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Doxorubicin Weekly Low Dose Administration: *in vitro* Cytotoxicity Generated by the Typical Pharmacokinetic Profile

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The cytotoxic effects of prolonged exposure to low concentrations of doxorubicin or a doxorubicin bolus were examined *in vitro* on four human breast cancer cell lines to simulate the plasma concentration profile of weekly low-dose (WLD) doxorubicin in breast cancer patients. Cells were exposed to doxorubicin for various prolonged times (24, 72, 120 and 192 h) and with different drug concentrations (5, 10, 20, 50 and 80 nmol/l). In a series of parallel experiments, cell lines were placed in contact with the drug for short periods (1 h) before prolonged exposure to doxorubicin; the concentrations of these pulses were 150, 250 and 350 nmol/l. A constant decrease in tritiated thymidine incorporation was noted as a function of the drug concentration and the duration of the cell contact with the drug. Interestingly the lowest concentrations (5–10 nmol/l) produced marked cytotoxic effects. For equivalent concentration × time values, experiments including doxorubicin pulses resulted in greater cytotoxicity than continuous exposure alone, in a dose-related manner. This finding was related to differences in intracellular doxorubicin concentrations. Results suggest that the rather empirically designed WLD doxorubicin schedule can generate greater cytotoxic effects than continuous doxorubicin administration alone.
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INTRODUCTION

DOXORUBICIN IS one of the major drugs used in cancer chemotherapy. Despite its impressive and broad spectrum of antineoplastic activity, use of this drug is hampered by several limiting toxic effects. Acute toxic effects such as myelosuppression and mucositis are manageable for the most part. However, cumulative doses of doxorubicin can result in severe cardiomyopathy, limiting the total dose that may be administered [1]. Attempts to reduce doxorubicin induced side effects include the use of low toxicity analogues [2], administration of haematopoietic growth factors [3], and modification of the administration schedule [4, 5]. Weekly low dose (WLD) doxorubicin has been suggested as a means of reducing the maximum initial plasma concentration, which is assumed to be directly related to the severity of myocardial injury [6]. This hypothesis was clinically confirmed by Torti *et al.* [7], in endomyocardial biopsy specimens; for these authors, doxorubicin administered on a weekly schedule was associated with less cardiac damage than doxorubicin delivered by the conventional 3-week schedule. In 8/24 patients we treated by WLD doxorubicin, the total cumulative doxorubicin dose was over 900 mg/m^2; none had any clinically evident cardiac toxicity [8]. A recent randomised study concluded that WLD doxorubicin is as effective for advanced breast cancer as a combination of vincristine, doxorubicin and cyclophosphamide administered every 3rd week and is considerably less toxic [9]. In a recent pharmacokinetic study of WLD doxorubicin in breast cancer patients [10], we found that the initial peak doxorubicin value (CO_{ext}) dropped in proportion to the dose reduction compared with a control group. Interestingly, approximately 50% of the residual drug levels on day 7 of successive weekly injections were between 1 and 20 nmol/l. WLD doxorubicin can thus be assimilated to continuous exposure to low drug levels with intermittent pulses; this represents an original pharmacological profile for doxorubicin. The present study analysed the cytotoxic effect of prolonged exposure of human breast cancer cell lines to low doxorubicin concentrations with a doxorubicin bolus in order to evaluate the cytotoxic effects generated by this original pharmacokinetic profile.

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MATERIALS AND METHODS

Chemicals

Doxorubicin was obtained from Roger Bellon (Neuilly/Seine) as a pure powder. Drug solutions were prepared before use. Dulbecco's modified Eagles medium (DMEM), medium 199, phosphate buffered saline (PBS), L-glutamine, trypsin EDTA, and fetal bovine serum (FBS) were from Gibco. Penicillin and streptomycin were from Merieux (Lyons). [^{3}H] thymidine (16.7 GBq/mmol) was from the CEA (Paris). The scintillation liquid, Dynagel, was from Baker.

Cell cultures

Four cell lines derived from breast cancers were used. Cell lines MCF 7-SR 7, ZR 75-1, and T 47 D were kindly provided by Dr Rochefort (INSERM Montpellier); CAL 18 B was isolated at our institute [11]. Cells were routinely cultured in a humidified incubator (Sanyo) at 37°C with an atmosphere containing 8% CO_2 in air. Cells were grown in DMEM supplemented with 10% FBS, penicillin (50 000 IU/l), streptomycin (86 μmol/l), and L-glutamine (2 mmol/l). Cells were grown in 24-well plates

for thymidine incorporation studies, and in 25 cm^2 culture flasks for drug incorporation studies. Cells were exposed to doxorubicin for various times (24, 72, 120 and 192 h) and at different low drug concentrations (5, 10, 20, 50 and 80 nmol/l). Parallel experiments were conducted in which short high concentration doxorubicin exposures (1 h) were used before prolonged exposure to low doxorubicin concentrations. This was done in order to simulate the specific pharmacokinetic profile generated by WLD doxorubicin [10]. We preliminary tested a range of doxorubicin pulses matching those found in WLD doxorubicin treated patients (100–2000 nmol/l). Taking into account the proper sensitivity of each tumour cell line which was evaluated during the preliminary step this led to finally test 150, 250 and 350 nmol/l as doxorubicin pulses.

Evaluation of cytotoxicity

Cytotoxic effects were assessed 8 days after the first drug exposure. The assay method was essentially that described by Volm *et al.* [12, 13]. Cells were washed with medium 199 (500 μl/well) and incubated in the same medium (500 μl/well) supplemented with 5% FBS. 50 μl [^{3}H]thymidine was added (final concentration 11.1 KBq/ml) and incubation was pursued overnight (16 h). Plates were then cooled on ice, washed twice with cold PBS; 500 μl/well trichloroacetic acid (TCA) (150 mg/ml) was added and left 1 h at 0°C. TCA was then removed, and cells were washed sequentially, first with 500 μl TCA (150 mg/ml), then twice with 500 μl cold PBS. Cells were then hydrolysed for 30 min at 37°C with 300 μl NaOH 1 mol/l. Well contents were then neutralised with 50 μl HCl 6 N, rinsed with 150 μl H_2O, and transferred to a scintillation counting vial. After adding 3 ml of scintillation liquid, the radioactivity was counted on a Packard Tricarb liquid scintillation counter. Results (mean of quadruplicate experiments) were expressed as the relative percentage of radioactivity incorporation compared with controls without the drug.

Measurement of intracellular doxorubicin concentration

Cells (1.5–6 × 10^6) were washed twice with 5 ml PBS, and trypsinised for 5 min at 37°C. This was done at the same time that 24-well plates were starting [^{3}H]thymidine incorporation. After stopping trypsinisation with 1 ml DMEM plus 10% FBS, cells were counted and centrifuged for 5 min at 1800 rpm. The cell pellet was stored at −20°C until assayed. At the time of doxorubicin measurement, to the cell pellet was added one volume of 0.2 mol/l borate buffer pH 9.8 spiked with 25 μl of internal standard (daunorubicin, 1 nmol/ml). After vigorous agitation (2 times, 30 sec) the mixture was treated by 18 volumes of chloroform–methanol (4/1, v/v) under vigorous agitation (45 s). The organic phase was evaporated under a stream of nitrogen at 30°C. The residue was diluted in 250 μl of high performance liquid chromatography (HPLC) buffer, centrifuged for 10 min at 4°C, and injected into the HPLC system. Analysis was performed on an HPLC column filled with μ Bondapak phenyl 30 × 0.4 (Millipore-Waters) with a CH_3CN formiate (33.5/66.5) buffer, pH 4, at a flow rate of 2.5 ml/min. Fluorescence detection was performed with a spectrofluorimeter (Kontron SFM 25) at λ ex = 470 nm and λ em = 600 nm. Results were expressed as molecules of doxorubicin per cell.

RESULTS

Figure 1 shows the dose–response curves for all four cell lines. Although the intrinsic sensitivities of cells to doxorubicin appear to be different, the doxorubicin concentration (C) and the

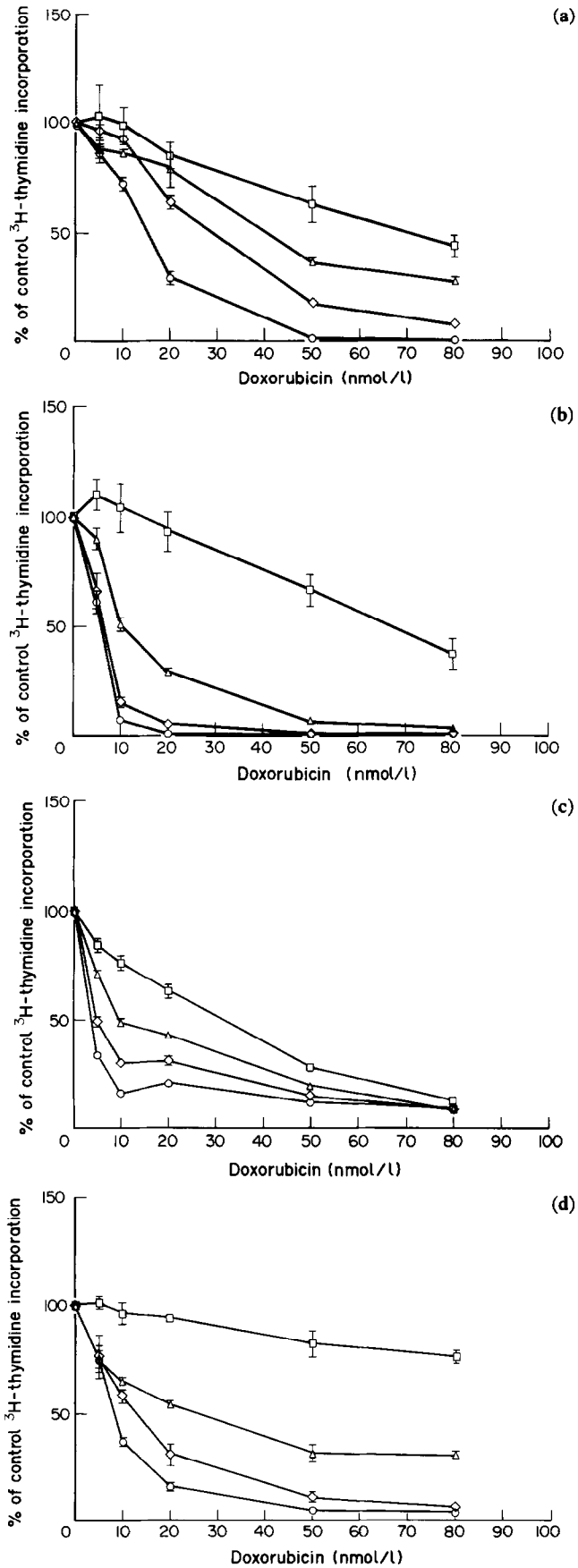

Fig. 1. Dose–response curve of breast cancer cell lines to doxorubicin. (a) = MCF 7-SR cells; (b) = ZR 75-1 cells; (c) = T 47 D cells; and (d) = CAL 18 B cells. Duration of drug exposure: squares, 24 h; triangles, 72 h; diamonds, 120 h and circles, 192 h.

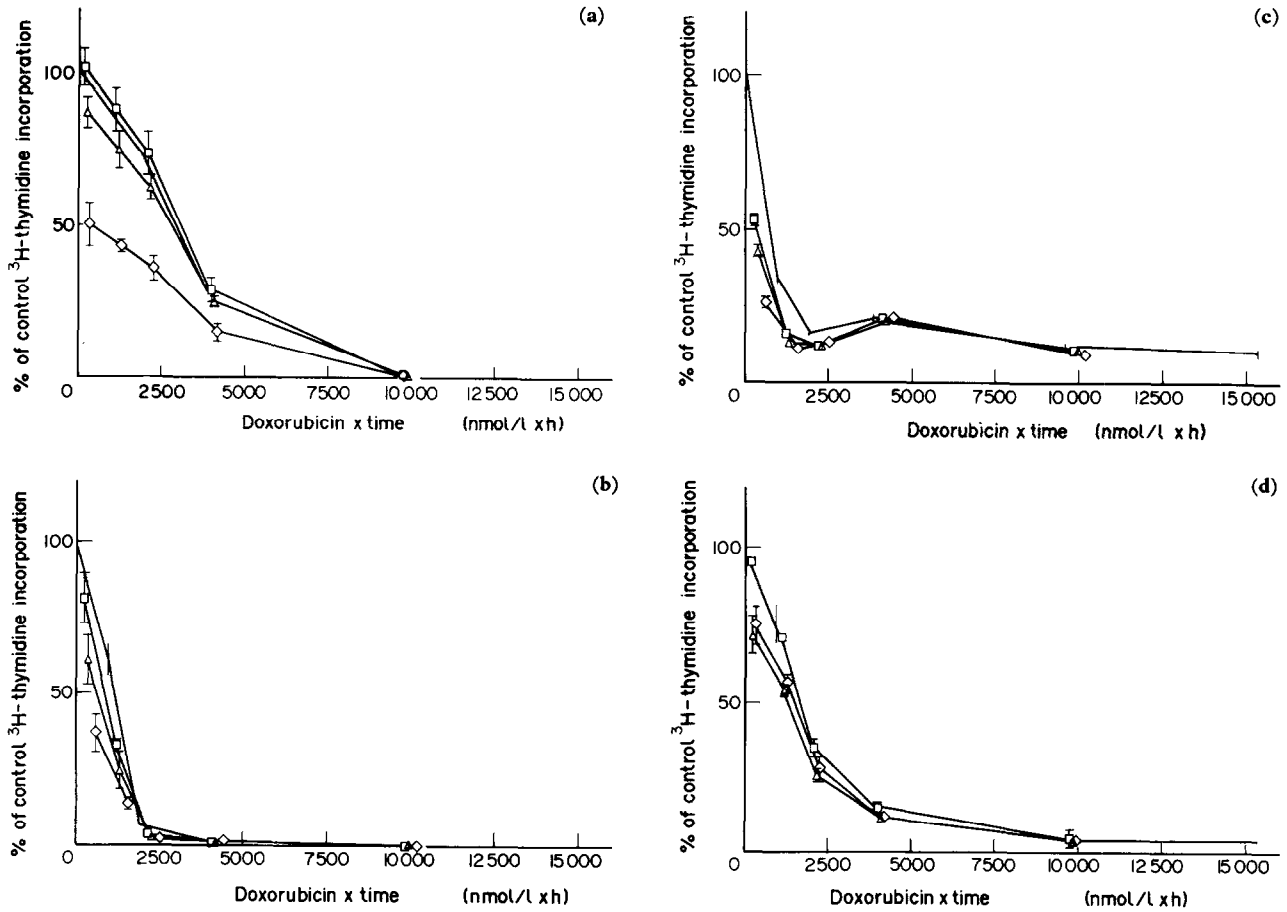

Fig. 2. Percentage of cell survival as a function of the parameter $C \times T$. (a) = MCF 7-SR cells; (b) = ZR 75-1 cells; (c) = T 47 D cells; and (d) = CAL 18 B cells. The duration of drug exposure was 192 h. No symbol = continuous exposure only (5, 10, 20, 50 and 80 nmol/l for 192 h); squares = pulse (150 nmol/l, 1 h) plus continuous exposure (5, 10, 20, 50 and 80 nmol/l for 192 h); triangles = pulse (250 nmol/l, 1 h) plus continuous exposure (5, 10, 20, 50 and 80 nmol/l for 192 h); diamonds = pulse (350 nmol/l, 1 h) plus continuous exposure (5, 10, 20, 50 and 80 nmol/l for 192 h).

duration (T) of drug exposure had a similar effect for cell lines: a constant decrease in tritiated thymidine incorporation was noted as a function of the drug concentration and the duration of the cell contact with the drug. Interestingly, the lowest doxorubicin concentrations (5–10 nmol/l) were able to generate marked cytotoxic effects; this was particularly evident for MCF 7 cells and ZR 75 cells.

The cytotoxic effects of doxorubicin were related to the increase in the concentration × time value (Fig. 2). This was true for all four cell lines. For equivalent concentration × time values, experiments in which doxorubicin pulses were used generated greater cytotoxicity than continuous exposure alone, in a dose-related manner.

Figure 3 illustrates the evolution of the intracellular doxorubicin concentration as a function of the extracellular concentration × time value for CAL 18B cells. The increase in cellular drug levels paralleled the elevation of concentration × time. However, for a given concentration × time value, the intracellular doxorubicin content varied depending on the drug exposure schedule: the highest cellular drug concentrations were obtained with a combination of short term plus continuous drug exposure.

Figure 4 shows the relationship between the intracellular drug concentration and cytotoxicity for CAL 18B cells. The increase in cytotoxic effects paralleled the elevation in cellular doxorub-

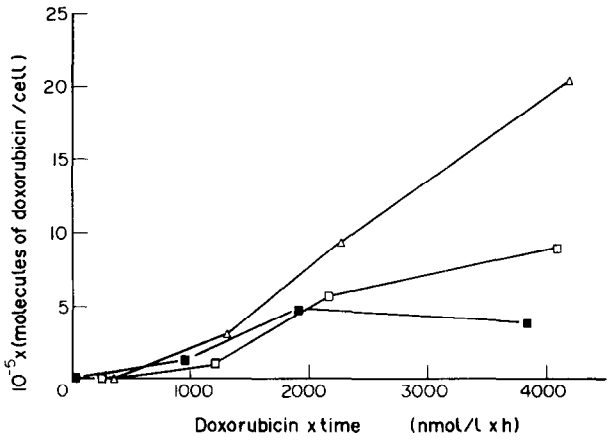

Fig. 3. Intracellular doxorubicin concentration as a function of the parameter $C \times T$ for CAL 18 B cells. The duration of drug exposure was 192 h. Solid squares = continuous exposure only; open squares = pulse (250 nmol/l, 1 h) plus continuous exposure; open triangles = pulse (350 nmol/l, 1 h) plus continuous exposure.

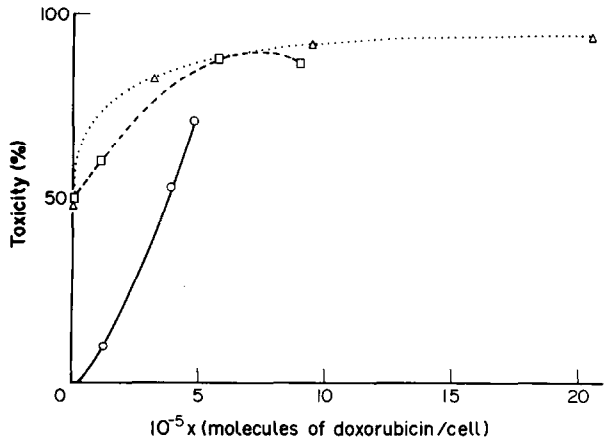

Fig. 4. Percentage of cytotoxicity as a function of intracellular doxorubicin concentration for CAL 18 B cells. Circles = continuous exposure only; squares = pulse (250 nmol/l, 1 h) plus continuous exposure; triangles = pulse (350 nmol/l, 1 h) plus continuous exposure.

icin levels. Interestingly, for equivalent cellular drug concentrations, cytotoxic damage differed with the drug exposure schedule. Associations of a short pulse with continuous exposure generated the highest cytotoxicity for a given cellular drug level.

DISCUSSION

One approach for improving the therapeutic index of doxorubicin consists in modifying the classical 3-week bolus injection schedule. Both weekly schedules and infusions lasting 24 h or longer have been proposed [5]; these protocols offer reduced cardiac toxicity without impairing clinical response compared with classical bolus administration [7, 14]. Because WLD doxorubicin also allows patients to be treated on an outpatient basis, it is a potential means for substantially lowering treatment costs. Like other authors [5, 9], we successfully used WLD doxorubicin for the treatment of advanced breast cancer patients [8]. Interestingly, new responses were observed in patients being resistant to the 3-week bolus injection schedule. We then initiated pharmacological investigations to improve our understanding of this treatment schedule. Pharmacological studies covering both clinical pharmacokinetics and cellular toxicology have previously been published for continuous administration of doxorubicin [15–18]. In an initial study, we investigated the pharmacokinetics of WLD doxorubicin [10]. In this study, the initial peak doxorubicin value was reduced in proportion to the dose reduction; approximately 50% of the drug levels on day 7 of successive weekly injections were between 1 and 20 nmol/l. WLD doxorubicin was thus assimilated to continuous treatment characterised by low baseline drug concentrations associated with intermittent pulses. In the present study, which is the second part of our pharmacological investigations on this treatment schedule, the characteristic profile of WLD doxorubicin was evaluated in terms of toxicity and cellular drug concentrations. Maintenance of doxorubicin concentrations as low as 5 nmol/l for several days resulted in significant cytotoxicity for most of the cell lines investigated. Low residual drug concentrations in the blood of patients treated by weekly low-dose doxorubicin may thus suffice to induce an antitumour effect. As well this could select for drug resistant tumour cells, but our clinical experience has shown tumour responses with WLD doxorubicin in patients refractory to previous 3-week schedule [8].

Confirming the data reported previously by Eichholtz-Wirth [19], we found that the cytotoxic effects of doxorubicin were linked to an increase in the product of the extracellular drug concentration (C) and exposure time (T). Careful analysis revealed that, for equivalent $C \times T$, the cytotoxicity generated differed for continuous exposure and association of short term plus continuous exposure. The latter schedule was the most active. Data concerning intracellular dox concentrations corroborate and may explain this observation. Cellular drug levels were linked to the elevation in $C \times T$ values, but at equivalent $C \times T$ values doxorubicin concentrations were higher when cells had been pretreated by short term contact plus prolonged drug exposure than after prolonged exposure only. Increases in cellular doxorubicin concentrations were correlated with increases in cytotoxic effects. These data corroborate the recent report by Luk and Tannok [20]; using seven cell lines of rodent and human origin, these authors found that cell killing was correlated with the intracellular drug concentration, with an r value of 0.88. In the present study, for equivalent cellular drug levels, the greatest cytotoxic effects were observed for associations of a pulse plus continuous contact with the drug. One explanation for these last observations is that the cellular targets reached by the drug may depend on the external doxorubicin concentration and conditions of cell exposure to the drug. At low extracellular concentrations, doxorubicin may remain mainly within the cellular membrane, whereas at higher concentrations the drug may penetrate the intracellular space and reach its main DNA targets. For an equivalent global cellular concentration, the effect would thus depend on the cellular doxorubicin localisation. This hypothesis concurs with the findings of Tritton and Yee [21], who demonstrated that doxorubicin can exert its cytotoxic action solely by interaction at the cell surface. More precisely, this anthracyclin was found to penetrate deep into the phospholipid bilayer and bind at an angle of 55°, severely disrupting the ordered arrangement of the fatty acyl chains [22]. In addition, it has been recently shown that doxorubicin when incubated with human erythrocyte membrane vesicles produced an elevation of inositol-1,4, 5-triphosphate levels [23]. However, we agree with Cummings [24] in not considering the cell membrane as the primary locus of action of doxorubicin, other well studied interactions with DNA and topoisomerase II being more probably responsible for the clinical activity of the drug. Overall, the experimental conditions in this study, which reproduce blood drug behaviour during WLD doxorubicin, indicate that this rather empirically constructed schedule can produce interesting cytotoxicity which is greater than that achieved with continuous drug administration alone. As WLD doxorubicin results in an objective reduction in cardiotoxicity compared to single bolus doses, further controlled clinical studies appear justified.

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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-