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Synergistic Antiproliferative Activity of Tamoxifen and Cisplatin on Primary Ovarian Tumours

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We looked for the presence of the so-called type II oestrogen binding sites (EBS), in four oestrogen (ER) and progesterone (PR) receptor negative primary ovarian tumours. Moreover, the colony-forming assay was used to evaluate the response of ovarian cancer cells from these primary tumours to tamoxifen and cisplatin used alone or in combination. All tumours contained type II EBS, and tamoxifen was able to compete for [^{3}H] oestradiol binding to these sites. Cisplatin and tamoxifen exhibited a dose-dependent inhibition of colony formation in a range of concentrations between 10 and 1000 μg/l and 37 and 3710 μg/l, respectively. The combination of the two drugs resulted in a synergistic antiproliferative activity, with a potentiation up to and beyond 50-fold. Our results show that in ovarian cancer tamoxifen interacts with type II EBS with an affinity consistent with the concentration effective both in inhibition of colony formation and in synergising cisplatin activity. Based on the experiments performed the action of tamoxifen on cell growth is independent of ER expression, and could be mediated by type II EBS. The possibility that the association of tamoxifen and cisplatin may result in an improved clinical response in ovarian cancer should be investigated.
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INTRODUCTION

ALTHOUGH THE development of new cytotoxic agents and combination chemotherapy protocols have resulted in improved survival, cures in patients with advanced ovarian cancer remain in the minority.

Cisplatin is the most active agent against ovarian cancer. However, the development of resistant clones as well as the neurological and kidney toxicity of the drug represent the major limitations of cisplatin treatment. The identification of agents able to act synergistically with cisplatin may be useful to overcome resistance at non-toxic doses. We have recently reported that quercetin, a flavonoid with growth-inhibitory activity [1–3], greatly enhances the antiproliferative activity of cisplatin on human ovarian cancer cells probably by an interac-

tion with the so-called type II oestrogen binding sites (type II EBS) [4, 5]. These sites were originally described by Markaverich and Clark [6] in rat uterus. While displaying the same steroid and tissue specificity they are distinct from the 'classical' oestrogen receptor (ER). They are reported to be present in higher concentrations than ER but to have a lower apparent affinity dissociation constant (K_d 10–20 nmol/l) for E_2 than ER (K_d 0.1–1 nmol/l) [7, 8]. It has also been demonstrated that in two human cancer cell lines [9, 10] tamoxifen interacts with type II EBS, and its binding affinity correlates well with its growth inhibitory effect.

On the other hand Geisinger *et al.* [11] recently reported preliminary evidence suggesting that in the human ovarian carcinoma cell line BG-1 synergistic activities were produced when tamoxifen was combined with either cisplatin or doxorubicin. Moreover, clinical data obtained in human metastatic melanoma [12] indicate that tamoxifen synergises with cisplatin, carmustine and dacarbazine in inducing objective tumour regression in approximately 50% of treated patients.

These observations prompted us to verify whether tamoxifen can synergise the inhibitory action of cisplatin on clonogenic activity of ER negative, type II EBS positive cancer cells from primary ovarian tumours.

MATERIALS AND METHODS

Drugs and chemicals

Cisplatin solutions, made in distilled water, were used at concentrations varying from 10 to 1000 μg/l. Tamoxifen, at concentrations ranging from 37 to 3710 μg/l, was added from an absolute ethanol stock solution. The control cells were treated with the same amount of vehicle alone. The final ethanol concentration never exceeded 1% in either control or treated samples.

Tissue samples and preparation of tumour cells

Tissue samples were obtained from 4 previously untreated patients with advanced ovarian cancers.

Tumour specimens, obtained immediately after surgery, were mechanically dissociated under aseptic conditions in a laminar flow hood. Tumours were washed in Ham's F-12 medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin and were finely minced. The small cubes were then incubated for 24 h at 37°C with Ham's F-12 medium supplemented as above with 0.1% collagenase IV (Sigma). Cells were then filtered through sterile gauze to remove cell clumps, passed through 25-gauge needles, and then washed by centrifugation. Tumour cells were separated on a Ficoll–Hypaque gradient and the cells collected from the interface were washed twice in Hanks' balanced salt solution. After washing, the tumour cells were resuspended in Ham's F-12 medium and the viability was determined in a haemocytometer count with Trypan Blue. The cellular concentration was adjusted to 2×10^5 viable cells per ml for clonogenic assay.

Clonogenic assay

Cells were cultured as described by Hamburger and Salmon [13]. Briefly, 1 ml under-layer, constituted by 0.5% agar in Iscove's modified Dulbecco's medium (IMDM) with 10% FCS, 200 U/ml penicillin and the compounds to be tested, was plated in 35 mm plastic Petri dishes.

The tumour cells processed as prior described, were suspended at a density of 2×10^5 viable cells per ml on 0.3% agar in IMDM supplemented as above and exposed continuously to tamoxifen (37, 371, 3710 μg/l) and cisplatin (10, 100, 500 μg/l). In the combination experiments tamoxifen was used at 1 μmol/l concentration. Cultures were incubated at 37°C in a 7.5% CO_2 humidified atmosphere. The colonies (aggregates of 30 or more cells) appeared 10–20 days after plating.

Evaluation of drug interaction

In synergy experiments dose–response curves were first generated for the single agents, the extent of the effect of the combination treatment was analysed by the isobole method [14] for a combination of drugs A and B by the equation:

$$A_c/A_e + B_c/B_e = D$$

where A_c and B_c correspond to concentrations of drugs used in the combination treatment, and A_e and B_e correspond to concentrations of drugs able to produce, alone, the same magnitude of effect. If D (combination index) < 1 the effect of the combination was synergistic, whereas if $D = 1$ or $D > 1$ the effects were additive and antagonistic, respectively. Drug protentiation was calculated as the amount of drug which when used alone would produce the same effect as when used in combination (A_e) divided by the amount of drug used in the combination (A_c). Each experiment was performed in triplicate to allow the calculation of the statistical significance (P) of the combination indices (D) compared to the additive combination index of $D = 1$ by the one-sided t-test.

Type II EBS analysis in tumour specimens

Fresh tissue specimens from primary tumours were finely minced and homogenised in 5 volumes of ice-cold buffer consisting of 10 mmol/l Tris, 1.5 mmol/l EDTA, 5 mmol/l NaN_3 (TEN) by applying three or four 10 s bursts of an Ultra-Turrax homogeniser with intermittent cooling. The crude homogenate was centrifuged at 105.000 g for 30 min at 0°C, and the resulting supernatant was used for type II EBS analysis. Briefly, 250 μl of cytosol were incubated at 30°C for 30 min with increasing concentrations of [^{3}H]E_2 (4–50 nmol/l) with or without a 300-fold molar excess of diethylstilbestrol (DES)(Sigma). Bound and free steroids were separated by the hydroxylapatite method [15]. Specific binding was calculated as the difference between binding in the absence (total binding) and in the presence (non-specific binding) of unlabelled DES. Protein concentration was determined by the method of Bradford [16].

Oestrogen and progesterone receptor analysis

ER and progesterone receptor (PR) were assayed according to EORTC protocols [17]. ER and PR status was defined using a cut-off value of 5 fmol/mg protein.

RESULTS

Table 1 shows the clinico-pathological characteristics and receptor status of the four ovarian tumours studied. All tumours were ER and PR negative but contained type II EBS with values ranging from 324 to 1446 fmol/mg protein and K_d values from 10 to 20 nmol/l.

Figure 1 shows the saturation curve and Scatchard analysis of type II EBS in an ovarian tumour specimen (case B of Table 1).

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Table 1. Patient characteristics and receptor status of ovarian tumours

Case	Histology	Figo stage	Grading	Type II EBS*	(K_d, nmol/l)†	ER	PR
A	Serous	IIIC	G2	864	14	2.5	3
B	Serous	IIIC	G2	324	10	3.7	0
C	Serous	IIIC	G3	761	15	0	0
D	Serous	IV	G2	1446	20	0	3.3

*fmol/mg of protein calculated from the saturation curve at maximum binding.
†Calculated as the $[^3H]E_2$ concentration required for half-saturation.

The curve of $[^3H]E_2$ binding to cytosolic sample was sigmoidal, with saturation occurring at a ligand concentration of about 45 nmol/l [Fig. 1(a)]. As predicted by the biphasic nature of the saturation curve, Scatchard analysis of the binding values yielded a concave plot (Fig. 1b). Since an accurate estimate of both the K_d and the no. of type II EBS cannot be made from a curvilinear Scatchard plot, reasonable estimates of these parameters have been obtained from the saturation curve. Thus for the experiment shown, at maximum binding, the amount of type II EBS was 324 fmol/mg of protein. The K_d value determined from the $[^3H]E_2$ concentration required for half saturation was 10 nmol/l (Fig. 1a). In the presence of 10 nmol/l dithiothreitol (DTT) the binding of $[^3H]E_2$ to type II EBS was reduced to approximately 30% of the control (without DTT) value (data not shown). This sensitivity to reducing agents is similar to that previously observed for type II EBS in rat uterus [18]. Among the steroids tested, only oestrogenic compounds competed for $[^3H]E_2$ binding to type II EBS in ovarian cancer (data not shown). Furthermore, in all cases tamoxifen competed with $[^3H]E_2$ for type II EBS (Fig. 2).

Figure 3 shows the inhibitory effects of tamoxifen and cisplatin alone or in combination on the clonogenic efficiency of cells obtained from primary ovarian tumours. Both drugs exhibited a dose-dependent inhibition of colony formation: cisplatin in a range of concentrations between 10 and 500 μg/l and tamoxifen between 37 and 3710 μg/l. When cisplatin was used in combination with tamoxifen (371 μg/l) there was a higher inhibitory effect on colony formation with respect to that observed at the corresponding dose of the drug used alone (Fig. 3). The combination index obtained by the isobole method showed a significant synergistic activity of cisplatin and tamoxifen in all cases with a cisplatin potentiation up to and beyond 50-fold (Table 2).

DISCUSSION

Our results show that the antiestrogen tamoxifen inhibits the clonogenic efficiency of cells from 4 ER negative primary ovarian tumours and that the treatment of these cells with combined tamoxifen and cisplatin results in a marked synergistic antiproliferative activity. The antiproliferative properties of tamoxifen on clonogenic cells of primary ovarian tumours is consistent with previous studies [11, 19]. Moreover, our results support the hypothesis that the action of tamoxifen on cell growth may be in some cases independent of ER expression.

The mechanism of this ER independent activity of tamoxifen remains to be clarified. One possibility is that tamoxifen action may be mediated by interaction with type II EBS. These sites are present in primary ovarian tumours and ovarian cancer cell lines, and binds to them with an affinity consistent with the concentration effective in synergising cisplatin activity. Moreover, we have also shown that quercetin interacts with type II EBS, inhibits colony formation and enhances cisplatin inhibitory activity on cell growth in cancer cells from primary ovarian tumours [5].

Although not mutually exclusive, two alternative hypotheses should also be taken into account.

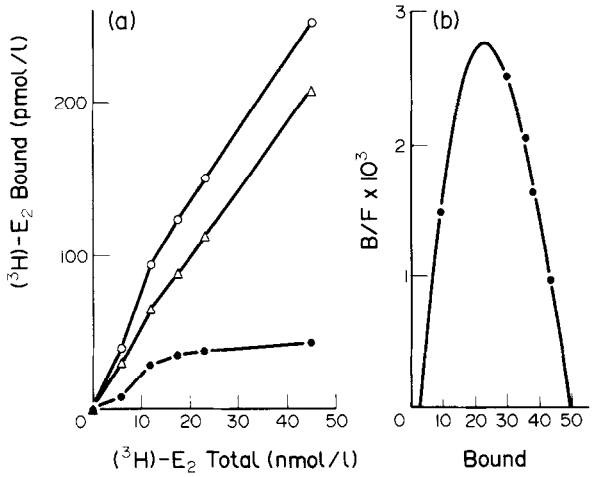

Fig. 1. (a) Specific binding of $[^3H]E_2$ as a function of tracer concentration (from 4 to 50 μmol/l) in a representative case of ovarian tumour (case B of Table 1). (○) total, (△) non-specific, (●) specific binding. Specifically bound $[^3H]E_2$ was measured as detailed in Materials and methods. (b) Scatchard analysis of data from (a).

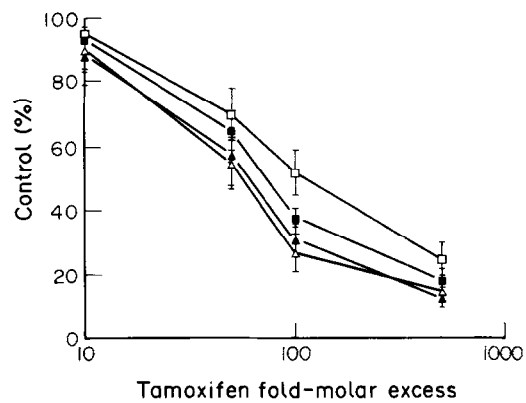

Fig. 2. Competition of tamoxifen for type II EBS in four ovarian tumours. Cases A–D are as in Tables 1 and 2. Tamoxifen was used at 10-, 50-, 100-, and 500-fold molar excess with respect to $[^3H]E_2$ (40 nmol/l). Results are expressed as the percentage of $[^3H]E_2$ bound in the absence (100%) or presence of competitors. Each value represents the mean (S.D.) of triplicate experiments performed in duplicate.

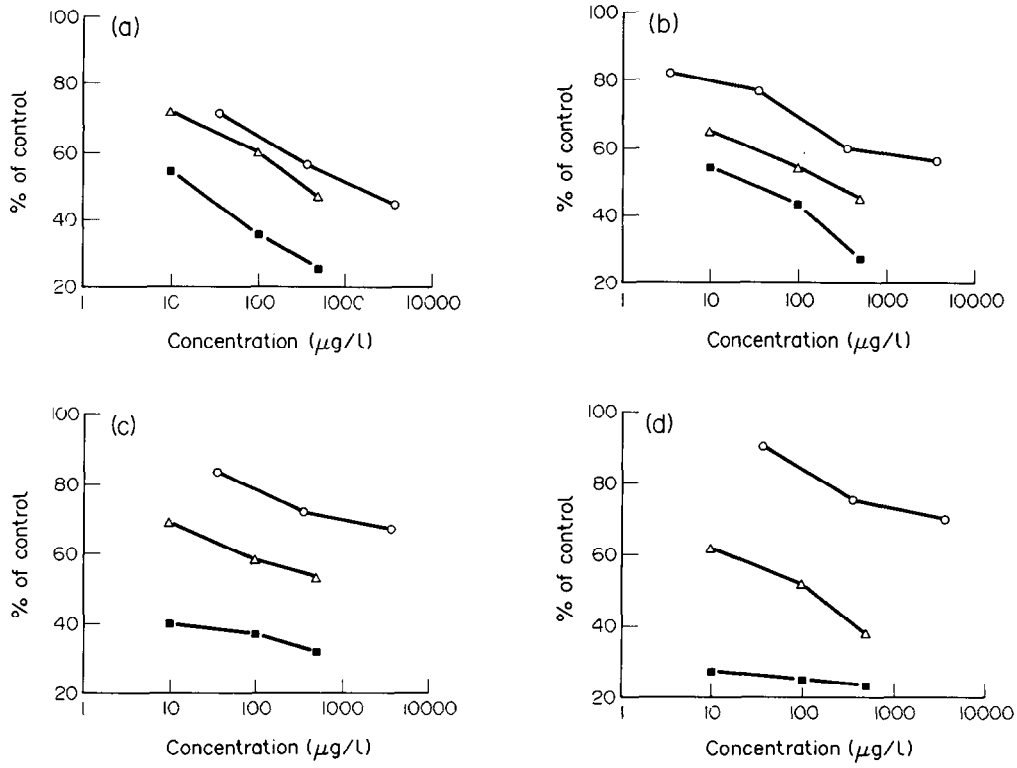

Fig. 3. Combined effect of tamoxifen (from 37 to 3710 μg/l) and cisplatin (from 10 to 500 μg/l) on clonogenic cells from four ovarian cancer specimens. Cases A–D are as in Tables 1 and 2. The symbols are tamoxifen (○), cisplatin (△), cisplatin + 371 μg/l tamoxifen (■). Cells were continuously exposed to tamoxifen and cisplatin as described in Materials and methods. Each point represents the mean of three separate plates. S.D.s were less than 10%.

First, it has been demonstrated, at least in the cisplatin resistant murine L1210 leukaemia cell lines [20], that the calcium channels from cisplatin resistant cell lines are larger and open for a longer period of time than cisplatin sensitive cell lines and that the activity of cisplatin is potentiated by calcium antagonists. Taking into account these observations one could hypothesise that tamoxifen may be acting in sinergy with cisplatin by virtue of its calcium channel-blocking properties [12]. Moreover, alters Ca^{2+} dependent processes by interacting directly with calmodulin [21], thus producing a calcium antagonistic activity similar to that demonstrated by the other calmodulin inhibitors.

Second, the action of tamoxifen could be due to an inhibition of protein kinase C activity [22]. However, we observed a

Table 2. Synergistic antiproliferative combination of cisplatin and tamoxifen in primary ovarian tumours

Case	Cisplatin (A_c) (μg/l)	Tamoxifen (B_c) μg/l	% of control growth	Cisplatin (A_e) μg/l	Tamoxifen (B_e) μg/l	Combination Index (D)	Cisplatin potentiation
A	10	371	54	260	500	0.78	26
	100	371	35	>500	>3710	<0.30*	>5
	500	371	25	>500	>3710	<1.1	>1
B	10	371	54	250	>3710	0.14*	25
	100	371	43	600	>3710	0.26*	6
	500	371	27	>500	>3710	<1.1	>1
C	10	371	40	>500	>3710	<0.13*	>50
	100	371	37	>500	>3710	<0.30*	>5
	500	371	32	>500	>3710	<1.1	>1
D	10	371	27	>500	>3710	<0.13*	>50
	100	371	25	>500	>3710	<0.30*	>5
	500	371	23	>500	>3710	<1.1	>1

Case numbers are as in Table 1 and Fig. 3.
*P <0.01, by a two-tailed one-sided t-test.

synergising activity at a concentration of 371 μg/l that is at least 1 log lower than that minimally inhibitory on protein kinase C.

From a clinical point of view the association of tamoxifen and cisplatin could be useful. Since no significant toxicity has been demonstrated for tamoxifen it could be employed with standard cytotoxic chemotherapy without reduction of the dose of the latter agents. The addition of tamoxifen produced a potentiation of cisplatin activity up to 50 times. This tamoxifen-induced increase of cisplatin sensitivity could be clinically relevant since clinical and experimental evidence [23–28] indicates that the level of resistance to cisplatin *in vivo* is from 2- to 4-fold, which is sufficient for treatment failure.

At present encouraging results have been reported in human melanoma with a multi-agent regimen including cisplatin and tamoxifen [12]. In ovarian cancer the combination of tamoxifen and cisplatin has been used in only one prospective randomised clinical trial. Schwartz *et al.* [29] compared two regimens (cisplatin and doxorubicin vs. cisplatin, doxorubicin and tamoxifen) as first-line chemotherapy in patients with stage III–IV disease and showed that the addition of tamoxifen did not improve overall and progression-free survival. However, in this study tamoxifen was administered at a dose of 20 mg/day. With this schedule tamoxifen blood steady-state levels are reached only after 16 weeks of administration and blood levels of tamoxifen are below 1 μmol/l (371 μg/l) [30].

On the basis of our *in vitro* data it is conceivable that tamoxifen should be administered at doses higher than those conventionally used in order to achieve the cisplatin synergising effect. Prospective clinical trials are now needed to verify whether the addition of tamoxifen to cisplatin-containing regimens will result in an improved clinical response in ovarian cancer.

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