The anti-oestrogen drug tamoxifen is an elongation inhibitor of eukaryotic protein biosynthesis

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The drug tamoxifen is widely used in the chemotherapy of breast cancer but its action is not explained completely by its anti-oestrogen properties. We now present evidence indicating that it is also a potent inhibitor of eukaryotic protein synthesis as demonstrated in *Xenopus* oocytes, intact reticulocytes and reticulocyte lysates. The inhibition affects general protein synthesis, is transient in oocytes and not reversed by oestrogen. The drug appears to act by inhibiting polypeptide chain elongation. This action of tamoxifen is independent of oestrogen receptors and may explain its therapeutic effectiveness in oestrogen-independent tumours.

Tamoxifen *Anti-estrogen* *Protein synthesis* *Polypeptide elongation* *Breast cancer*

1. INTRODUCTION

The non-steroidal anti-oestrogen drug tamoxifen (Nolvadex)* has been used to treat breast cancer for over a decade. Recently, two extensive reviews [1,2] have examined the hypothesis that the anti-tumour activity is due to binding of tamoxifen to the oestrogen receptor of cells thereby blocking the response to the hormone. Both articles conclude that there is evidence, for instance the inhibition of an oestrogen receptor-negative cell line [3], suggesting the existence of another mechanism of action of tamoxifen in addition to its anti-oestrogen activity.

Whilst using tamoxifen as an anti-oestrogen in *Xenopus* oocytes we made the fortuitous observation that the drug inhibits the incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material. Since active messenger RNA could be isolated from oocytes after treatment with tamoxifen it seemed likely that the observed effect was due to inhibition of RNA translation. In this paper we report that protein synthesis is inhibited by tamoxifen in intact oocytes and reticulocytes, as well as in reticulocyte lysates, at concentrations which are similar to those obtained in vivo with therapeutic doses of the drug. The inhibition is rapid in all systems and appears to involve a block at elongation as shown by the absence of polyribosome disaggregation in the presence of the drug. Also, since inhibition is observed in systems lacking the oestrogen receptor it seems to be independent of the anti-oestrogen activity of tamoxifen.

* Tamoxifen is (*Z*)-2-[4-(1,2-diphenylbut-1-enyl)phenoxy]-*N,N*-dimethylethanamide, which is produced by Imperial Chemical Industries under the tradename Nolvadex

2. MATERIALS AND METHODS

2.1. *Preparation and use of reticulocyte lysates*

Reticulocytes were obtained from the blood of New Zealand white rabbits made anaemic by subcutaneous injections of neutralized phenylhydrazine and lysates were prepared as described [4]. Incubation mixtures for measuring cell-free protein synthesis by the incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable radioactivity were set up and used as in [4]. Where indicated tamoxifen in ethanol was

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added, giving appropriate amounts of the drug and a final concentration of ethanol of 1%. Radioactivity was measured in an LKB minibeta scintillation spectrometer.

2.2. *Preparation and culture of rabbit reticulocytes*

Washed reticulocytes were prepared as for lysis [4] and cultured at 10^8 cells/ml in NCTC-135 growth medium supplemented with 10% foetal calf and 2% rabbit serum (Gibco-Biocult), ferrous ammonium sulphate (10 μM), glutamine (2 mM) and containing 100 U/ml of a penicillin-streptomycin solution. Preincubation of a cell suspension (20 ml) was carried out in an 80 cm^3 Nunclon bottle (Gibco-Biocult) in a humidified incubator at 36.5°C in an atmosphere containing 5% CO_2. After 4 h, aliquots of the suspension (2 ml) were similarly incubated in 25 cm^3 Nunclon bottles after treatment as described in the figure legends. Cells were harvested by centrifugation, washed with 130 mM NaCl, 5 mM KCl, 7.5 mM $MgCl_2$ (2 × 10 ml) and lysed with water (150 μl). Aliquots of the lysate (5 μl) were assayed for trichloroacetic acid-precipitable radioactivity as above.

2.3. *Preparation and culture of* Xenopus *oocytes*

An ovary lobe was removed from a female *Xenopus laevis*, large healthy oocytes were selected after mechanical stripping and incubated at 21°C overnight in MBS medium [5]. Groups of 5 oocytes were then incubated under the stated conditions in fresh MBS medium (50 μl) in Nunclon 96-well microtitre plates. Extraction of oocyte proteins was carried out according to Colman [5] and trichloroacetic acid-precipitable radioactivity assayed as above. Proteins were analysed by SDS-PAGE [6] and revealed by fluorography [7].

2.4. *Analysis of polyribosomes*

After incubation of reticulocyte lysates (0.2 ml) for 12 min at 30°C under optimal protein synthesizing conditions, 1 vol. cold RS buffer (10 mM Tris-Cl, pH 7.6, 10 mM KCl, 1.5 mm $MgCl_2$) containing 10 μg/ml cycloheximide was added. The mixtures were then loaded immediately onto 15–30% isokinetic sucrose gradients prepared in RS buffer and centrifuged in a Beckman SW40 rotor at 185000 × g_{av} for 2 h at 4°C. Gradients were fractioned by upward displacement through an ISCO fractionator and analysed by monitoring the absorbance at 254 nm.

2.5. *Materials*

[^{35}S]Methionine (1120 Ci/mmol) was purchased from New England Nuclear Corporation. Tamoxifen was a gift from Dr B. Furr (ICI Pharmaceuticals). A stock solution in absolute ethanol was prepared daily. Cycloheximide and 17β-oestradiol were from Sigma and all other chemicals were of Analar grade as supplied by BDH.

3. RESULTS

3.1. *Inhibition of protein synthesis by tamoxifen*

The effect of different tamoxifen concentrations on cell-free protein synthesis in reticulocyte lysate is shown in figs 1 and 2. The inhibition observed was rapid. At tamoxifen concentrations of 18 μM and 180 nM, which include those found in tumours and other tissues, protein synthesis was

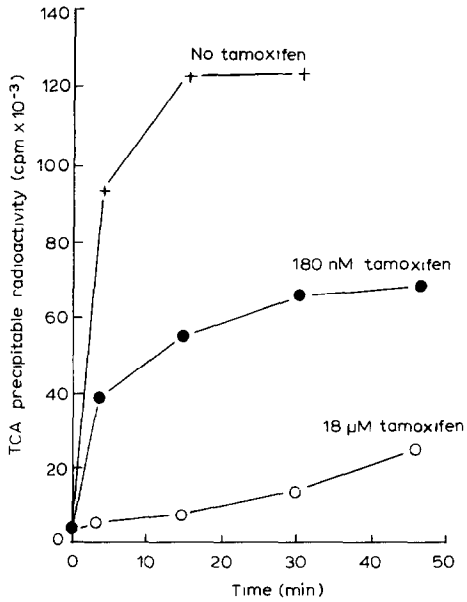


Fig.1. Inhibition of protein synthesis by tamoxifen. An untreated reticulocyte lysate was incubated under conditions of optimal protein synthesis with [^{35}S]methionine (17 μCi) at 30°C for up to 45 min with or without tamoxifen. Ethanol (1%) was included in the control. At the times shown aliquots (5 μl) were removed and assayed for trichloroacetic acid-precipitable radioactivity.

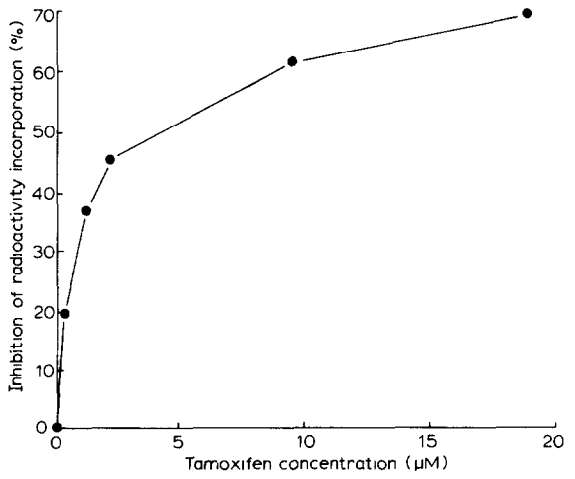


Fig.2. The effect of various concentrations of tamoxifen upon protein synthesis in reticulocyte lysates. Reticulocyte lysate was incubated at 30°C with [^{35}S]methionine (17 μCi) in the presence of various amounts of tamoxifen or 1% ethanol (control). After 30 min aliquots were removed from the solution and assayed for trichloroacetic acid-precipitable radioactivity. In the absence of tamoxifen the level of incorporation was 179385 counts/min.

decreased by more than 90% and 50%, respectively, after 3 min and a very low rate of protein synthesis continued throughout the 45 min. The range of concentrations over which tamoxifen inhibited protein synthesis extends at least as low as 180 nM, but at no concentration could synthesis be stopped completely. Treatment of lysates with tamoxifen and various concentrations of 17β-oestradiol (10 nM–20 μM), at the start of or during incubation failed to reverse the inhibition. Inhibition also occurred in whole reticulocytes (fig.3).

When *Xenopus* oocytes were incubated in the presence of 1.5 μM (fig.4) or 7 μM tamoxifen (not shown), 40–50% inhibition of protein synthesis

Fig.4. Tamoxifen inhibits protein synthesis in *Xenopus* oocytes. Oocytes were removed from a single female *Xenopus*. After mechanical separation groups of 5 were incubated at 21°C in MBS medium (50 μl) containing either ethanol (1%) or 1.5 μM tamoxifen in ethanol. At the times shown the oocytes were labelled for 2 h with [^{35}S]methionine (8.4 μCi), then homogenised in buffer containing protease inhibitors (100 μl) and the homogenate was centrifuged to pellet cell debris. Aliquots of the supernatant (5 μl) were assayed for trichloroacetic acid-precipitable radioactivity.

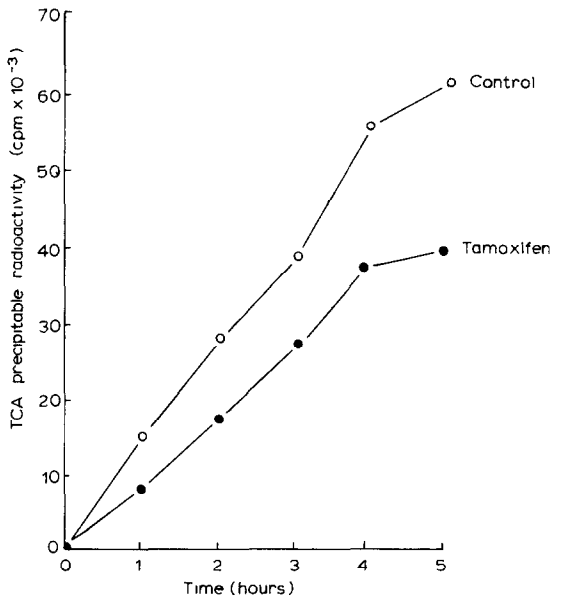
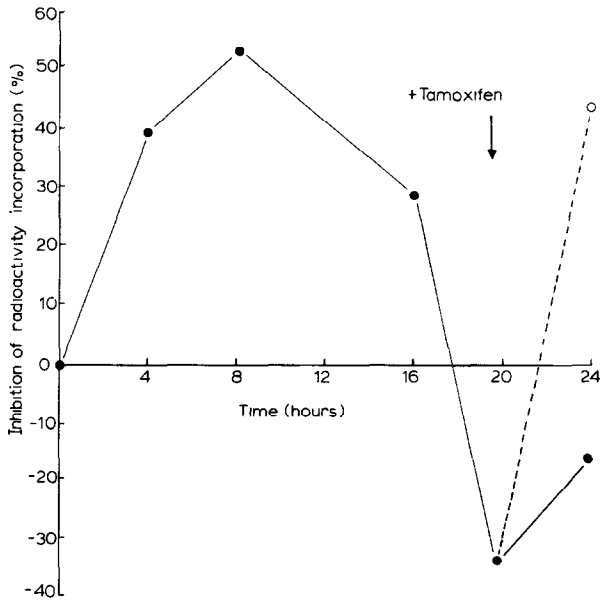


Fig.3. Inhibition of protein synthesis by tamoxifen in intact reticulocytes. Reticulocytes (2 × 10^9 cells) were preincubated in NCTC-135 medium (20 ml) for 4 h at 35°C. The cell suspension was divided into 10 portions (2 ml), which were incubated with [^{35}S]methionine (17 μCi) with or without tamoxifen (7 μM). Ethanol (1%) was included in the control flasks. At 1 h intervals cells were collected, washed with Borsook saline and lysed with water (150 μl). Aliquots (5 μl) of the lysates were then assayed for trichloroacetic acid-precipitable radioactivity.



was observed in both cases in 4 h. This inhibition was then reversed after 16 h but re-established by a further addition of the drug.

Qualitative analysis of the proteins made in oocytes during exposure to tamoxifen by SDS-PAGE showed no differences (not shown).

3.2. *Polyribosome profiles during tamoxifen treatment*

Sucrose-gradient analyses of reticulocyte lysates containing 0, 0.5 or 7 μM tamoxifen are shown in fig.5. Incubation times were chosen to allow ribosomes to run off the mRNA in controls. This

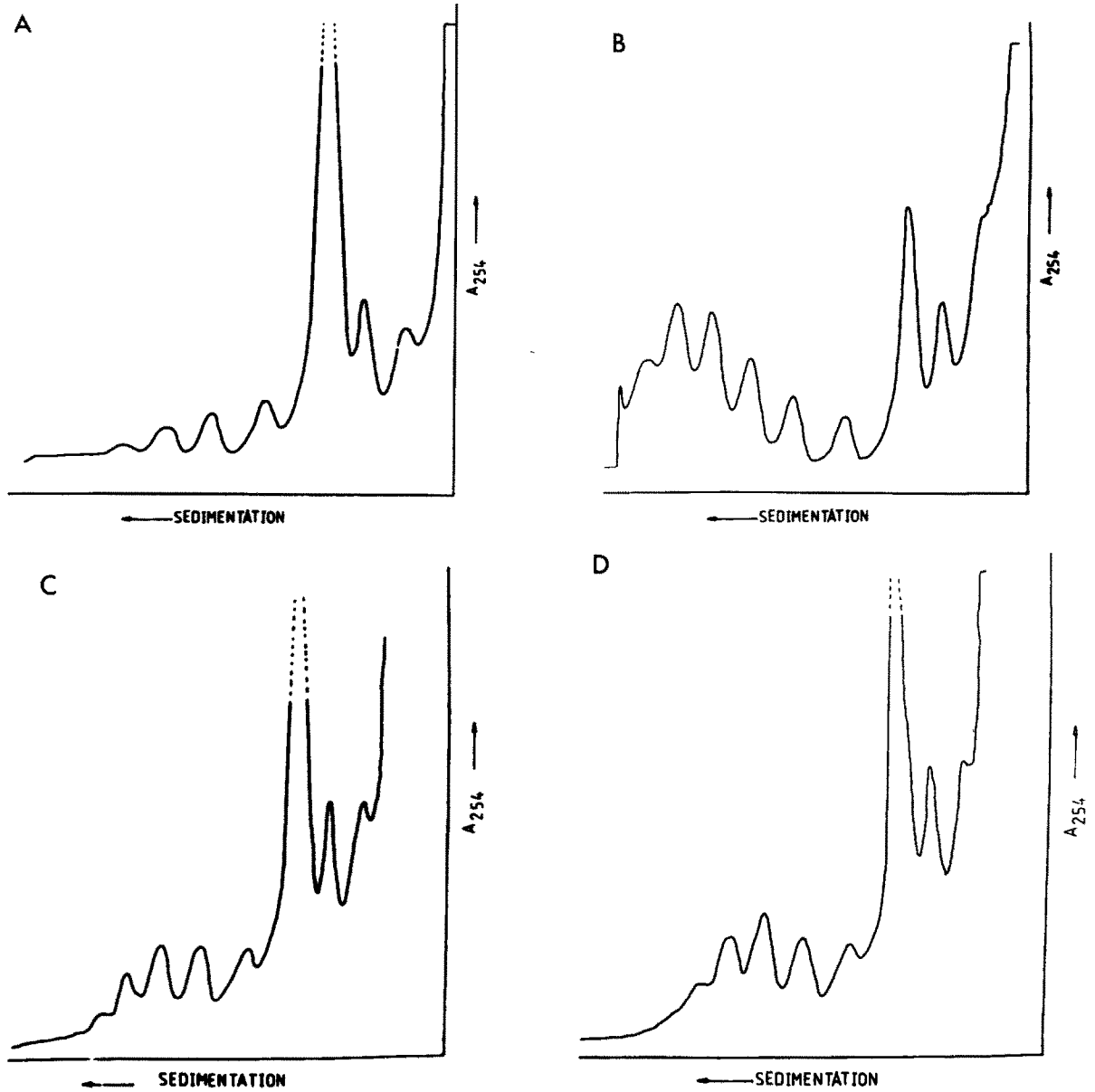


Fig.5. Polyribosome profiles from tamoxifen-treated reticulocyte lysates. Reticulocyte lysates were incubated at 30°C with [^{35}S]methionine in two separate experiments. Protein synthesis was stopped after 12 min by addition of 1 vol. RS buffer containing cycloheximide (10 μg/ml). The solutions were loaded immediately onto 15–30% isokinetic sucrose gradients, centrifuged at 185000 × g for 2 h and fractionated by upward displacement through an ISCO fractionator. (A) Control lysate (1% ethanol); (B) lysate incubated in the presence of tamoxifen (7 μM); (C) control lysate (1% ethanol); (D) lysate incubated in the presence of tamoxifen (0.5 μM).

process was stopped by 7 μM tamoxifen which produced a pattern identical with that of an unincubated lysate (not shown). Run off was also slowed by 0.5 μM tamoxifen.

Tamoxifen (7 μM) produced no change in the binding of [^{35}S]methionine to the 48 S preinitiation complex compared with controls (not shown).

4. DISCUSSION

In our experiments tamoxifen inhibited protein synthesis both in reticulocyte lysates and intact reticulocytes. Additionally it decreased protein synthesis in *Xenopus* oocytes, either by a direct effect or as a result of a decrease in the synthesis of other macromolecules such as mRNA. In all cases, the inhibition was rapid and observed at concentrations found in tumours and other tissues during chemotherapy with this drug [2]. Oestradiol neither prevented nor reversed the inhibition and this, together with the fact that oocytes [8] and presumably reticulocytes have no oestrogen receptor, suggests that this effect is unrelated to the anti-oestrogen properties of the drug.

No concentration of tamoxifen up to 18 μM appeared to inhibit protein synthesis completely in reticulocyte lysates. This may be due to specific protein(s) being unaffected by the drug or to a general continuation of synthesis as observed with some other inhibitors [10]. The latter explanation is favoured for 2 reasons. First, globin synthesis accounts for 90% of protein synthesis in reticulocytes and no other component would be able to account for the 30% of residual synthesis in the presence of tamoxifen. Secondly, analysis of proteins made by oocytes indicated that tamoxifen does not alter significantly the proportions in which different proteins are synthesized.

Analysis of the size classes of polysomes present in control and tamoxifen-treated reticulocyte lysates, together with the fact that there was no decrease in the binding of [^{35}S]methionine to the 48 S preinitiation complex, suggests that the drug acts on the elongation step in protein synthesis rather than on initiation.

The recovery of protein synthesis in oocytes treated with tamoxifen after 16 h suggests that this cell may metabolize or sequester the drug within a considerably shorter time than the 7 day half-life reported for whole animals.

The inhibition of protein synthesis in reticulocyte lysates, reticulocytes and *Xenopus* oocytes evidently does not require the presence of the oestrogen receptor. In reticulocyte lysates the drug acts upon elongation and the effect is not reversible by oestrogen. This general effect of protein synthesis may be similar to that reported by Stewart and Stern [9], who showed that 100 μM tamoxifen slows protein synthesis in foetal rat bone. Despite the different cell types, inhibition of protein synthesis may provide a possible explanation for the action of tamoxifen as an anti-tumour agent against oestrogen receptor-negative cells. Should tamoxifen inhibit protein synthesis in breast cancer cells in a manner similar to that observed here, the differences in drug concentrations between tumour and non-tumour tissues discussed in recent reviews [1,2] may account for its selective anti-tumour action. At a concentration of 6.7 μM, which is found in tumours, tamoxifen would cause a 70% reduction in protein synthesis slowing growth markedly or causing cell death. In contrast, the 0.53 μM concentrations found in other tissues would reduce protein synthesis by only 20%, which is likely to have a much less profound effect.

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