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Original Paper

Tamoxifen Inhibits Particulate-associated Protein Kinase C Activity, and Sensitises Cultured Human Glioblastoma Cells not to Etoposide but to γ-Radiation and BCNU

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We investigated the potential mechanisms of tamoxifen cytotoxicity in the U-373, U-138, and U-87 human glioblastoma cell lines, namely interference with protein kinase C (PKC) activity, the oestrogen receptor, and/or the production of transforming growth factor β1 (TGF-β1). We further examined the effects of tamoxifen on the cytotoxicity exerted by γ-radiation, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), and etoposide in this cell line panel. Thus, the cells were treated for 4 days with tamoxifen, γ-radiation, purified recombinant human TGF-β1 (rhTGF-β1), BCNU, or etoposide, either alone or at certain combinations. Cellular responses were evaluated with the sulphorhodamine B assay, as well as by multiple drug effect analysis, and related to PKC activities in particulate and cellular fractions; cellular oestrogen receptor contents; and the influence of rhTGF-β1 on cell growth. Tamoxifen inhibited cell proliferation as well as the phosphorylation capacity of the particulate, but not of the cytosolic fractions dose-dependently, at comparable kinetics, and at IC_{50} values of approximately 15 μM. At these concentrations, tamoxifen acted synergistically with γ-radiation (4- to 6-fold) and additively with BCNU (approximately 2-fold), but did not affect etoposide cytotoxicity. The cells were negative to immunostaining for the oestrogen receptor, and rhRGF-β1 did not influence their growth up to 100 nm. Our data suggest that tamoxifen can sensitise cultured glioblastoma cells not to etoposide but to γ-radiation and BCNU, possibly through interference with membrane PKC, supporting its evaluation in experimental protocols for primary malignant gliomas. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: glioblastoma, cell culture, tamoxifen, protein kinase C, γ-radiation, BCNU, etoposide, drug interaction
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INTRODUCTION

GLIOBLASTOMAS ARE the most frequent primary tumours of the central nervous system (CNS), comprising approximately 50% of cerebral gliomas [1, 2]. Despite advances in surgical techniques and the development of new protocols in radiation therapy and chemotherapy, the prognosis for patients suffering from these malignancies remains poor [1, 2]. Thus, more effective therapeutic strategies are needed in these tumour types.

Converging lines of evidence suggest a critical role for alterations in mitogenic signalling and apoptotic cascades in the development and progression of primary malignant brain tumours [3, 4]. One of the most widely studied elements of these pathways is the protein kinase C (PKC) system. In normal cells, PKCs play a key role in the regulation of cell cycle progression by relaying growth factor-mediated mitogenic signals from the plasma membrane to the nucleus

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[3, 4]. In addition, PKCs have been implicated in the protection of cells from apoptosis, amongst others by inhibiting the production of ceramide—a second messenger in various apoptotic subprograms—from sphingomyelin hydrolysis [5].

In contrast to non-transformed glia, PKC activity is significantly upregulated in many malignant gliomas [6–8]. For instance, in established human glioblastoma cell lines higher PKC activity correlated with more rapid growth [6–8]. These findings suggest the involvement of an aberrant PKC system in the hyperproliferative state of these tumours, as well as in their resistance to undergo apoptosis in response to treatment with cytotoxic agents and/or ionising radiation. Thus, pharmacological manipulation of PKC activity may restrain tumour cell proliferation and/or restore susceptibility to apoptosis, representing a promising approach in the treatment of primary brain malignancies.

To date, several PKC-inhibiting agents have been evaluated in CNS tumours [3]. Among these is the triphenylethylene tamoxifen, an anti-oestrogen with low toxicity that is widely used for the treatment of oestrogen-dependent breast carcinoma [9]. In these cases of breast cancer, tamoxifen probably acts by competitively inhibiting oestrogen receptor-mediated signalling for cell proliferation [9]. Of interest, a mechanism involving anti-oestrogen action has been also suggested for tamoxifen cytotoxicity in certain meningiomas [10, 11]. Besides its anti-oestrogen activity, tamoxifen can indirectly inhibit cell growth by stimulating the autocrine production of negative growth factors from the transforming growth factor β (TGF-β) family [12]. Such a mechanism may mediate tamoxifen cytotoxicity in oestrogen receptor-negative breast cancer cells and malignant melanoma cells [12].

At high doses, orally administered tamoxifen can be of benefit in some patients with malignant glioma [13–15]. Since tamoxifen has been found to inhibit PKC activity and the proliferation of cultured glioma cells at comparable kinetics [16, 17], it is possible that clinical responses to tamoxifen are related to interference with this enzyme activity. However, a possible contribution of the above-mentioned, non-PKC-related mechanisms to the apparent antiglioma activity of tamoxifen is uncertain.

In the present study, we evaluated the roles of PKC inhibition as well as non-PKC-related mechanisms in tamoxifen cytotoxicity in a panel of human glioblastoma cell lines. In addition, the apparent efficacy of tamoxifen in gliomas suggests a rationale for its combination with radiation therapy and/or active chemotherapeutic drugs in malignant gliomas [1, 2]. Therefore, we also assessed tamoxifen for its cytotoxicity together with γ-radiation, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), or etoposide in this glioma model.

MATERIALS AND METHODS

Drugs and chemicals

Tamoxifen was obtained from Sigma (St Louis, Missouri, U.S.A.). BCNU and etoposide were obtained from Bristol-Myers Squibb (São Paulo, SP, Brazil). Purified recombinant human TGF-β1 (rhTGF-β1) was obtained from Gibco BRL-Life Technologies (Gaithersburg, Maryland, U.S.A.). The drug preparations were first diluted with, or dissolved in cell culture-grade dimethylsulphoxide (DMSO; Sigma), or HPLC-grade absolute ethanol, and subsequently further diluted with cell culture medium, achieving final concentrations of DMSO or ethanol of at the most 0.1% (v/v). rhTGF-β1 was dissolved in 50 mM sodium acetate (pH 4.5) containing 1% (w/v) bovine serum albumin before diluting with cell culture medium. Except where indicated, all other chemicals used were from our laboratory stock, and were of the highest grade available.

Cell lines and cell line maintenance

The U-373 MG and U-138 MG human glioblastoma cell lines, and the U-87 MG human glioblastoma-astrocytoma cell line, were from the American Type Culture Collection (Rockville, Maryland, U.S.A.). The cell lines were maintained in Eagle's Minimum Essential Medium containing 2% (w/v) L-glutamine, and supplemented with 15% (v/v) fetal calf serum (Cultilab, Campinas, SP, Brazil), in 25-cm^2 flasks, at a temperature of 37°C, a minimum relative humidity of 95%, and an atmosphere of 5% CO_2 in air.

For experiments, exponentially growing cells were detached from the culture flasks either using ethylenediamminetetraacetic acid (EDTA)–trypsin, or by scraping with a rubber policeman. Cell viability greater than 95% was confirmed by trypan blue exclusion.

Drug exposure and irradiation

The effects of the drug treatments and/or γ-irradiation on cell proliferation were examined using triplicate cultures which were inoculated onto 96-well microplates at densities of 2×10^3 to 5×10^3 cells per 100 μl medium per well, depending on the cell line. The cultures were allowed to stabilise for 24 h before being submitted to the various treatments.

For the chemosensitivity studies, cell cultures were exposed to serial dilutions of tamoxifen, rhTGF-β1, BCNU, or etoposide, either alone, or at certain combinations. The experiments with rhTGF-β1 were also performed with serum-free medium. Incubations were carried out for a total of 4 days, in final volumes of 200 μl per well.

For the irradiation studies, untreated cells, or cells that were exposed for 24 h to tamoxifen, were γ-irradiated in the presence of the drug at various single doses (dose rate 1.14 Gy per minute), using a Telecobalt Theretron Phoenix SR 7510 linear accelerator (Philips, Eindhoven, The Netherlands), at a source-to-target distance of 70 cm. The extent of cell growth inhibition achieved was evaluated after culturing for 3 additional days in the presence of tamoxifen in final volumes of 200 μl.

Cellular responses

Cellular responses following the above-mentioned treatments were assessed with the sulphorhodamine B (SRB) assay [18]. Briefly, the cell cultures were fixed *in situ* with 10% (w/v) trichloroacetic acid, and stained with SRB solution (0.4% (w/v) in 1% (v/v) acetic acid). Unbound SRB was removed with 1% (v/v) acetic acid, and cell-bound SRB was solubilised with 10 mM Trizma base pH 10.5.

Absorbances at a wavelength of 515 nm were measured and plotted against drug concentrations or radiation doses after correction for background absorption. The latter was determined from control wells which had received either medium alone or drug-containing medium, but no cells. Dose–response profiles were constructed, from which IC_{50} values or D_0 values were derived, i.e. drug concentrations resulting in 50% inhibition of cell growth when compared with untreated controls, or radiation doses yielding 37% survival with respect to untreated controls, respectively.

Multiple drug effect analysis

Dose–response interactions between drugs, and between drugs and radiation, were assessed with the aid of a computer software for multiple drug effect analysis developed by Chou and Talahay [19]; Elsevier-Biosoft, Cambridge, U.K. Data were expressed as combination indices (CIs), which were calculated by the formula:

$$CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2$$

where $(D_x)_1$ and $(D_x)_2$ are the concentrations of tamoxifen alone or BCNU alone, or the doses of radiation alone, giving x% growth inhibition, and $(D)_1$ and $(D)_2$ the drug concentrations and radiation doses in combination inhibiting cell growth also x%. $(D_x)_1$ and $(D_x)_2$ were calculated by the median-effect equation described by Chou [20]:

$$D_x = D_m[FA/(1 - FA)]^{1/m}$$

where D_m is the median-effect dose (or IC_{50}), FA is the fraction affected (reflected by the degree of cell growth inhibition), and m the slope of the median-effect plot.

Data were evaluated by calculation of the means of the CIs at FAs 0.50, 0.75, 0.90, and 0.95, according to the latest update of the program. CI < 1, CI = 1, and CI > 1 indicates synergism, additivity, or antagonism, respectively.

PKC activity

Cellular PKC activity was assessed as described [6], using the incorporation of [γ-^{32}P]ATP into lysine-rich Type IIIS histone as a measure of PKC phosphorylation. Thus, untreated or 4 day tamoxifen-treated samples of 2×10^7–5×10^7 cells were sonicated in the presence of 2 ml of homogenisation buffer (50 mM Tris–HCl (pH 7.4), 2 mM dithiotreitol, 2 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), 5 mM $MgCl_2$, and 1 mM phenylmethylsulphonyl fluoride). The homogenates were centrifuged for 60 min at 100,000 ***g***, and the supernatants were used as the cytosolic cellular fractions. The pellets were stirred for 30 min in homogenisation buffer containing 1.0% (v/v) Triton X-100 and again ultracentrifuged, yielding the particulate cellular fractions.

PKC activities were assessed in 4-μl enzyme preparations with a reaction mixture consisting of 50 mM Tris–HCl (pH 7.4), 250 μg/ml of lysine-rich Type IIIS histone (Sigma), 100 mM $CaCl_2$, 500 μg/ml of phosphatidylserine, and 25 μg/ml of diacylglycerol, in final volumes of 40 μl. After 2 min pre-incubation at 30°C, the phosphorylation reaction was initiated by adding 10 mM $MgCl_2$ and 10 μM ATP mixed with [γ-^{32}P]ATP (specific activity 1000 cpm/pmol; Amersham, Bucks, U.K.; 0.5×10^6 to 1×10^6 cpm).

The reactions were terminated after 1 min by placing a 25 μl aliquot of each mixture onto a Whatman P81 phosphocellulose filter (Gibco BRL-Life Technologies), which was then transferred to 75 mM phosphoric acid. The filters were dehydrated with absolute methanol, and the amounts of radioactivity bound were determined by liquid scintillation counting.

Negative blanks consisted of the reaction mixture minus Ca^{2+}, phosphatidylserine, and diacylglycerol, but in the presence of EDTA and EGTA. The PKC-dependent reactions were calculated as the difference between histone phosphorylation in the presence and absence of phosphatidylserine and diacylglycerol. Results were expressed as pmoles ATP transferred per minute per mg of protein. Protein contents were determined by the method of Lowry and colleagues [21].

Immunohistochemistry of the oestrogen receptor

Oestrogen receptor expression was assessed in glioblastoma cells that were plated in phenol red-free RPMI 1640 medium containing 2% (w/v) L-glutamine (Sigma) and supplemented with 15% (v/v) fetal calf serum (Gibco BRL-Life Technologies) which was stripped of steroids by treatment with dextran-coated charcoal as previously described [22]. Approximately 1.5×10^7 cells were fixed for 1 h with absolute ethanol, 40% formol, and glacial acetic acid (85:10:5, v/v/v), snap-frozen, and embedded in paraffin. Sections of a thickness of 4 μm were cut, which were mounted on slides.

After clearing in xylene, hydration through graded alcohols to PBS, and microwaving in nitric acid pH 6.0, the sections were overnight incubated at 4°C with the prediluted 1D5 mouse anti-human monoclonal antibody (DAKO Carpinteira, California, U.S.A.). Rabbit anti-mouse IgG, diluted 1:20, was used as secondary antibody. For detection, the DAKO kit streptABComplex/HRP mouse/rabbit, diluted 1:800, and diaminobenzidine (Sigma) were used. The streptABComplex/HRP mouse/rabbit is streptavidin complexed with biotinylated peroxidase and with a biotinylated goat antibody reacting with mouse and rabbit immunoglobulins. Normal mouse IgG1 was used as a control primary antibody. Identically processed and treated malignant cells collected from the pleural effusion of a patient with histopathologically confirmed oestrogen receptor-positive breast cancer served as positive control. The slides were counterstained with haematoxylin, examined under a light microscope, and immunoreactivity was determined from staining intensity.

Statistics

All experiments were carried out at least three times in triplicate or quadruplicate, and means ± S.D.s are presented. Student's *t*-test for paired samples was used to compare the results between the untreated and the different treatment groups. P values < 0.05 were taken to indicate statistical significance.

RESULTS

Tamoxifen cytotoxicity

The cytotoxicity of tamoxifen was examined in the human glioblastoma cell lines U-373, U-138, and U-87. To this end, the cells were exposed to serial dilutions of tamoxifen, and after 4 days assessed for growth inhibition using the SRB assay [18]. As shown in Table 1, tamoxifen inhibited the growth of all three cell lines at IC_{50} concentrations of approximately 15 μM.

Effect of tamoxifen on PKC activity

In order to evaluate the effect of tamoxifen on PKC activity, we assessed the cytosolic and particulate fractions of cells that were treated with tamoxifen as mentioned above, for their capacity to phosphorylate lysine-rich histone [6]. The data obtained were compared with those found with untreated cells.

As shown in Figure 1, phosphorylation by the cytosolic fractions was not significantly affected by treatment with tamoxifen at concentrations up to 35 μM. In contrast, phosphorylation by the particulate fractions decreased in the three

Table 1. *IC_{50} values (μM; means ± S.D.s; n ≥ 3) of tamoxifen; BCNU in the absence or presence of tamoxifen* 15 *μM; and etoposide in the absence or presence of tamoxifen* 15 *M, in the human glioblastoma cell lines U-*373*, U-*138*, and U-*87

	U-373	U-138	U-87
Tamoxifen alone	15.4 ± 2.1	14.0 ± 1.6	13.4 ± 2.1
BCNU alone	30.0 ± 8.7	32.3 ± 7.2	37.7 ± 9.1
BCNU + tamoxifen	14.3 ± 7.8*	18.3 ± 4.2*	16.3 ± 8.1*
Etoposide alone	1.0 ± 0.6	4.3 ± 0.9	2.4 ± 0.5
Etoposide + tamoxifen	0.8 ± 0.3†	2.7 ± 1.9†	1.8 ± 0.2†

*Significantly different from BCNU alone ($P < 0.05$, Student's *t*-test); †Not significantly different from etoposide alone.

cell lines with increasing concentration of tamoxifen (Figure 1). Like the inhibition of cell growth (Table 1), the inhibition of the phosphorylation capacity occurred at 50%-inhibitory concentrations of approximately 15 μM (Figure 1). Moreover, the dose-dependent kinetics of phosphorylation inhibition was comparable with that for the tamoxifen-mediated cell growth inhibition (Figure 1, insert). These data suggest a functional relationship between particulate-localised PKC activity, and tamoxifen cytotoxicity in our glioblastoma model.

Oestrogen receptor expression

We further examined a possible contribution of interference with oestrogen receptor-mediated signalling to tamoxifen cytotoxicity in our glioblastoma cells. To this end, the cells were immunohistochemically assessed for oestrogen receptor expression, using an oestrogen-receptor-positive clinical breast cancer specimen as a positive control. To avoid background staining, cells were used that had been cultured in phenol red-free medium supplemented with charcoal-stripped, steroid-free serum [22]. The analyses revealed intense staining of the nuclei of the breast cancer cells, whereas those of the glioblastoma cells remained essentially unstained (data not shown). These findings suggest that tamoxifen cytotoxicity in our model is not related to oestrogen receptor-mediated mechanisms.

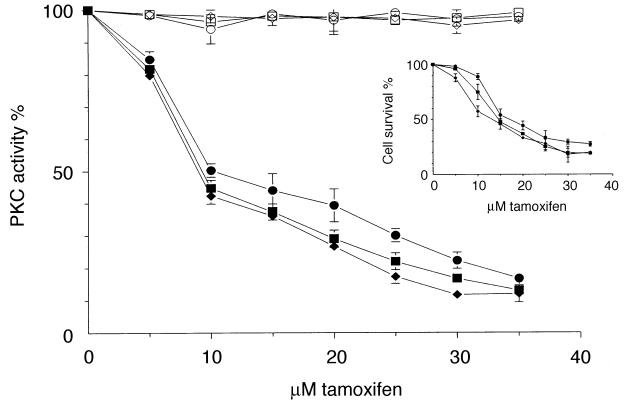

Figure 1. Effects of tamoxifen 0–35 μM on phosphorylation of lysine-rich Type IIIS histone by cytosolic (open symbols) and particulate cellular fractions (solid symbols) in the human glioblastoma cell lines U-373 (●), U-138 (■), and U-87 (◆). Data points are means ± S.D.s (vertical bars; $n \geq 3$). Total PKC activity in untreated U-373, U-138 or U-87 cells was 2145.7 ± 145.2, 1340.3 ± 96.9, and 1082.7 ± 140.8 pmoles ATP transferred per minute per mg protein, respectively. Particulate-associated PKC activity in U-373, U-138 or U-87 was 1074.3 ± 25.6, 499.3 ± 19.9, and 371.7 ± 7.7 pmoles ATP transferred per minute per mg protein, respectively. Insert: Inhibition of cell growth by tamoxifen 0–35 μM in the human glioblastoma cell lines U-373 (●), U-138 (■), and U-87 (◆). Data points are means ± S.D.s (vertical bars; $n \geq 3$). IC_{50} values are given in Table 1.

Influence of rhTGF-β1 on cell growth

To assess the possibility of tamoxifen inhibiting cell growth indirectly by stimulating autocrine TGF-β production, we examined the effects of rhTGF-β1 on the growth of the cells, in serum-containing medium as well as in serum-free medium. Under both conditions, cell proliferation was only minimally affected by rhTGF-β1 at concentrations up to 100 nM (data not shown). This suggests no significant role of TGF-β1-mediated processes in tamoxifen cytotoxicity in our glioblastoma model.

Effect of tamoxifen on γ-irradiation-produced cytotoxicity

We also examined the effects of tamoxifen on the cytotoxicity exerted by γ-radiation, a treatment modality currently employed in primary malignant gliomas, either alternatively, or subsequent to surgery [1, 2]. To this end, the cells were exposed for 24 h to tamoxifen at IC_{50} concentrations (approximately 15 μM), then γ-irradiated in the presence of the drug. The cells were allowed to grow for 3 more days in the presence of tamoxifen before assessment of growth inhibition. The resulting surviving fractions were compared with those found upon treatment with radiation alone on the basis of D_0 values.

Irradiation with 2 or 5 Gy alone did not have a significant effect on the growth of the cells. A slight inhibition of cell proliferation (at most 10%) was only seen upon irradiation with 10 Gy, yielding D_0 values of approximately 13, 16, and 10 Gy in the U-373, U-138, and U-87 cells, respectively (Table 2). However, irradiation with concomitant tamoxifen treatment, led to D_0 values of approximately 2.5, 4, and 2 Gy, respectively (Table 2). This suggests that tamoxifen had sensitised the cells by approximately 5-fold, 4-fold, and 6-fold, respectively, to γ-radiation.

Multiple drug effect analysis [19, 20] of the interactions between tamoxifen and radiation showed for all three cell lines CIs that were at all dose- and effect-levels significantly lower than 1 (Figure 2a). This suggests synergism between both treatment modalities under our experimental conditions.

Effect of tamoxifen on BCNU cytotoxicity

Next, we investigated a possible influence of tamoxifen on the cytotoxicity of BCNU, an active agent in primary CNS tumours [1, 2]. For this purpose, the cells were pretreated for 24 h with tamoxifen 15 μM, and then exposed for 3 days to BCNU in the presence of tamoxifen at the same concentration. The extent of cell growth inhibition observed was compared with that found with BCNU alone.

Table 2. *D_0 values (Gy; means ± S.D.s; n ≥ 3) produced by γ-irradiation of the human glioblastoma cell lines U-*373*, U-*138*, and U-*87 *in the absence or presence of tamoxifen* 15 *μM*

	U-373	U-138	U-87
γ-radiation alone	13.5 ± 0.5	16.0 ± 1.1	10.2 ± 1.3
γ-radiation + tamoxifen	2.5 ± 1.1	4.3 ± 2.0	1.6 ± 0.5

BCNU alone inhibited the growth of the three cell lines at IC_{50} concentrations of approximately 35 μM (Table 1). These values decreased about 2-fold upon pre- and co-treatment with tamoxifen (Table 1), indicating potentiation of BCNU cytotoxicity. Multiple drug effect analysis [19, 20] showed CIs > 1 for tamoxifen together with BCNU at effect-levels ≤ 0.3, and CIs of approximately 1 with BCNU at effect-levels ≥ 0.5 (Figure 2b). This apparent biphasic interaction suggests antagonistic effects of tamoxifen at low dose-levels of BCNU (< 20 μM), but additive interactions between both drugs at higher BCNU doses (≥ 20 mM), yielding clinically relevant surviving fractions below 50%.

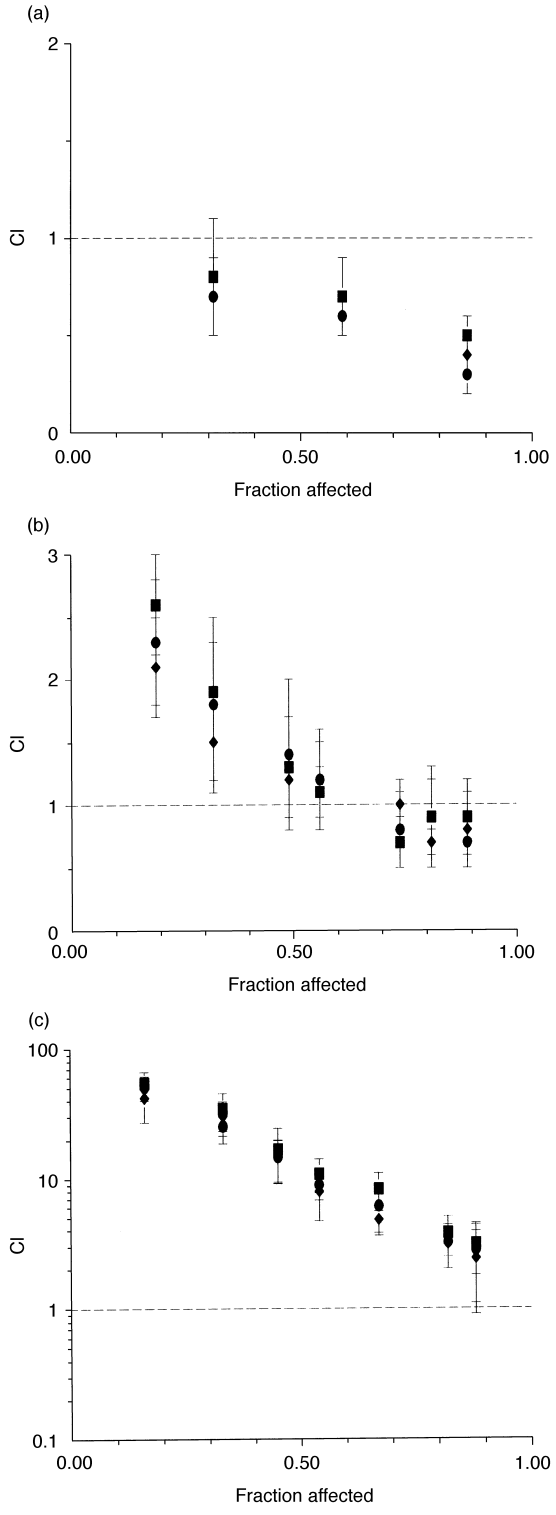

Figure 2. Mutually exclusive combination indices (CIs) for combinations of 15 μM tamoxifen plus radiation 0–10 Gy (a), BCNU 0–200 μM (b), or etoposide 0–20 μM (c) in the human glioblastoma cell lines U-373 (●), U-138 (■), and U-87 (◆). Data points are means ± S.D.s (vertical bars; $n \geq 3$). CI < 1, CI = 1, and CI > 1 indicates synergism, additivity, or antagonism, respectively.

Effect of tamoxifen on etoposide cytotoxicity

We also examined the effects of tamoxifen on the cytotoxicity of etoposide, which may be employed in the experimental treatment of CNS tumours [1, 2]. To this end, we compared the effects on cell growth of 24 h tamoxifen 15 μM followed by 3 days etoposide, with those of 4 days etoposide alone.

The IC_{50} values for etoposide alone in the U-373, U-138, and U-87 cells were approximately 1 μM, 4 μM, and 2 μM, respectively, which were not significantly affected by tamoxifen (Table 1). Multiple drug effect analysis [19, 20] of these interactions in the three cell lines resulted in CIs greater than 1 (Figure 2c), suggesting that the drugs had antagonised each other's cytotoxicity.

DISCUSSION

In this study, we found strong evidence that tamoxifen cytotoxicity in the U-373, U-138, and U-87 human glioblastoma cell lines is mainly mediated through inhibition of PKC activity. Furthermore, our data do not support a significant role of oestrogen receptor- and/or TGF-β-associated mechanisms in the antiproliferative effects of tamoxifen, as previously suggested for certain meningiomas [10, 11] and oestrogen receptor-negative tumours [12]. This is indicated by the absence of detectable oestrogen receptor expression in the cells, and the lack of an effect on their proliferation by an excess of rhTGF-β.

Rather, the cell growth inhibition by tamoxifen appeared to involve interference with membrane PKC activity. This can be derived from the similar kinetics of tamoxifen-mediated inhibition of the proliferation as well as the particulate-associated PKC activity in the three cell lines studied (Figure 1). The 50%-inhibitory concentrations of approximately 15 μM correlate well with those found with other glioma cell lines [16, 17]. More importantly, with the common administration schedule of tamoxifen 20 mg daily, plasma levels of 0.3–0.4 μM have been achieved [23], with approximately 40 times higher levels in brain tissue [24], supporting the clinical relevance of our data.

Tamoxifen did not have an apparent effect on the phosphorylation capacity of the cytosolic cellular fractions. A reduction in particulate-associated PKC activity with no concomitant rise in cytosolic PKC activity, has also been noted in several glioma cell lines treated with tamoxifen 5–20 μM [17]. These results were interpreted to indicate that tamoxifen had selectively interfered with membrane PKC, in accordance with the concept that this PKC fraction represents the active form of the enzyme [3, 4].

In more recent studies with non-glial cell lines [25–27], tamoxifen was also suggested to inhibit cell growth by interfering with PKC activity, but through a mechanism assumed for a variety of structurally and functionally distinct PKC-modulating agents [3, 4]. Thus, tamoxifen would induce alterations at the transmembrane signalling level, leading to

the activation of, and a decrease in cytosolic PKC activity, followed by its translocation to the plasma membrane, where it would be rapidly down-regulated [25–27].

Preliminary findings in our laboratory tend to favour the latter hypothesis. Using Western blotting and pan-PKC antibody, down-regulation of PKC was observed with tamoxifen 15 μM for 24 h, generating a pattern similar to that found for PKC translocation and down-regulation by long-term phorbol 12-myristate 13-acetate (PMA) treatment (data not shown). Additional support for this suggestion comes from the observation that adding tamofixen directly to the *in vitro* PKC enzyme assay did not significantly affect PKC-mediated phosphorylation (data not shown).

Nevertheless, inhibition of PKC activity by tamoxifen or other PKC-interfering agents [3, 4, 28, 29] can lead to cell growth inhibition and/or cytotoxicity, possibly through perturbations in key phosphorylation reactions regulating vital cellular functions. Indeed, PKC inhibition has been associated with the inappropriate expression and activation of transcription factors [30], as well as cyclins and cyclin-dependent kinases [31, 32]. These alterations may not only account for the antiproliferative effects of tamoxifen itself, but may also modulate cellular responses to ionising radiation and chemotherapeutic agents, potentially improving tumour cell kill. We tested the latter hypothesis for γ-radiation, as well as for BCNU and etoposide.

As shown in Table 2, more than 10 Gy of radiation was required to kill 63% of the cells (D_0 values of 10 to 16 Gy), suggesting that they are radioresistant. However, prior treatment with 15 μM tamoxifen sensitised the cells 4–6-fold to radiation, which probably involved a synergistic interaction. The resulting D_0 values of 2–4 Gy are in the range of (cumulative) doses of γ-radiation that are feasible in the clinic [33].

Such modest, but significant radiosensitising effects were also seen upon PKC inhibition in C6 rat glioma cells using tamoxifen [34] or other PKC-interfering agents [30, 35, 36], and may be attributable to tamoxifen-mediated, PKC-related perturbations in the above-mentioned biochemical pathways. For instance, changes in $p34^{cdc2}$ kinase [31, 32], may impede normal cell cycle passage [36], and disrupt checkpoints and DNA repair mechanisms [33], enhancing the cytotoxic effects of radiation. As noted in cultured U-373 cells [36] and in nude mice-U373 xenografts [37], radiation may further directly affect total PKC activity, which may account for the observed synergism with tamoxifen.

Tamoxifen was also able to potentiate the cytotoxicity of BCNU at higher, therapeutically relevant BCNU dose-levels (Table 1), probably through an additive interaction, reducing its IC_{50} concentrations to those that are achievable in the CNS with common clinical protocols [38]. Such effects of tamoxifen were also seen with BCNU or with the BCNU analogue 3-[(4-amino-2-methyl-5-pyrimidinyl) methyl]-1-[2-chloroethyl]-1-nitrosourea (ACNU) in rat and human glioma cells [34, 39].

These observations may be attributed to the same molecular alterations suggested to underlie the potentiation of γ-radiation by tamoxifen. Thus, the adducts formed by the nitrosoureas with DNA [38] may have been improperly removed, leading to the increased cell kill by BCNU (Table 1; Figure 2b).

Surprisingly, tamoxifen elicited no significant effect on the cytotoxicity of etoposide, but produced antagonism with this agent in multiple drug effect analysis. These data are not in accordance with the potentiation of etoposide cytotoxicity noted in, amongst others, HeLa cells following PKC inhibition by staurosporine [40]. However, interference with PKC activity may not always affect etoposide cytotoxicity. Manipulation of PKC activity using a phorbol ester, calphostin C [40], or tamoxifen [41], for instance, was without significant effect on etoposide-mediated cell kill.

The mechanism of action of etoposide involves interference with the DNA strand-breakage and -rejoining action of DNA topoisomerase II [42]. The activation of this enzyme complex presumably requires phosphorylation by several kinases including PKC [42], which may be differentially affected by different classes of kinase-interfering agents [43]. The lack of effect of tamoxifen on etoposide cytotoxicity in our model may therefore be tentatively explained by inappropriate phosphorylation of topoisomerase II following tamoxifen-induced PKC inhibition, impairing its DNA binding activity, thus counteracting etoposide cytotoxicity.

Taken together, our results suggest that tamoxifen inhibits cell growth in the glioma panel under study by interfering with membrane PKC, which may underlie its potential to sensitise the cells to γ-radiation and BCNU, but not to etoposide. These data warrant further evaluation of PKC-inhibiting agents together with therapeutic radiation and/or certain cytotoxic agents in the experimental treatment of high-grade gliomas. In this respect, concurrent administration of tamoxifen and BCNU has indeed been found to produce durable responses in patients with recurrent grade 3 or grade 4 astrocytoma [44].

Unfortunately, the majority of the currently available PKC-interfering agents lack PKC specificity, having affinity to a broad array of kinases [3–5]. Many of these enzymes play very specific roles in intricately regulated biochemical reactions, being switched on or off in a highly timed manner [45]. Random interference with kinase activity can be envisaged to induce multiple, and sometimes unpredictable and undesirable effects which may oppose each other, as exemplified by our results with the tamoxifen–etoposide combination. Optimum clinical use of PKC-interfering agents will therefore require improved insights into (tumour) cell biology, PKC biochemistry, as well as anticancer drug pharmacology, and continued efforts dedicated to the development of more (PKC isoform-) specific drugs.

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