGF receptor in MCF-7 and RL-3 cells. Cells were withdrawn and then treated with 10^{-9} mol/l oestradiol, 10^{-6} mol/l tamoxifen or 10^{-7}mol/l ICI 164,384. Type I IGF receptor binding was measured as described in the Materials and Methods.

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Antiproliferative Activity of Thermosensitive Liposome-encapsulated Doxorubicin Combined with 43°C Hyperthermia in Sensitive and Multidrug-resistant MCF-7 Cells

Jean-Louis Merlin, Sophie Marchal, Carole Ramacci, Dominique Notter and Claude Vigneron

Thermosensitive liposome-encapsulated doxorubicin (TLED) was compared to free doxorubicin, at 37°C or combined with 43°C hyperthermia, in sensitive and multidrug-resistant MCF-7 human tumour cells using clonogenic assays. In the resistant subline, TLED was found to partly circumvent multidrug resistance (MDR). The reversal was comparable to that obtained when verapamil was added to free doxorubicin. When hyperthermic treatment was applied, no difference in thermosensitivity was found between sensitive and resistant cells. The combination of hyperthermia with free doxorubicin did not reverse MDR. Hyperthermia and TLED yielded additive effects in the resistant cells while potentiation was observed in the sensitive cells. These results confirmed the usefulness of the liposome encapsulation of doxorubicin in reversing MDR. The possibility of obtaining additive cytotoxicity using TLED combined with hyperthermia may represent an alternative way of intensification of doxorubicin cytotoxicity concomitant with the circumvention of MDR without using MDR reversing agents, which often generate limiting toxic side-effects.
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INTRODUCTION

BECAUSE OF its implication in the failure of some chemotherapeutic treatments, drug resistance has been largely studied in the last two decades [1–4] and is now known to occur through different mechanisms, including the increase in activity of enzymes such as glutathione-S-transferase [5, 6], glutathione peroxidase [7, 8], superoxide dismutase [7], as well as the alteration of topoisomerase II activity [9, 10] or hexose phosphate metabolism [11]. Beside all these mechanisms of resistance, the so-called multidrug resistance (MDR) phenotype involves the overproduction of a 170 kD transmembrane glycoprotein called P-glycoprotein (Pgp) which works as a drug extruding pump and which is associated with the overexpression of *mdr*1 gene [4]. The MDR phenotype is found to induce resistance to several unrelated compounds including anthracyclines, vinca alkaloids, epipodophylotoxins and dactinomycin, as well as mitoxantrone and taxol derivatives [4]. Pgp is detected

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