ELSEVIER

Biochimica et Biophysica Acta 1266 (1995) 215–222

BBA Biochimica et Biophysica Acta

Inhibitors of protein and RNA synthesis block the cytotoxic effects of non-steroidal antiestrogens

Peter L. Hwang *, Mei Y. Shoon, Yoke L. Low, Li Lin, Hwee L. Ng

Department of Physiology, National University of Singapore, 10 Kent Ridge Crescent, Singapore City, 0511 Republic of Singapore

Received 18 July 1994; revised 28 October 1994; accepted 27 January 1995

Abstract

Non-steroidal antiestrogens such as tamoxifen are known to exert cytotoxic effects against various cell lines in culture. When the antiestrogens are present at sufficiently high concentrations, their cytotoxicity cannot be reversed by estrogens and is demonstrable even with cell lines which lack the estrogen receptor. The mechanism of this cytotoxicity, which is clearly independent of estrogen antagonism, remains unknown. Using two murine cancer cell lines (the K36 leukemia and the EL4 lymphoma cell line), the human breast cancer cell line MCF7, and two non-steroidal antiestrogens (tamoxifen and clomiphene), our laboratory attempted to determine whether the cytotoxic action of non-steroidal antiestrogens was mediated by a mechanism requiring protein or RNA synthesis. In the case of K36 and EL4 cells, inclusion of tamoxifen or clomiphene in the culture medium regularly caused the viable call count to fall below 20–30% of control in 36–48 h. Under these conditions, the addition of inhibitors of protein or RNA synthesis consistently increased viable cell count in a dose-dependent manner. With cultures of K36 cells grown in the presence of 10 μM tamoxifen, for example, the addition of appropriate concentrations of emetine, cycloheximide, puromycin, or actinomycin D increased the percentage of viable cells to 5.0, 2.4, 4.0, and 4.0 times that of control, respectively. Additional experiments revealed that the macromolecular synthesis inhibitors, while effective in inhibiting protein or RNA synthesis to varying degrees, did not affect the cellular uptake of [^{3}H]tamoxifen, suggesting that their ability to protect cells against antiestrogen-induced cell death was not due to an inhibition of cellular uptake of antiestrogens. In the case of MCF7 cells, however, inhibition of protein synthesis did not protect the cells against the cytotoxic effect of tamoxifen. These observations suggest that non-steroidal antiestrogens may exert their cytotoxic effect by at least two different mechanisms; only one of these require de novo protein synthesis. The effect of antiestrogens on K36 and EL4 cells may provide a useful system for the identification of proteins involved in cell death.

Keywords: Antiestrogen; Cytotoxicity

1. Introduction

It is well recognized that non-steroidal antiestrogens such as tamoxifen and clomiphene are known to exert antiproliferative and cytotoxic effects against breast cancer cell lines in culture (reviewed in Ref. [1]). For cells containing estrogen receptors, the antiproliferative effect can be accounted for, at least partly, by antagonism against the effect of estrogens since the effect can be reversed by excess estrogen if the antiestrogens are present at moderate concentrations [2]. When the antiestrogens are present at sufficiently high concentrations, however, their cytotoxic effects cannot be reversed by estrogens [3] and are demonstrable even in the absence of estrogens [4] or with cell lines which lack the estrogen receptor [5]. The mechanism of this estrogen receptor-independent antiproliferative and cytotoxic effect of non-steroidal antiestrogens is not understood.

In the studies reported here, we examined the possibility that the cytotoxic effect of non-steroidal antiestrogens in estrogen-receptor-negative cells is mediated by a mechanism requiring protein or RNA synthesis. Using two murine lymphoma cell lines (K36 and EL4) and two non-steroidal antiestrogens (tamoxifen and clomiphene), we demonstrated that inhibitors of protein and RNA synthesis can block the cytotoxic effect of the antiestrogens, thus suggesting that the cytotoxic action of these compounds may

Abbreviations: AEBS, antiestrogen binding site; MTT, tetrazolium salt.

* Corresponding author. Fax: (+65) 7788161.

Elsevier Science B.V.
SSDI 0167-4889(95)00017-8

be mediated by mechanisms requiring de novo protein or RNA synthesis. With the breast cancer cell line MCF7, however, this phenomenon was not observed. A portion of this work was presented in abstract form at the 1994 Annual Meeting of the Endocrine Society at Anaheim, USA.

2. Experimental procedures

2.1. Materials

[5,6-^{3}H]Uridine (spec. act. 35 Ci or 1.3 TBq/mmol) and [N-methyl-^{3}H]tamoxifen (spec. act. 82 Ci or 3.03 TBq/mmol) were obtained from Amersham International (Buckinghamshire, UK). L-[^{35}S]Methionine (spec. act. > 1000 Ci or 37.0 TBq/mmol) was obtained from New England Nuclear (Du Pont, Wilmington, Delaware, USA). Cycloheximide, puromycin, emetine, tamoxifen citrate, and clomiphene were obtained from Sigma (St. Louis, MO, USA). Actinomycin D was from Fluka (Switzerland). Newborn calf serum was purchased from Waitaki (New Zealand) while cell culture media, diethylstilbestrol and steroids were obtained from Sigma. Other chemicals were of analytical grade and were obtained from conventional sources.

2.2. Cell cultures

The mouse lymphoma cell line EL4, and the AKR T-cell leukemia line K36 were generously provided by Dr. K.M. Hui (Institute of Molecular and Cellular Biology, National University of Singapore). MCF7 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were propagated in RPMI-1640 medium supplemented with 5% (v/v) charcoal-stripped [6], lipoprotein-deficient newborn calf serum which was prepared, with minor modifications, as described by Kirsten and Watson [7]. Briefly, after charcoal stripping [6], anhydrous potassium bromide was added to the newborn calf serum to a concentration of 32% (w/v). After centrifugation at 258 000 × g at 15° C for 24 h, the lower (lipoprotein-depleted) fraction was removed by aspiration and extensively dialysed against 6 changes of 100 volumes of 0.15 M sodium chloride solution over 72 h at 4° C. After sterilization by filtration through a 0.2 μm membrane, the lipoprotein-deficient serum was added to the culture medium in an amount adjusted to be equivalent to supplementation by 5% (v/v) of the original newborn calf serum. Running stock cultures were propagated in 75-cm^2 flasks (Costar, USA) at 37° C in an atmosphere of 5% CO_2 in humidified air. Subcultures were carried out when cell densities reached 2.5–3.5 · 10^6 cells/ml for K36 and EL4 cells. For MCF7 cells, media change was carried out every 2–3 days; otherwise culture conditions were similar to that of K36 and EL4 cells.

2.3. Cytotoxicity assays

EL4 or K36 cells in the logarithmic phase of growth were harvested and plated on to 24-well plates (Costar, USA) at an initial cell density of 0.8–1.2 · 10^6 cells/ml. Cell viability of stock was routinely determined by Trypan blue exclusion and only cultures with viabilities > 90% were used. In preliminary assays, varying concentrations of tamoxifen and clomiphene were added to determine the optimal cytotoxic concentrations to use for subsequent studies. These compounds were initially dissolved in ethanol and the ethanolic solutions were added directly to the culture medium. The final ethanol concentration was usually 0.1–0.2% and never exceeded 0.5%. Control wells received only ethanol. In assays designed to test the effects of cycloheximide, puromycin, emetine, or actinomycin D, these inhibitors were first dissolved in either ethanol (cycloheximide, emetine and actinomycin D) or phosphate-buffered saline or RPMI-1640 (puromycin) and then added to the culture medium at varying concentrations. At specified times after plating, aliquots of cell suspensions were removed for cell counting using an improved Nebauer hemocytometer while cell viabilities were simultaneously determined by means of Trypan blue exclusion. The viable cell count was calculated by multiplying the total cell count by the percent of cells which were viable. In some experiments, the viable cell count was quantitated by the MTT assay which depends on the conversion of 3(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide to colored formazan [8]. In the case of MCF7 cells, 10 000–15 000 cells per well were plated on to 96-well plates. After overnight attachment, fresh medium containing varying concentrations of antiestrogens and inhibitors were added. After one medium change on day 3, the assay was terminated on day 5. Viable cell count was determined by the MTT method [8].

2.4. Other procedures

For statistical analysis, unpaired Student's t-test or Bonferroni's multiple comparison test [9] was employed. Other procedures were carried out as described in the appropriate figure and table legends.

3. Results

Preliminary studies were carried out to determine the concentrations of tamoxifen and clomiphene required to reduce cell viability to less than 30% of control (usually in 36–48 h for K36 and EL4 cells and in 120 h for MCF7 cells). It was found that 5–10 μM of tamoxifen and 0.5 μM of clomiphene would be adequate. These culture conditions were used in all subsequent cytotoxicity studies, except where indicated otherwise, to examine the effect of protein or RNA synthesis inhibitors.

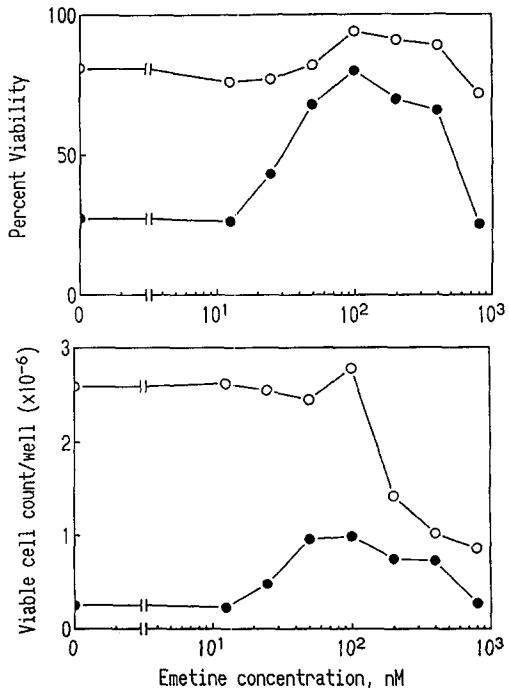

Fig. 1. Effect of emetine on the cytotoxic effect of tamoxifen on K36 cells. K36 cells were cultured with varying concentrations of emetine in the absence (○) or presence (◐) of 10 μM tamoxifen. After 48 h, aliquots of the cultures were taken for cell count and viability determination using the Trypan blue exclusion technique. Viable cell count was calculated by multiplying the total cell count by the percent of total cells which excluded Trypan blue (percent viability). The data were representative of three separate experiments.

Parenthetically, we also noted that diethylstilbesterol, estradiol, cortisol, progesterone, and testosterone, when added to cell cultures at concentrations up to 10 μM had no discernible effect on cell viability.

Fig. 1 shows the effect of the protein synthesis inhibitor emetine on cell viability (as measured by Trypan blue exclusion) of K36 cells after 48 h of culture in the presence or absence of 10 μM tamoxifen. In the absence of tamoxifen, emetine itself had little effect on percent cell viability at concentrations up to 800 nM (upper panel). In the presence of tamoxifen, however, percent cell viability was clearly affected by emetine in a dose-dependent manner, increasing from 27% in the absence of emetine to a maximum of 80% in the presence of 100 nM emetine. Higher concentrations of emetine reduced cell viability, probably because of a direct cytotoxic effect of emetine itself. The lower panel of Fig. 1 depicts the total viable cell counts observed in the same experiment. It is clear that emetine, at concentrations between 25 and 400 nM, protects K36 cells against the cytotoxic effects of tamoxifen. Similar results were obtained when the MTT method [8] was used to quantitate viable cell count (not shown).

Fig. 2 shows the effect of emetine on K36 cell viability in the presence of tamoxifen as a function of time. In the presence of 10 μM tamoxifen alone, there was a rapid decline in percent cell viability, falling from 97% to 14% and 0% at 48 and 72 h respectively (upper panel). The addition of 0.1 μM emetine to the incubation medium markedly improved cell viability so that at 48 and 72 h the percent viabilities remained at 94% and 84%, respectively. The lower panel of Fig. 2 depicts the total viable cell counts observed as a function of time in the same experiment. The increased cell viability, whether expressed as percent viability or as total viable cell count, were highly significant at both 48 h and 72 h ($P < 0.001$).

Similar studies were carried out with two other protein synthesis inhibitors, cycloheximide and puromycin, and with the RNA synthesis inhibitor actinomycin D. The results of representative experiments with these inhibitors are shown in Fig. 3. In all instances, under conditions where the presence of 10 μM tamoxifen reduced viable cell count to 20% or less of control, the presence of cycloheximide, puromycin, or actinomycin D clearly increased viable cell count in a dose-dependent manner. Cycloheximide, puromycin, and actinomycin D showed maximum protective effects at concentrations of 0.25 μM, 3.12 μM, and 6.25 nM, respectively. Fig. 3 also shows the effect on viable cell count of cycloheximide, puromycin, or actinomycin alone. As in the case of emetine, all these agents are cytotoxic by themselves at higher concentrations. However, for all these macromolecular synthesis

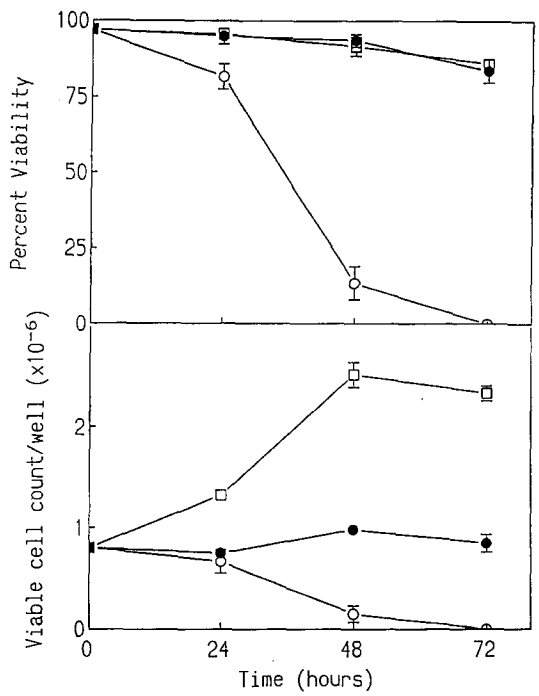

Fig. 2. Time course of changes in percent cell viability and total viable cell count in K36 cell cultures in the presence of tamoxifen with and without emetine. Cell cultures were set up as described in Section 2. Control wells (□) contained 0.2% ethanol. Other wells contained either 10 μM tamoxifen alone (○), or 10 μM tamoxifen plus 0.1 μM emetine (●). Viable cell counts were determined at 24, 48, and 72 h. Each point represents the mean ± S.D. of triplicate determinations.

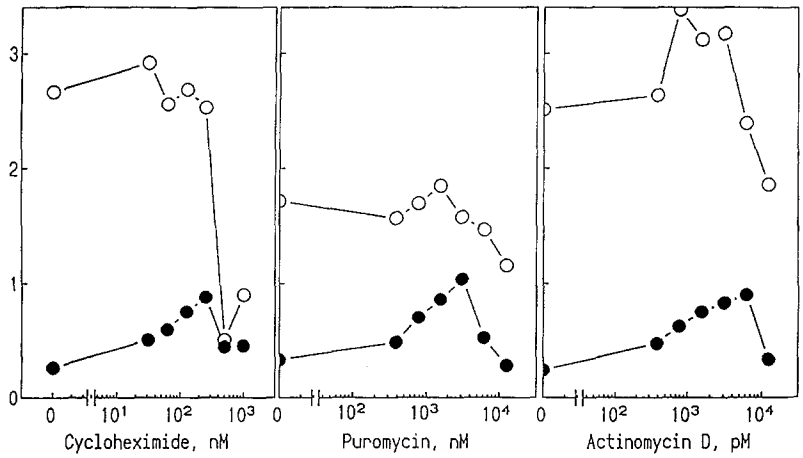

Fig. 3. Effect of cycloheximide, puromycin, and actinomycin D on the cytotoxic effect of tamoxifen on K36 cells. Cultures were incubated with varying concentrations of cycloheximide, puromycin, or actinomycin D in the absence (○) or presence (●) of 10 μM tamoxifen. Total cell count and percent viability were determined after 48 h. The data were representative of three separate experiments.

inhibitors, there exists a 'window' in which the inhibitor exerted a protective action without being cytotoxic.

Fig. 4 summarizes the effect of 0.1 μM emetine, 0.25 μM cycloheximide, 1.56 μM puromycin, and 2.4 nM actinomycin D on K36 cell viability after 42–48 h of culture in the presence of 10 μM tamoxifen. All four compounds significantly and, in some instances, dramatically increased the percent cell viability (upper panel) as well as the total viable cell count (lower panel). Among the four inhibitors, emetine appeared to be most efficacious in protecting the K36 cells against the cytotoxic action of tamoxifen, increasing percent cell viability from 19% to 95% and viable cell count from 0.12 to 1.13 million per well. These changes were highly significant ($P < 0.001$) as were the changes observed with all the other macromolecular synthesis inhibitors ($P < 0.05$ in all instances). The percent viabilities observed with the addition of cycloheximide, puromycin and actinomycin D were 2.4, 4.0, and 4.0 times that of control, while the viable cell counts were 4.2, 4.8, and 4.5 times that of control, respectively.

In addition to tamoxifen, another non-steroidal antiestrogen, clomiphene, was also studied in experiments similar to those performed for tamoxifen. The results, shown in Table 1, were similar to those obtained with tamoxifen. Emetine, cycloheximide, puromycin and actinomycin D, at the concentrations indicated, increased the percent viability to 4.2, 3.7, 2.4, and 2.9 times that of control, and increased the viable cell count to 5.3, 5.1, 2.8, and 3.4 times that of control, respectively. All the increases were highly significant ($P < 0.001$).

To determine whether the observations with K36 cells also apply to other cell lines, we carried out similar studies with the murine lymphoma cell line EL4 and the breast cancer cell line MCF7. Table 2 shows the effect of the four inhibitors on the cytotoxic effect of tamoxifen against EL4 cells. All four metabolic inhibitors significantly increased cell viability in the presence of 5 or 10 μM tamoxifen. It would appear that the ability of protein and RNA synthesis inhibitors to protect cells against the cytotoxic effects of antiestrogens applies also to EL4 cells and may represent a general phenomenon not restricted to one particular cell type. However, with MCF7 cells, we failed to observe any protection with the protein synthesis inhibitor emetine over a wide range of concentrations (Fig. 5). Emetine itself, at concentrations above 25 nM, was cytotoxic to MCF7 cells.

Table 1
Effect of macromolecular synthesis inhibitors on clomiphene-induced cytotoxicity in K36 cells

Inhibitor	Conc. (μM)	Viable cell count/well	P [a]	Viability (%)	P [a]
Control	–	0.21 ± 0.01	–	21.3 ± 1.5	–
Emetine	0.1	1.06 ± 0.10	< 0.001	90.0 ± 1.0	< 0.001
Cycloheximide	0.25	1.03 ± 0.07	< 0.001	78.1 ± 1.2	< 0.001
Puromycin	1.56	0.56 ± 0.05	< 0.001	52.0 ± 2.0	< 0.001
Actinomycin D	1.25×10^{-3}	0.68 ± 0.09	< 0.001	62.3 ± 4.1	< 0.001

K36 cell cultures were set up as described in the experimental procedures in the absence or presence of emetine (0.1 μM), cycloheximide (0.25 μM), puromycin (1.56 μM), or actinomycin D (1.25 nM). All cultures contained 0.5 μM clomiphene. After 42 h, viable cell count and percent cell viability were determined by Trypan blue exclusion. The values represent the mean ± S.D. of triplicate determinations.
a P-values were obtained by comparing the observed data with control (no inhibitor added) using the Bonferroni multiple comparison test.

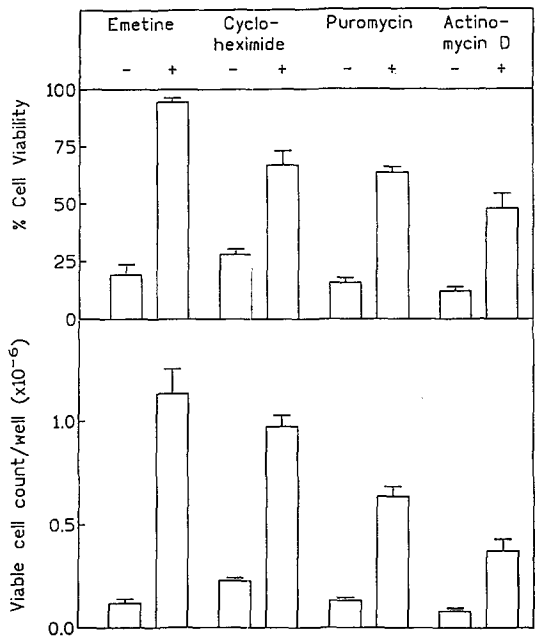

Fig. 4. Effect of protein or RNA synthesis inhibitors on the viability of K36 cells cultured in the presence of tamoxifen. Cell cultures were set up as described in Section 2. All cultures contained 10 μM tamoxifen with (+) or without (−) emetine (0.1 μM), cycloheximide (0.25 μM), puromycin (1.56 μM), or actinomycin D (2.4 nM). Percent cell viability and total viable cell count were determined by Trypan blue exclusion after 42–48 h. The data represent mean ± S.D. of quadruplicate determinations. The observed increases in percent cell viability (upper panel) or total viable cell count per well (lower panel) in the presence of inhibitor were statistically significant in all cases ($P < 0.05$).

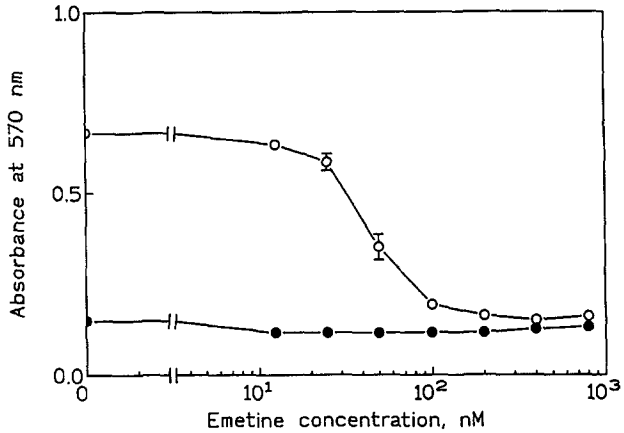

Fig. 5. Effect of emetine on the cytotoxic effect of tamoxifen on MCF7 cells. MCF7 cells were plated on to 96-well plates at an initial cell density of 10000–15000 cells per well. After overnight attachment, fresh media containing varying concentrations of emetine and either 0 (○) or 10 μM (●) tamoxifen were added. After one change of media on day 3, the assay was terminated on day 5 using the MTT technique to quantitate viable cell count. The data represent ± S.D. of triplicate determinations; where error bars are not seen, the S.D. is less than the diameter of the symbol.

The marked difference observed between the effect of emetine on K36 cells on the one hand (Fig. 1), and MCF7 cells on the other hand (Fig. 5), suggests that antiestrogens kill cells by two or more mechanisms, not all of which are dependent on protein synthesis.

We next examined the extent to which inhibition of protein and RNA synthesis in intact K36 cells was inhibited by the metabolic inhibitors at concentrations which were cytoprotective (Fig. 6). Emetine (0.1 μM), cycloheximide (0.25 μM), puromycin (3.12 μM) and actinomycin D (5 nM) inhibited protein or RNA synthesis by 52%, 33%, 10%, and 55%, respectively. Higher concentrations of the inhibitors inhibited macromolecular synthesis to a greater degree, but they also tended to be cytotoxic (Figs. 1 and 3).

Since protection against antiestrogen-induced cell-killing could be observed even when the inhibition of protein or RNA synthesis was only modest, as in the case of puromycin, we considered the possibility that the cytoprotective effect of the metabolic inhibition might be unrelated to their ability to inhibit protein or RNA synthesis. One possibility we examined was that these inhibitors could interfere with the cellular uptake of the antiestrogens. Accordingly, the study shown in Table 3 was carried out. [^{3}H]Tamoxifen was incubated with K36 cells in the

Table 2
Effect of macromolecular synthesis inhibitors on tamoxifen-induced cytotoxicity in EL4 cells

Inhibitor	Conc. (μM)	Viability (%)	P	Viable cell count per well ($\times 10^{-6}$)	P
Emetine	0	1.5 ± 0.4	< 0.01	0.05 ± 0.01	< 0.05
	0.25	46.7 ± 4.3		0.77 ± 0.08	
Cycloheximide	0	5.0 ± 0.8	< 0.01	0.06 ± 0.01	< 0.05
	0.25	18.0 ± 1.4		0.21 ± 0.03	
Puromycin	0	0.5 ± 0.1	< 0.05	0.02 ± 0.01	< 0.05
	3.12	12.9 ± 2.1		0.29 ± 0.08	
Actinomycin D	0	0.3 ± 0.4	< 0.01	0.01 ± 0.01	< 0.05
	0.1	10.6 ± 0.3		0.25 ± 0.04	

EL4 cell cultures were set up in the absence or presence of emetine, cycloheximide, puromycin or actinomycin D. All cultures contained 5 or 10 μM tamoxifen. After 42–72 h, percent cell viability and viable cell count were determined by Trypan blue exclusion. The values represent mean ± S.D. The P-values were obtained by the unpaired Student's t-test.

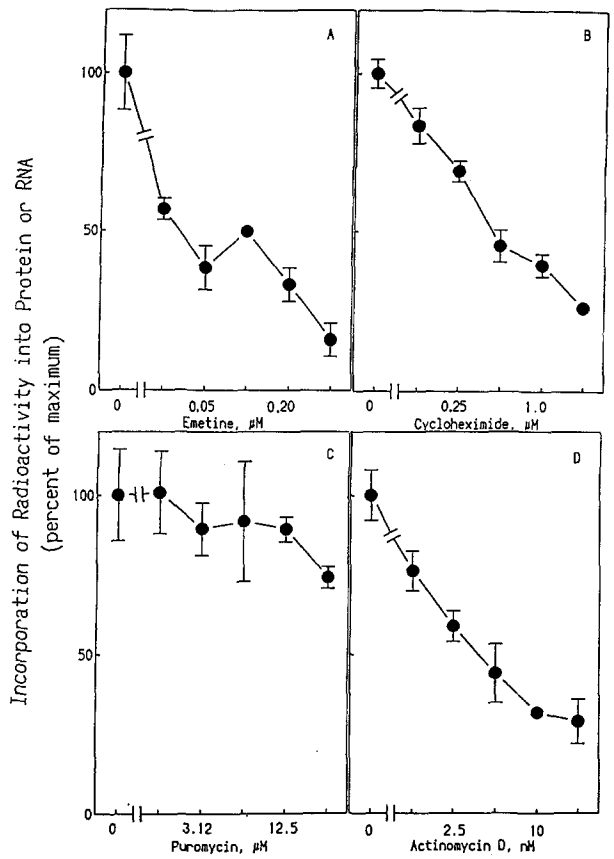

Fig. 6. Effect of protein and RNA synthesis inhibitors on [^{35}S]methionine incorporation into protein or [^{3}H]uridine incorporation into RNA in intact K36 cells. Cell cultures containing approximately 1.0×10^6 cells/ml were set up in 96-well plates and preincubated for 3 h at 37° C with varying concentrations of emetine (A), cycloheximide (B), puromycin (C) or actinomycin D (D). [^{35}S]methionine was then added to A, B, and C to a final concentration of 0.4 μCi/ml while [^{3}H]uridine was added to D to a final concentration of 0.2 μCi/ml. After a further 5 h of incubation, cells were trapped on scintillant-coated glass microfiber filters (Beckman, USA) in a multiple-sample cell harvester (Brandel, USA) and washed in turn with 2×2 ml of phosphated-buffered saline, 10% (w/v) trichloroacetic acid and 95% (v/v) ethanol. The filters were dried and counted in a Beckman scintillation counter. Results were expressed as a percentage of counts observed in control cultures which contained no inhibitor. Each point represents mean ± S.E.M. of triplicate determinations.

absence and presence of the inhibitors. None of the inhibitors, at the concentrations indicated, had any significant effect on the cellular uptake of [^{3}H]tamoxifen. These inhibitors also did not significantly reduce [^{3}H]tamoxifen uptake by EL4 cells (not shown). These observations make it unlikely that the protective effect of the inhibitors is due to an ability to reduce the cellular uptake of antiestrogens.

4. Discussion

Although it is generally known that non-steroidal antiestrogens have antiproliferative and cytotoxic effects, the mechanism of these effects is incompletely understood. In estrogen target tissues, at least part of this effect is believed to be due to estrogen antagonism. However, for cells with no estrogen receptors, the mechanism by which non-steroidal antiestrogens exert their cytotoxic action is obscure.

The studies reported here demonstrate that the cytotoxic effects of non-steroidal antiestrogens are clearly blocked by inhibitors of protein and RNA synthesis in K36 and EL4 cells. Since the observations on these two cell lines were obtained with two different antiestrogens and four different macromolecular synthesis inhibitors, it is reasonable to infer that the cell death induced by non-steroidal antiestrogens in K36 and EL4 cells may require protein or RNA synthesis. This possibility is particularly attractive because the four metabolic inhibitors studied are known to inhibit protein synthesis via distinct mechanisms: cycloheximide inhibits peptidyl transferase activity, puromycin causes premature peptide-chain termination, emetine inhibits ribosomal translocation along the mRNA template, while actinomycin D inhibits RNA synthesis [10,11].

We were somewhat surprised to observe that, unlike K36 and EL4 cells, the antiestrogen-induced cell death of MCF7 cells was not blocked by emetine, a protein synthesis inhibitor (Fig. 5). The breast cancer cell line MCF7 contains estrogen receptors; its responses to antiestrogen

Table 3
Uptake of [^{3}H]tamoxifen by K36 cells

Inhibitor used	Uptake of [^{3}H]tamoxifen (% of control)	P
Control	100 ± 10.0	–
Cycloheximide (0.25 μM)	94.6 ± 4.1	> 0.05
Puromycin (1.5 μM)	106.1 ± 12.2	> 0.05
Emetine (50 nM)	100.4 ± 4.3	> 0.05
Actinomycin D (2 nM)	106.2 ± 11.3	> 0.05

K36 cells in the absence or presence of inhibitors of protein or RNA synthesis were incubated with 100000 cpm of [^{3}H]tamoxifen (10 μM). At 2 h, the cell suspensions were filtered on glassfiber filters (Whatman GF/A) and washed with 4 × 3 ml of phosphate-buffered saline. The filters were air-dried and counted. The values indicated are means ± S.D. of quadruplicate determinations expressed as percent of control uptake. The P-values were obtained by Bonferroni's multiple comparison test comparing the test values against control. Parallel cultures indicated that the cell count and viability were not changed significantly by the presence of the inhibitors during the 2 h of incubation. The uptake of [^{3}H]tamoxifen in the control wells amounted to 14.6% of the number of counts added at the beginning of the experiment.

treatment have been extensively studied [1]. The fact that emetine had no protective effect against tamoxifen-induced cell death in MCF7 cells (Fig. 5) but exhibited clear protection for K36 cells (Fig. 1) would suggest that there are at least two different mechanisms by which non-steroidal antiestrogens kill cells; only one of these can be blocked by protein synthesis inhibitors. It is possible that antiestrogens kill 'target' cells (those with estrogen receptors) in a different manner from non-target cells, such as K36 and EL4 cells, which do not contain estrogen receptors. This possibility remains to be explored further.

We have examined the possibility that the metabolic inhibitors might interfere with the cellular uptake of antiestrogens, thereby reducing their apparent cytotoxic potency. Using the conditions described in Table 3, we were unable to show that any of the inhibitors reduced [^{3}H]tamoxifen uptake. We have also considered an alternative interpretation of our data: that the inhibition of protein synthesis reduces the energy requirement of the cells and thus prolongs their survival in the face of a limited energy supply provided by the culture medium. This possibility is plausible only when the culture medium is not changed for prolonged periods of time and seems unlikely under our experimental conditions where cell death was induced in about 48 h by the antiestrogens. It would seem, therefore, reasonable to suggest that antiestrogen-induced cell death, at least in K36 or EL4 cells, is likely to involve mechanisms requiring protein synthesis.

Nevertheless, we have some reservations about this interpretation. In the first place, although protective concentrations of emetine, cycloheximide, puromycin and actinomycin D were also effective in inhibiting protein or RNA synthesis, the degree of inhibition of macromolecular synthesis was highly variable. In the case of puromycin, for example, a maximally protective concentration inhibited protein synthesis by only about 10%. It is difficult to visualize how a 10% reduction in protein synthesis could block the cytotoxicity of antiestrogens if indeed protein synthesis is an obligatory step in the mechanism of antiestrogen-induced cell death. This could be the case only if the putative protein required to mediate cell death is rate-limiting, a point which cannot be tested directly at present.

The non-steroidal antiestrogens are known to bind with high affinity not only to the estrogen receptor but also to a ubiquitously distributed microsomal protein, the antiestrogen binding site (AEBS) which was identified as a distinct entity more than a decade ago [12]. Our laboratory has earlier shown that, among compounds which bind to the AEBS and which exhibit cytotoxic effects, the cytotoxic potency (as measured by the EC_{50} values) broadly paralleled their binding affinities for the AEBS [13]. On the basis of this observation, as well as the studies of other investigators [14,15], we suggested that the AEBS might mediate the cytotoxic effects of its ligands. In particular, other classes of ligands of the AEBS, such as diphenylmethane derivatives [14] and synthetic analogues of triphenylethylene antiestrogens [15], have also been found to have cytotoxic potencies which parallel their binding affinities for the AEBS. Nevertheless, currently there is no direct evidence for the hypothesis that AEBS mediates the cytotoxicity of any of its ligands. It is possible that the AEBS may be one of the proteins involved antiestrogen-induced cell death and that the inhibition of its synthesis may partly explain the protective effect of inhibitors of protein and RNA synthesis. Further studies are needed to examine this possibility.

Finally, it should be emphasized that the studies described here demonstrate primarily an in vitro phenomenon, namely the dependence of antiestrogen-induced cell death on protein synthesis. Whether this in vitro biological phenomenon has any in vivo counterpart, for example in breast cancer patients treated with tamoxifen, is not known. Indeed, the results obtained with MCF7 cells (Fig. 5) would suggest that tamoxifen kills such 'target' cells via a different mechanism which is *not* dependent on protein synthesis. Nevertheless, the studies with K36 and EL4 cells revealed an interesting biological phenomenon which suggests that at least one of the cytotoxic mechanisms of non-steroidal antiestrogens appears to depend on de novo protein synthesis. This cytotoxic effect of antiestrogens on K36 or EL4 cells could provide a potentially useful model for the identification of proteins involved in the regulation of cell death and survival.

Acknowledgements

The authors are grateful for the superb secretarial assistance of Ms Asha Das. This study was supported by the National University of Singapore.

References

[1] Sutherland, R.L. and Jordan, V.C. (1981) Non-steroidal Antioestrogens: Molecular Pharmacology and Antitumour Activity. Academic Press, New York.
[2] Murphy, L.C. and Sutherland, R.L. (1983) J. Clin. Endocrinol. Metab. 57, 373–379.
[3] Sutherland, R.L., Watts, C.K.W., Hall, R.E. and Ruenitz, P.C. (1987) J. Steroid Biochem. 27, 891–897.
[4] Vignon, F., Bouton, M.M. and Rochefort, H. (1987) Biochem. Biophys. Res. Commun. 146, 1502–1508.
[5] Hoh, Y.K., Lim, E.H., Ooi, S.O. and Kon, O.L. (1990) Experientia 46, 1032–1037.
[6] Westley, B. and Rochefort, H. (1980) Cell 20, 353–362.
[7] Kirsten, E.S. and Watson, J.A. (1974) J. Biol. Chem. 249, 6104–6109.
[8] Hansen, M.B., Nielsen, S.E. and Berg, K. (1989) J. Immunol. Methods 119, 203–210.
[9] Bailar III, J.C. and Mosteller, F. (1986) Medical Uses of Statistics, New Engl. J. Med. Books, MA, USA.

[10] Gupta, R.S. and Siminovitch, L. (1977) Biochemistry 16, 3209–3214.
[11] Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. (1989) in Molecular Biology of the Cell, 2nd edn., p. 218, Garland, New York.
[12] Sutherland, R.L., Murphy, L.C., Foo, M.S., Green, M.D., Whybourne, A.M. and Krozowski, Z.S. (1980) Nature 288, 273–275.
[13] Hwang, P.L.H. (1990) Biochim. Biophys. Acta 1033, 154–161.
[14] Fargin, A., Bayard, F., Faye, J.C., Traore, M., Poirot, M., Klaebe, A. and Perie, J.J. (1988) Chem.-Biol. Interactions 66, 101–109.
[15] Tang, B.L., Teo, C.C., Sim, K.Y., Ng, M.L. and Kon, O.L. (1989) Biochim. Biophys. Acta 1014, 162–172.