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Effects of gamma-linolenic acid and oleic acid on paclitaxel cytotoxicity in human breast cancer cells

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Abstract

It has been suggested that dietary interventions may improve the effectiveness of cancer chemotherapy. We have examined the combined *in vitro* cytotoxicity of paclitaxel and the fatty acids gamma-linolenic acid (GLA, 18:3n-6) and oleic acid (OA, 18:1n-9) in human breast carcinoma MDA-MB-231 cells. The effect of fatty acids on paclitaxel chemosensitivity was determined by comparing IC_{50} and IC_{70} (50 and 70% inhibitory concentrations, respectively) obtained when the cells were exposed to IC_{50} and IC_{70} levels of paclitaxel alone and fatty acids were supplemented either before or during the exposure to paclitaxel. The 3-4,5-dimethylthiazol-2-yl-2,5-diphenyl-tetrazolium bromide (MTT) assay was used to determine cell growth inhibition. GLA by itself showed antiproliferative effects, and a possible GLA–paclitaxel interaction at the cellular level was assessed by the isobologram and the combination-index (CI) methods. Isobole analysis at the isoeffect levels of 50 and 70% revealed that drug interaction was predominantly synergistic when GLA and paclitaxel were added concurrently for 24 h to the cell cultures. Interaction assessment using the median-effect principle and the combination-index (CI) method showed that exposure of MDA-MB-231 cells to an equimolar combination of concurrent GLA *plus* paclitaxel for 24 h resulted in a moderate synergism at all effect levels, consistent with the results of the isobologram analysis. When exposure to GLA (24 h) was followed sequentially by paclitaxel (24 h) only an additive effect was observed. The GLA-mediated increase in paclitaxel chemosensitivity was only partially abolished by Vitamin E, a lipid peroxidation inhibitor, suggesting a limited influence of the oxidative status of GLA in achieving potentiation of paclitaxel toxicity. When OA (a non-peroxidisable fatty acid) was combined with paclitaxel, an enhancement of chemosensitivity was found when OA was used concurrently with paclitaxel, although less markedly than with GLA. Pretreatment of MDA-MB-231 cells with OA for 24 h prior to a 24 h paclitaxel exposure produced greater enhancement of paclitaxel sensitivity at high OA concentrations than the concurrent exposure to OA and paclitaxel. The OA-induced sensitisation to paclitaxel was not due to the cytoxicity of the fatty acid itself. When these observations were extended to three additional breast carcinoma cell lines (SK-Br3, T47D and MCF-7), simultaneous exposure to GLA and paclitaxel also resulted in synergism. GLA preincubation followed by paclitaxel resulted in additivity for all cell lines. Simultaneous exposure to paclitaxel and OA enhanced paclitaxel cytotoxicity in T47D and MCF-7 cells, but not in SK-Br3 cells, whereas preincubation with OA failed to increase paclitaxel effectiveness in all three cell lines. For comparison, the effects of other fatty acids on paclitaxel chemosensitivity were examined: GLA was the most potent at enhancing paclitaxel cytotoxicity, followed by alpha-linolenic acid (ALA; 18:3n.3), eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), whereas linoleic acid (LA; 18:2n-6) did not increase paclitaxel toxicity. These findings provide experimental support for the use of fatty acids as modulators of tumour cell chemosensitivity in paclitaxel-based therapy.

Keywords: Paclitaxel; Gamma-linolenic acid; Oleic acid; Breast cancer; Chemotherapy

1. Introduction

Some experimental studies have reported the ability of exogenous fatty acids to modulate the cytotoxic activity of several anticancer drugs either in cell culture [1] and/or in tumour-bearing animals [2]. A precise

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mechanism of action of fatty acid modulation of chemotherapeutic effectiveness has not been determined, but two major hypotheses have been proposed. The first suggests that the enhanced cytotoxicity of anticancer drugs could result from changes in the biophysical properties and functions of tumour cell membranes brought about by fatty acid supplementation [3,4]. Membrane fluidity and drug transport are influenced by the composition ratio of saturated fatty acids to unsaturated: the greater the percentages of unsaturated fatty acids, the greater membrane fluidity so that the inward diffusion of drug is increased. This is particularly relevant to many cytotoxic drugs which are thought to enter the cell by passive diffusion. The second hypothesis is related to lipid peroxidation, which has been involved in the modulation of drug efficacy by fatty acids, especially by polyunsaturated fatty acids (PUFAs). Some anticancer drugs yield oxygen-reactive species that could react with the PUFA double bounds and induce the lipid peroxidation process [5]. An increased membrane unsaturation index would provide more abundant targets for peroxidation events generated by anticancer drugs and, thus, increase drug efficacy [1,2].

Little is known about the modulation by fatty acids of the antitumour activity of drugs usually considered to be unable to induce *in vitro* peroxidation events, such as paclitaxel. Paclitaxel is a microtubule-binding drug that has antitumour activity against a range of human tumours and, in particular, breast and ovarian carcinomas [6,7]. However, a major difficulty in developing paclitaxel as a chemotherapeutic agent is its poor water solubility. Presently, paclitaxel is formulated for clinical use in ethanol and Cremophor EL (polyethoxylated castor oil), a solvent system associated with severe adverse effects. Thus, more extensive clinical use of this drug is somewhat delayed due to the lack of appropriate delivery vehicles. Development of alternative treatments which act by enhancing the effect of paclitaxel itself to allow the use of lower concentrations of paclitaxel may alleviate these problems. Thus, the aim of this study was to assess whether the interactions between a lipid-soluble antineoplastic drug such as paclitaxel and the dietary fatty acids gamma-linolenic acid (GLA; polyunsaturated fatty acid of the n-6 family, 18:3n-6) and oleic acid (OA; monounsaturated fatty acid of the n-9 family, 18:1n-9) may improve drug effectiveness. GLA is a naturally occurring PUFA (it is a major component of evening primrose oil) reported to be cytotoxic at micromolar concentrations for cancer cells *in vitro* and *in vivo* [8–11]. Moreover, the chemotherapeutic efficacy of its clinically useful form, the lithium salt of GLA, is being evaluated in Phases II and III clinical trials for a variety of tumours [12,13]. However, few attempts have been made in earlier studies to evaluate the efficacy of the combined use of GLA with some established anticancer drugs. Several dietary studies have suggested that olive oil and monounsaturated fat may reduce breast cancer risk. The strongest evidence comes from studies of southern European populations, in which intake of oleic acid, particularly from olive oil, appears protective [14,15]. Whether this monounsaturated fatty acid may act as a chemosensitiser is not established. However, the high liposolubility intrinsic to OA led us to address its role as a modulator of paclitaxel cytotoxicity.

In this study, we examined the ability of GLA, OA and other n-3 and n-6 PUFAs such as linoleic acid (LA, 18:2n-6), alpha-linolenic acid (ALA; 18:3n-3), eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) to modulate the sensitivity of breast cancer cells to paclitaxel.

In order to determine the best combination of fatty acids with paclitaxel, different schedules of administration of the agents were compared to elucidate whether there was schedule dependency. Since GLA was cytotoxic as a single agent, the interactions between GLA and paclitaxel were evaluated for synergism, additivity, or antagonism using both isobologram and median effect plot analyses, two methods employed for assessing the combined effect of antitumor drugs *in vitro* as preclinical screening tests. To investigate the relationship between the oxidative status of GLA and its effect on paclitaxel-induced cytotoxicity, we examined whether inhibiting lipid peroxidation altered this effect.

2. Materials and methods

2.1. Cell culture

MDA-MB-231, T47D and MCF-7 breast cancer cells were obtained from the American Type Culture Collection and they were routinely grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% heat-inactivated fetal bovine serum (FBS, BioWhittaker), 1% L-glutamine, 1% sodium pyruvate, 50 U/ml penicillin and 50 μg/ml streptomycin. SK-Br3 breast cancer cells were obtained from Dr H. Riese (Centro Nacional de Biotecnología, Madrid, Spain) and they were passaged in McCoy's 5 *A* medium containing 10% heat-inactivated FBS, 1% L-glutamine, 1% sodium pyruvate, 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were in the logarithmic phase of growth at the time of the drug sensitivity assays.

2.2. Chemicals

Gamma-linolenic acid (GLA; 18:3n-6), oleic acid (OA; 18:1n-9), linoleic acid (LA; 18:2n-6), alpha-linolenic acid (ALA; 18:3n-3), eicosapentaenoic acid (EPA; 20: 5n-3) and docosahexaenoic acid (DHA;2:6n-3), were

purchased from Sigma-Chemical and used as methyl esters (>99% purity). The fatty acids were dissolved in 99% ethanol and stored in the dark as stock solutions (1 g/ml) at −20°C. For experimental use, all fatty acids were prepared freshly from stock solutions and diluted with growth medium. Control cells were cultured in medium containing the same concentration of ethanol (v/v) as the experimental cultures with fatty acids. The ethanol solution had no noticeable influence on the proliferation of the experimental cells. Paclitaxel (Taxol®) was supplied by Bristol-Myers Squibb and kept as a stock solution of 6 mg/ml in Cremophor EL, stored at 4°C. Paclitaxel stock was freshly diluted in culture medium before any experiment. Vitamin E (dl-α-tocopherol; Sigma-Chemical) was prepared as a 100 mM solution in ethanol and stored at 4°C and a working solution in growth medium was prepared at a concentration of 100 μM. Wells not receiving vitamin E received the same concentration of ethanol (v/v).

2.3. Determination of cytotoxicity

Drug sensitivity was determined using a standard colorimetric MTT (3-4,5-dimethylthiazol-2-yl-2,5-diphenyl-tetrazolium bromide) assay. Briefly, cells were plated out at a density of 10^4 cells/100 μl/well in 96-well microtitre plates and allowed an overnight period for attachment. Then the medium was removed and fresh medium, along with various concentrations of paclitaxel, fatty acids or combinations of compounds, were added to cultures in parallel. Agents were studied in combination concurrently (fatty acid *plus* paclitaxel 24 h) or sequentially (fatty acid 24 h followed by paclitaxel 24 h) with the first agent washed out prior to the introduction of the second drug. Control cells without agents were cultured using the same conditions with comparable media changes. Following treatment, cells were fed with drug-free medium (100 μl/well) and MTT (10 μl/well, 5 mg per ml in PBS) and incubation was prolonged for 3 h at 37°C. After removing the supernatants, the MTT-formazan crystals were dissolved in dimethyl sulphoxide (100 μl/well) and the absorbance was measured at 570 nm in a multi-well plate reader (Model Anthos Labtec 2010 1.7 reader).

The growth inhibitory effects from exposure of cells to each compound alone and their combination for a particular schedule were analysed generating concentration-effect curves as a plot of the fraction of unaffected (surviving) cells versus drug concentration. Growth inhibition was expressed as a percentage of the untreated controls that were processed simultaneously, using the following equation: (A_{570} of treated sample/A_{570} of untreated sample)×100. The IC_{50} (cytostatic condition) and IC_{70} (cytotoxic condition) values were defined as the drug concentrations inhibiting cell growth by either 50% (50% reduction of absorbance) or 70% (70% reduction of absorbance), compared with untreated controls, respectively. It was established that optical density was directly proportional to the cell number up to the density reached by the end of the assay. The degree of sensitisation to paclitaxel by fatty acids was determined by dividing both IC_{50} and IC_{70} values of control cells by those obtained when cells were exposed to fatty acids before or during exposure to paclitaxel.

To evaluate whether oxidative stress was involved in the sensitisation to paclitaxel by GLA, one set of plates received the antioxidant Vitamin E simultaneously with GLA. Unsupplemented and GLA-supplemented cultures incubated in the presence or absence of Vitamin E were examined together after identical incubation periods. Cell viability was determined according to the procedure described above.

2.4. Data analysis for the combination treatments

The interaction between GLA and paclitaxel was evaluated by the isobologram technique, a dose-oriented geometric method of assessing drug interactions [16]. In the isobologram method, the concentration of one agent producing a desired (e.g. 50 or 70% inhibitory) effect is plotted on the horizontal axis, and the concentration of another agent producing the same degree of effect is plotted on the vertical axis; a line is drawn connecting these two points. The experimental isoeffect points are the concentrations (expressed relative to the IC_{50} or IC_{70} concentrations) of the two agents which kill 50 or 70% of the cells when combined. When the experimental isoeffect points fall below that line, the combination effect of the two drugs is considered to be supra-additive or synergistic, whereas antagonism occurs if the experimental isoeffect points lie above it.

In addition, the multiple drug effect analysis of Chou and Talalay, which is based on the median-effect principle, was used to examine the nature of the interaction observed between GLA and paclitaxel. Details of this methodology have been published [17]. Briefly, the log $[f_a/f_u]$ (f_a and f_u are the fractions of cells affected or unaffected, respectively) was plotted against log (drug dose). From the resulting median effect lines, the x-intercept (log IC_{50}) and slope m were calculated for each agent. These parameters were then used to calculate doses of the component agents (and combinations) required to produce various cytotoxicity levels according to Eq. (1). For each level of cytotoxicity, combination index (CI) values were calculated according to Eq. (2) where $(D_x)_1$ is the dose of agent 1 required to produce x% effect alone, and $(D)_1$ is the dose of agent 1 required to produce the same x% effect in combination with $(D)_2$. Similarly $(D_x)_2$ is the dose of agent 2 required to produce x% percent effect alone, and $(D)_2$ is the dose

required to produce the same effect in combination with $(D)_1$. If the agents are mutually exclusive (e.g. similar mode of action), then α is 0 (i.e. CI is the sum of the two terms); if the agents are mutually non-exclusive (e.g. independent mode of action), α is 1 (i.e. CI is the sum of the three terms). If it is uncertain whether the agents act in a similar or an independent manner, the formula may be solved both ways.

$$\text{Dose}_1 = \text{Dose IC}_{50}[(1-f)/f]^{1/m} \quad (1)$$

$$\text{CI} = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 + \alpha(D)_1(D)_2/(D_x)_1(D_x)_2 \quad (2)$$

CI values of <1 indicate synergy (the smaller the value, the greater the degree of synergy), values >1 indicate antagonism and values equal to 1 indicate additive effects. The conformity of the experimental data to the median-effect principle of the mass-action law is automatically provided by the computer printout in terms of the linear correlation coefficient (r value) of the median-effect plots. In this study, the r values for GLA, paclitaxel and their combinations were all greater than 0.95.

2.5. Statistical analysis

Statistical analysis was performed using the Student's t-test. Statistical significance levels were $P<0.05$ and $P<0.001$.

3. Results

3.1. Growth effects observed with single agents

To determine the cytotoxic activity of paclitaxel, dose–effect experiments were performed. The paclitaxel concentrations needed for 50% inhibition of cell growth (IC_{50}) over varying periods of exposure are shown in Fig. 1a. The results showed a marked time dependency for the activity of paclitaxel. The IC_{50} value against MDA-MB-231 cells after a 6 h exposure to paclitaxel (135±2 μM) was 5.4 times higher than that obtained with a 24 h exposure (25±1 μM). Prolonging the time of exposure for more than 24 h did not substantially increase the activity of paclitaxel; the IC_{50} value after a continuous 3-day exposure (10±3 μM) was only 2.5 times lower than that after 24 h exposure. These results were consistent with the known cell-cycle specificity of paclitaxel.

We also examined the effect of the polyunsaturated and monounsaturated fatty acids on the growth of MDA-MB-231 cells. GLA significantly reduced the growth rate of the tumour cells in a dose-dependent manner; time dependency was also evident, but less pronounced than was observed with paclitaxel exposure. The IC_{50} value after a 6 h exposure to GLA (277±5 μM) was 2.2 times higher than that obtained with a 24 h exposure (125±3 μM). Prolonging the time of exposure for more than 24 h did not substantially increase the activity of GLA; a continuous 3-day exposure to GLA resulted in an IC_{50} value of 79±4 μM that was only 1.6 times lower than that after 24 h exposure (Fig. 1a). The effect of OA at a variety of concentrations on the growth rate of MDA-MB-231 cells after a 24 h exposure is shown in Fig. 1b. A weak cytostatic effect

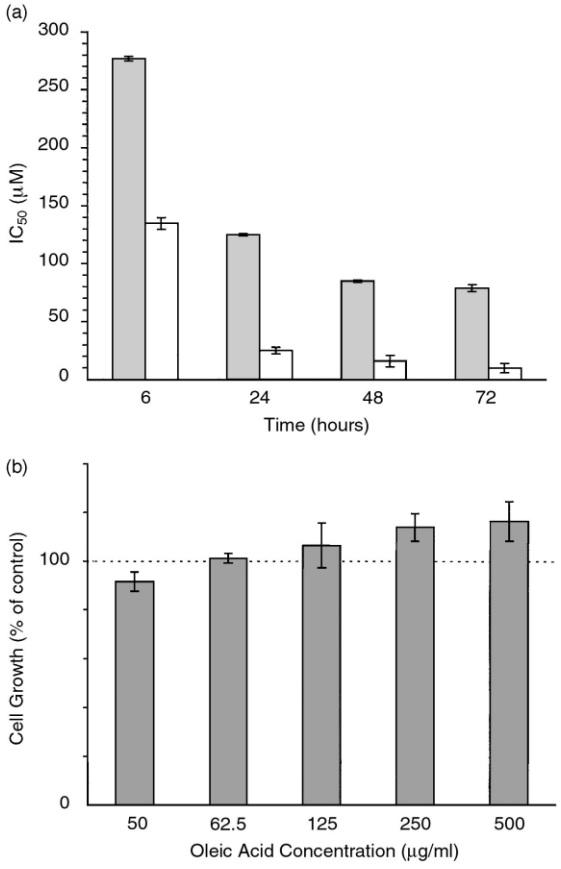

Fig. 1. (a) Exposure time dependency of anticellular activity of paclitaxel (□) and gamma-linolenic acid (GLA) (■). MDA-MB-231 cells (10^4 per well) were plated on day 0, and treated with each agent from day 1 for the indicated exposure time. Compounds were not renewed during the entire period of cell exposure. The IC_{50} values were calculated as described in the 'Materials and Methods'. The IC_{50} values for both paclitaxel and gamma-linolenic acid after a 24 h exposure allowed a constant ratio combination design (1:5) to be used for assessment of the drug combination employing the median-effect principle and the combination-index (CI) method; (b) effect of oleic acid (OA) on the growth of the MDA-MB-231 cell line. Cells (10^4 per well) were plated on day 0, and treated with increasing concentrations of OA on day 1. After 24 h, cell growth was measured using the (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) MTT assay and the results were expressed as a percentage of ethanol-only controls (dotted line). Results in each panel represent the means± standard deviation (bars) of at least four separate experiments carried out in triplicate.

was achieved at the lower concentration used (50 μg/ml; 168.6 μM) while cell growth was slightly stimulated when higher concentrations of OA (62.5–500 μg/ml; 210.7–1686.3 μM) were used. Longer exposure of the cells to OA showed a similar trend to that found in the 24 h exposure period (data not shown).

From these results, we decided to use exposure times for paclitaxel, GLA and OA of 24 h for simultaneous and sequential exposure.

3.2. Sensitivity of the MDA-MB-231 breast cancer cell line to paclitaxel in the presence of fatty acids

The effects of a 24-h co-exposure to sub-optimal doses of GLA (lower than the IC_{50}) on the sensitivity of the MDA-MB-231 breast cancer cell line to paclitaxel are shown in Table 1. Both IC_{50} and IC_{70} values were chosen in order to analyse the combination effect of GLA and paclitaxel in terms of cytostatic conditions as well as cytotoxic conditions. There were significant differences between the IC_{50} and IC_{70} values determined either in the presence or absence of GLA. In order to measure the increase in sensitivity, a 'sensitisation factor' was determined by dividing both the IC_{50} and IC_{70} values for the drug alone by those in the presence of GLA. GLA enhanced the growth-inhibitory activity of paclitaxel in a dose-dependent manner. As the concentration of GLA increased, the growth-inhibitory activity of paclitaxel was markedly enhanced. A concentration of 5 μg/ml of GLA (17.1 μM) was able to cause a weak potentiation of paclitaxel activity (1.8-fold decrease at IC_{50}; 1.6-fold decrease at IC_{70}) while 30 μg/ml of GLA (102.6 μM) exhibited a maximal sensitisation effect (8.3-fold decrease at IC_{50}; 7.2-fold decrease at IC_{70}).

Since the sensitisation observed using GLA may be due to the toxicity of the fatty acid itself, we evaluated the type of interaction between GLA and paclitaxel performing a series of isobologram transformations of multiple dose–response analyses. Representative transformations are presented graphically (isobolograms) in Fig. 2. The *dashed line* drawn between the IC_{50} or IC_{70} for paclitaxel alone and the IC_{50} or IC_{70} for GLA alone indicates the alignment of theoretical isoeffect data points for additive interactions between paclitaxel and GLA. The true IC_{50} and IC_{70} points (the experimental concentrations of GLA and paclitaxel which combined for 24 h produced either 50 or 70% reduction in survival

Table 1
Effects of fatty acids on the sensitivity of MDA-MB-231 breast cancer cells to paclitaxel[a]

	Fatty acid dose (μg/ml)	IC_{50} (μM)	Sensitisation factor[b]	IC_{70} (μM)	Sensitisation factor
A. Simultaneous schedule					
Paclitaxel alone	0	25±1	–	36±1	–
+ Gamma-linolenic acid (GLA)	5	14±3*	*1.8*	23±2*	*1.6*
	10	9±1†	*2.8*	17±1†	*2.1*
	20	5±1†	*5.0*	7±1†	*5.1*
	30	3±1†	*8.3*	5±1†	*7.2*
+ Oleic acid (OA)	62.5	19±1*	*1.3*	28±2*	*1.3*
	125	18±1*	*1.4*	23±2*	*1.6*
	250	17±1*	*1.5*	23±3*	*1.6*
	500	17±1*	*1.5*	20±1*	*1.8*
B. Sequential schedule					
Paclitaxel alone	0	27±2	–	38±1	–
+ Gamma-linolenic acid	5	21±2*	*1.3*	37±2	*1.0*
	10	17±1†	*1.6*	32±1*	*1.2*
	20	14±2†	*1.9*	28±1*	*1.4*
	30	3±1†	*9.0*	5±1†	*7.6*
+ Oleic acid	62.5	22±1*	*1.2*	30±2*	*1.3*
	125	17±1†	*1.6*	27±1*	*1.4*
	250	14±1†	*1.9*	22±1*	*1.7*
	500	9±1†	*3.0*	19±2*	*2.0*

[a] Data represent the effect of exposure schedule on fatty acids-induced sensitisation to paclitaxel. MDA-MB-231 cells were incubated in serial dilutions of paclitaxel in the absence or presence of a given concentration of fatty acid for 24 h (simultaneous schedule) or incubated with a given concentration of fatty acid for 24 h and then fatty acid was washed out prior to the introduction of serial dilutions of paclitaxel for 24 h (sequential schedule). IC_{50} and IC_{70} are the concentrations of paclitaxel which inhibit cell growth by either 50 or 70% respectively, measured as described in the 'Materials and Methods'. Values are means (±standard deviation) of four independent experiments carried out in triplicate. Statistically significant differences in sensitivity when compared with exposure to paclitaxel alone are shown by the asterisks (*$P < 0.05$; †$P < 0.001$).

[b] Sensitisation factors were obtained by dividing either IC_{50} or IC_{70} values of paclitaxel alone by those when fatty acids were supplemented either before or during exposure to paclitaxel.

of the cell line MDA-MB-231) were plotted and compared with the additive line. The experimental isoeffect data points fell to the left side of the line in the concurrent exposure experiments, suggesting a supra-additive or synergistic interaction of GLA and paclitaxel. In addition, a similar synergistic combined effect was observed in both the cytostatic (IC_{50}) and cytotoxic (IC_{70}) conditions.

In order to verify this synergistic cell-killing activity, the combined effect of GLA and paclitaxel was also assessed using the median-effect plot method. For the analysis, 1.5-fold dilutions of the paclitaxel (5.062–38.44 μM) and GLA (25.31–192.2 μM) were prepared and they were combined with each other from the lowest to the highest concentration. Thus, for two agents in combination (paclitaxel *plus* GLA), we varied the doses of the two compounds while monitoring the cell fraction affected (Fig. 3a). The doses were varied such that a constant molar ratio paclitaxel to GLA was maintained. The dose ratio of the drugs was fixed at 1:5 (paclitaxel:GLA), based on IC_{50} concentrations determined with the single agents so that the contribution of the effect for each drug in the mixture would be the same (i.e. equipotency ratio). When cells were exposed to a fixed 1:5 ratio of paclitaxel and GLA, more cells were killed than when cells were exposed to each agent alone, which suggested an interaction between GLA and paclitaxel. Using the mutually exclusive assumption of the combination-index (CI) method of Chou and Talalay [17], the CI values ranged from 0.703±0.012 (mean±standard deviation) to 0.949±0.030 for 5–95% cell kill levels, suggesting a moderate synergism for cell-killing activity in the simultaneous combination regimen (circles in Fig. 3b). These data were consistent with the results of the isobologram analysis. When the data were calculated using the mutually non-exclusive assumption, the CI values ranged from 0.799±0.018 to 1.170±0.043 for 5–95% cell kill levels, also suggesting a slight-to-moderate synergism or nearly additive interactions (asterisks in Fig. 3b).

To elucidate whether the synergistic interaction of paclitaxel with GLA was a general phenomenon

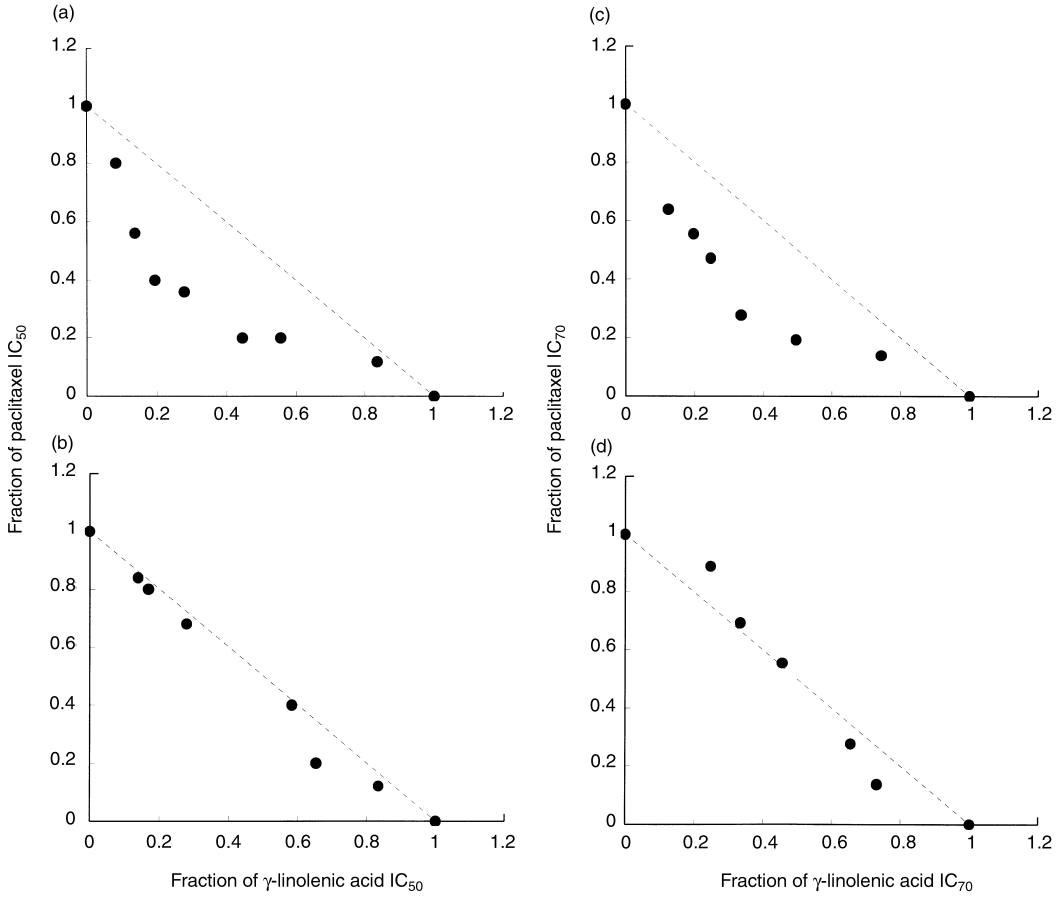

Fig. 2. Isobolograms of concurrent 24 h exposure of MDA-MB-231 cells to paclitaxel and gamma-linolenic acid (a and c) and sequential exposure to gamma-linolenic acid for 24 h followed by paclitaxel for 24 h (b and d) at 50% (a and b) and 70% (c and d) effect levels. The dashed line indicates the alignment of theoretical values of an additive interaction between the two compounds. Values above the dashed line indicate an antagonistic interaction, and values below indicate potentiation of toxicity (synergism). Experimental isoeffective points at 50 and 70% effect levels are signified as ●. Concurrent exposure of paclitaxel and gamma-linolenic acid yielded synergistic effects, whereas a sequential exposure to gamma-linolenic acid followed by paclitaxel showed no potentiation of toxicity. Results are representative of three independent experiments.

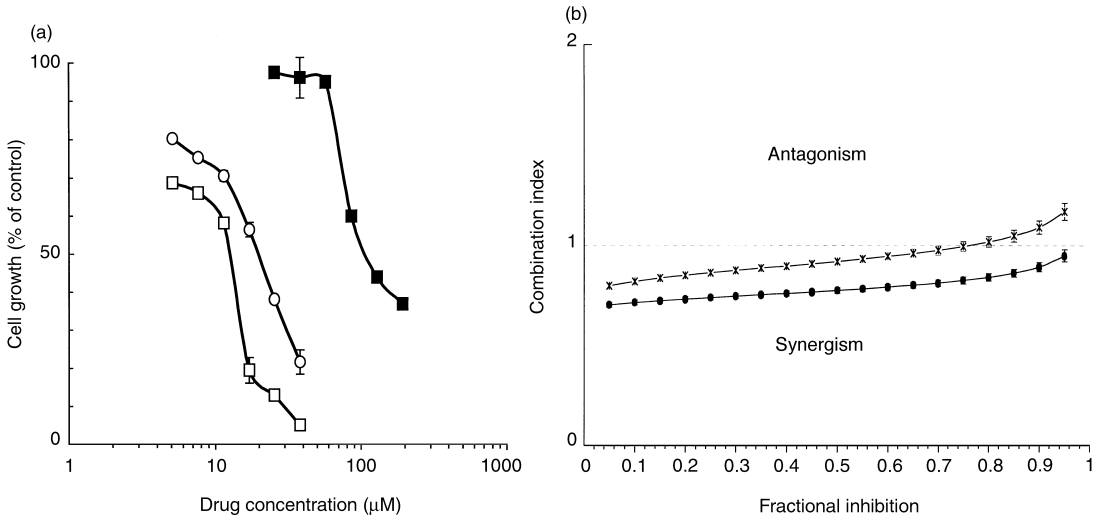

Fig. 3. (a) Percentage survival as a function of drug concentration for MDA-MB-231 cells incubated for 24 h with gamma-linolenic acid alone (■), paclitaxel alone (○), or a fixed 1:5 ratio of paclitaxel and gamma-linolenic acid (□). Cell viability was determined by the MTT assay and data are expressed as percentages of survival compared with untreated control cultures. Drug concentrations were expressed based on the paclitaxel concentration; (b) combination index values employing the results of a were calculated using assumptions for the drug action both mutually exclusive (circles) and mutually non-exclusive (asterisks) at effect levels ranging from 5 to 95% inhibition of cell growth (fractional inhibition), as described in 'Materials and methods'. The line across the CI value of 1 indicates additivity; CIs above and below indicate antagonism and synergism, respectively. Results in each panel are means±standard deviation (bars) of at least three independent experiments made in triplicate (some data points have deviation bars that are concealed by the symbol in both a and b).

applicable to other human breast cancer cell lines, the combination effect was further assessed in SK-Br3, T47D and MCF-7 breast cancer cells using the Chou–Talalay analysis (Table 2). In all three breast cancer cell lines, simultaneous exposure to both agents at their equipotent ratios for 24 h resulted in synergism. The maximum degree of synergism was achieved in SK-Br3 cells (CI = 0.276±0.022 at the IC_{50} level and 0.678±0.061 at the IC_{70} level). For T47D cells, simultaneous 24 h exposures to the two agents produced moderate synergism at low effects levels (CI = 0.783±0.070 at the IC_{50} level) or nearly additive interactions at higher levels of cell kill (CI = 1.243±0.123 at the IC_{70} level). Similar results were observed in MCF-7 cells (CI = 0.816±0.048 at the IC_{50} level and 1.171±0.115 at the IC_{70} level).

When OA was used in combination with paclitaxel in MDA-MB-231 cells, more modest changes in both cytostatic and cytotoxic values of paclitaxel against MDA-MB-231 breast cancer cells were found (Table 1). In contrast to GLA, the sensitising effect induced by OA was very similar over a 62.5–500 μg/ml concentration range (1.3- to 1.5-fold decrease at IC_{50}; 1.3- to 1.8-fold decrease at IC_{70}) showing that the potentiation of the growth-inhibitory activity of paclitaxel by OA is not dose-dependent. There was no significant potentiation of paclitaxel sensitivity when tumour cells were exposed to OA concentrations lower than 50 μg/ml (data not shown).

The potentiating effect exerted by the highest concentration used of OA (500 μg/ml) was examined in the

Table 2
Combination index (CI) values of the interaction between paclitaxel and GLA in human breast cancer cells[a]

Cell line	Simultaneous exposure			Sequential exposure		
	Combination index at			Combination index at		
	IC_{50}	IC_{70}	Interpretation	IC_{50}	IC_{70}	Interpretation
MDA-MB-231	0.776*	0.812*	Synergism	1.071	1.215	Additivity
SK-Br3	0.276†	0.678*	Strong synergism	1.167	1.316*	Moderate antagonism
T47D	0.783*	1.243	Moderate synergism	1.031	1.113	Additivity
MCF-7	0.816*	1.171	Moderate synergism	1.035	1.167	Additivity

[a] The combination index values (mutually exclusive case) were calculated on the multiple drug effect equation derived by Chou and Talalay. CI < 1, = 1 and > 1 indicate synergism, additive effect and antagonism, respectively. Each value represents the mean of at least four experiments carried out in triplicate. Student *t*-tests were computed to evaluate whether significant differences in the mean CI values occurred as compared with a null hypothesised CI of 1 (*$P < 0.05$; †$P < 0.001$). For simplicity the standard deviations (< 10%) were omitted.

breast cancer cell lines SK-Br3, T47D and MCF-7. The 24 h IC_{50} value of paclitaxel against MCF-7 cells decreased from 21±4 to 12±3 μM ($P < 0.05$) when paclitaxel was used in the presence of OA. For T47-D cells, co-addition of OA for 24 h significantly decreased the IC_{50} value of paclitaxel from 23±4 to 15±2 ($P < 0.05$). Such effects were not observed in SK-Br3 cells.

3.3. Effect of pre-incubation of MDA-MB-231 cells with fatty acids on the sensitivity to paclitaxel

The effects of a 24 h pre-incubation with GLA on the sensitivity of the MDA-MB-231 breast cancer cell line to paclitaxel are shown in Table 1. The lower concentrations of GLA used (5–20 μg/ml; 17.1–68.4 μM) had little effect on paclitaxel sensitivity (1.3- to 1.9-fold decrease at IC_{50}; 1.0- to 1.4-fold decrease at IC_{70}). However, pre-exposure to 30 μg/ml GLA (102.6 μM) for 24 h sensitised MDA-MB-231 cells to paclitaxel by 9.0-fold at the IC_{50} level and by 7.6-fold at the IC_{70} level. This magnitude of potentiation was greater than that with a concurrent 24 h exposure. The type of interaction between GLA and paclitaxel was again analysed by the isobologram method (Fig. 2). Under this experimental condition, most of the isoeffect data points fell near the theoretical additive line, suggesting that sequential exposure to GLA followed by paclitaxel produced additive effects. In addition, analysis of the combination by the Chou–Talalay method indicated only additive effects (CI = 1.071±0.040 at the IC_{50} level and 1.215±0.120 at the IC_{70} level).

Based on these results, the effect of a 24 h pre-exposure to GLA on the sensitivity to paclitaxel was examined in the three additional breast cancer cell lines. Similarly to what happened in MDA-MB-231 cells, the sequential exposure to GLA and paclitaxel in SK-Br3, T47D and MCF-7 produced additive interactions only, with CI values significantly higher (loss of the synergism) compared with those for simultaneous exposure (Table 2).

Sequential exposure with 24 h OA treatment prior to that of paclitaxel for 24 h produced a significant potentiation of the paclitaxel-induced cytotoxicity (Table 1). Interestingly, the degree of sensitisation achieved using OA concentrations ranging from 62.5 to 500 μg per ml was higher at the higher concentrations than the potentiation observed when OA was supplemented concurrently (1.2- to 3.0-fold decrease at IC_{50}; 1.3- to 2.0-fold decrease at IC_{70} for the whole concentration range). Furthermore, OA enhanced the growth-inhibitory activity of paclitaxel in a dose-dependent manner. There was no noticeable effects on paclitaxel sensitivity when tumor cells were pre-incubated with OA concentrations lower than 50 μg/ml (data not shown).

The pre-incubation with OA for 24 h followed by paclitaxel for 24 h failed to increase paclitaxel effectiveness in SK-Br3, T47D and MCF-7 breast cancer cell lines (data not shown).

3.4. Effect of Vitamin E on GLA-induced paclitaxel sensitisation

To examine whether oxidative stress was involved in the GLA-induced paclitaxel sensitisation, three independent experiments were performed using the antioxidant agent Vitamin E (dl-α-tocopherol) as a lipid peroxidation inhibitor. First, we measured cell viability in the MDA-MB-231 cells exposed to paclitaxel in the presence or absence of a non-cytotoxic concentration of Vitamin E. At 10 μM Vitamin E, a concentration proposed previously by Bégin and colleagues [18], no significant effect on paclitaxel cytotoxicity was observed in any experiment (data not shown). Secondly, we monitored the antioxidant role of Vitamin E in the cytotoxic effect of GLA. Thus, one set of plates received 10 μM Vitamin E at the time of fatty acid treatment. Vitamin E completely blocked the growth-inhibitory effects of low concentrations of GLA (5 and 10 μg/ml) and significantly prevented the cytotoxic effects of higher concentrations of GLA (Fig. 4a and c). Finally, if lipid peroxidation is associated with GLA-induced paclitaxel sensitisation, antioxidants like Vitamin E should inhibit it. Fig. 4b shows the changes in cell toxicity of 15 μM paclitaxel after a 24 h co-exposure to increasing concentrations of GLA alone (5–40 μg/ml) or combined with 10 μM Vitamin E. The dose-dependent enhancing effect of GLA on paclitaxel cytotoxicity was significantly reduced in the presence of Vitamin E only when low concentrations of GLA were tested (5–10 μg/ml). With higher concentrations of GLA (20–40 μg/ml), addition of the antioxidant agent did not significantly change the GLA-induced increase in paclitaxel toxicity. Interestingly, the effect of a 24 pre-exposure to GLA on paclitaxel cytotoxicity was more profoundly abolished when Vitamin E was used in the pre-treatment, being significant from 5 to 30 μg/ml of GLA (Fig. 4d).

3.5. Comparative effect of different fatty acids on paclitaxel-induced cell toxicity

The effects of other PUFAs were examined on paclitaxel-induced cell toxicity. Experiments using alpha-linolenic acid (ALA; 18:3n-3), eicosapentaenoic acid (EPA; 20:5n-3), docosahexaenoic acid (DHA; 22:6n-3) and linoleic acid (LA; 18:2n-6) were carried out under conditions identical to those used with a 24 h co-exposure to GLA. Fig. 5 shows the effects of the individual PUFAs on the sensitivity of MDA-MB-231 cells to paclitaxel, measured as the amount of paclitaxel necessary to kill

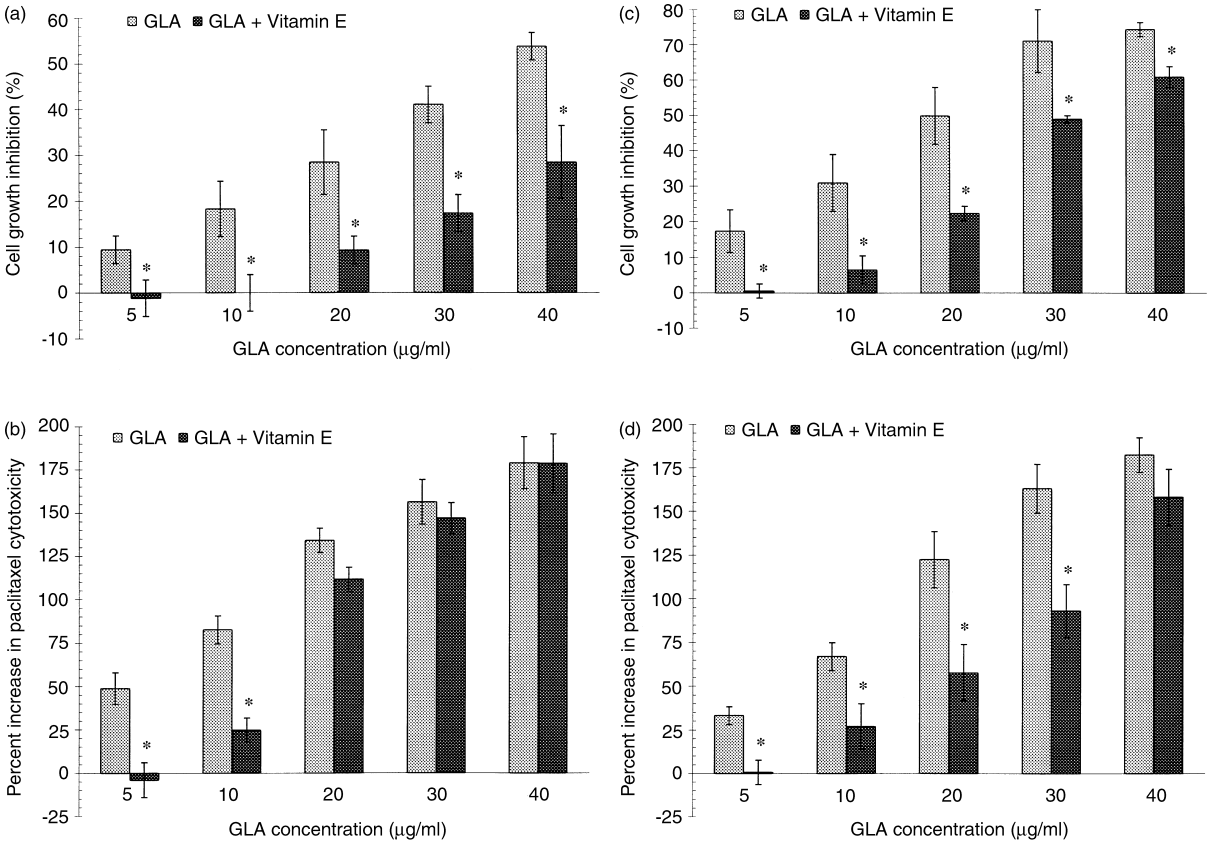

Fig. 4. (a and c) Effect of vitamin E supplementation on the growth inhibitory effects of GLA in MDA-MB-231 breast cancer cells. Cells were incubated with various concentrations of GLA in the presence or absence of vitamin E (final concentration in the medium 10 μM) for 24 h (a) or incubated with various concentrations of fatty acid for 24 h in the presence or absence of vitamin E and then experimental medium was washed out prior to the introduction of fresh medium for 24 h (c). Cell growth inhibition, is expressed as the per cent of untreated cells (grown in the absence of vitamin E and GLA). (b and d) Effect of the combination of GLA with vitamin E on the cytotoxicity of paclitaxel. MDA-MB-231 cells were incubated in the presence of a specified concentration of GLA alone with or without vitamin E (10 μM) added simultaneously with 15 μM paclitaxel (b) or incubated with various concentrations of fatty acid for 24 h in the presence or absence of vitamin E and then experimental medium was washed out prior to the introduction of 15 μM paclitaxel for 24 h (d). Cell toxicity of paclitaxel was measured in four separate experiments performed in triplicate and compared with cell toxicity of paclitaxel alone (0% of variation = cell viability measured for condition with paclitaxel alone). Results are expressed as per cent of change of paclitaxel-induced cytotoxicity. Results in each panel are means±standard deviation (bars). Statistically significant differences from GLA-supplemented cultures incubated in the presence or absence of vitamin E are shown by asterisks (*$P < 0.05$).

50% of cells (the IC_{50} value) in cultures containing either no PUFA or 20 μg/ml of the individual PUFA. GLA was the most potent at enhancing the cytotoxic effect of paclitaxel, decreasing the IC_{50} value by 80%. The enhancement in paclitaxel cytotoxicity was also evident employing ALA, EPA and DHA, which reduced the IC_{50} value by 69, 45 and 34%, respectively. Such effects were not observed with LA.

4. Discussion

There is considerable interest in exploiting the dietary effects of fatty acids in cancer prevention and also as an adjunct to conventional cancer therapy. The ability of fatty acids, especially PUFAs, to increase the cytotoxic activity of some anticancer drugs has been documented [1–4]. However, little is known about the involvement of fatty acids in the antitumour activity of drugs that do not induce *in vitro* peroxidation, such as paclitaxel, a microtubule-binding drug. Thus, the purpose of this study was to assess the interactions between paclitaxel and the dietary fatty acids GLA and OA in a human breast cancer model, with a view to defining a potential role of dietary fatty acids as modulators of paclitaxel cytotoxicity.

We first determined whether there is an acute effect of the fatty acids on cellular drug sensitivity. Co-incubation of breast cancer cells with paclitaxel and fatty acids showed that GLA enhanced up to approximately 8-fold the growth-inhibitory activity of paclitaxel in a dose-dependent manner. Since the cytotoxicity of GLA alone could lead to inappropriate speculations regarding fatty-acid–drug interactions, GLA–paclitaxel interactions were subject to a more critical analysis. The isobologram technique showed that the two agents given

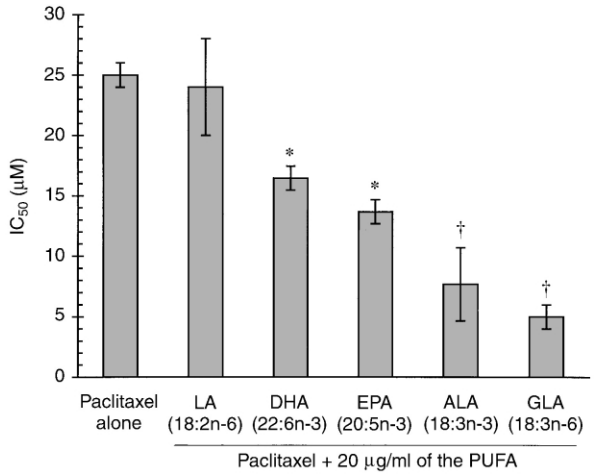

Fig. 5. Comparative effect of individual polyunsaturated fatty acids (PUFAs) on paclitaxel-induced cell toxicity. PUFAs are linoleic acid (LA; 18:2n-6), docosahexaenoic acid (DHA; 22:6n-3), eicosapentaenoic acid (EPA; 20:5n-3), alpha-linolenic acid (ALA; 18:3n-3) and gamma-linolenic acid (GLA; 18:3n-6). MDA-MB-231 cells were incubated with serial dilutions of paclitaxel in the absence or presence of the specified fatty acid (final concentration in the medium 20 μg/ml) for 24 h. The concentration of paclitaxel that inhibited cell growth by 50% (IC_{50} value) was measured in the absence or presence of the specified fatty acid as previously described. Results are means±standard deviation (bars) of at least three independent experiments performed in triplicate. Statistically significant differences from unsupplemented (paclitaxel alone) and PUFA-supplemented cultures are shown by asterisks (*$P < 0.05$; †$P < 0.001$).

concurrently demonstrate synergistic (supra-additive) toxicities. Synergism was also assessed using the median-effect principle and the CI method which takes into account both the potency of each drug and combinations of these drugs and the shapes of their dose–effect curves and quantitates the synergism or antagonism at different concentrations and at different effect levels. Exposure of breast cancer cells to an equimolar co-incubation of GLA *plus* paclitaxel resulted in a consistent synergistic effect (CIs were <1 over all of the range of cytotoxicities examined) when the equation was solved under the assumption of mutually exclusive drug interactions (similar modes of action). These results are in agreement with the conclusions resulting from the isobologram analysis. In SK-Br3, T47D and MCF-7 breast cancer cells, the Chou–Talalay analysis of the combination revealed that the interaction between paclitaxel and GLA was also synergistic.

The magnitude of the effects of OA on paclitaxel sensitivity was relatively small compared with those obtained with GLA, but the effect was large enough to be significant. The highest concentration of OA used sensitised MDA-MB-231 cells to paclitaxel by approximately 2-fold at the IC_{70} level. Unlike GLA, the OA-induced potentiation of paclitaxel cytotoxicity was not dose-dependent. An OA-induced increase in paclitaxel sensitivity was also noted for MCF-7 and T47D breast cancer cells. We were unable to detect any sensitising effect after incubation with OA concentrations lower than 50 μg/ml. Thus, it appears that only when an OA threshold has been overcome can paclitaxel-sensitivity be improved.

Our study was not designed to address the mechanism of GLA and OA in the modulation of paclitaxel efficacy. However, the enhanced killing effect of anticancer drugs could result from alterations in the biophysical properties of membranes brought about by fatty acid supplementation or by enhanced lipid peroxidation. Changes in the cell membrane fatty acid composition may be particularly relevant for paclitaxel, which is thought to enter the cell by passive diffusion. Thus, we attempted to enhance the sensitising effects of the fatty acids by pre-incubating cells with GLA and OA before exposure to paclitaxel. The rationale behind this approach was based on the assumption that pretreatment with fatty acids would allow fatty acid incorporation into the cellular lipids, altering membrane fluidity and increasing drug uptake. When the magnitude of the effect of GLA in paclitaxel sensitivity was estimated using IC ratios, a concentration of GLA of 30 μg/ml produced an increase in the apparent degree of 'sensitisation' to paclitaxel from 8.3- (simultaneous exposure) to 9.0-fold (sequential exposure) based on the IC_{50} level results. However, the application of the isobologram method to the sequential schedule GLA for 24 h followed by paclitaxel for 24 h showed clearly that the two agents demonstrate only additive cytotoxicities. Interestingly, under this sequential schedule, the synergism was also completely lost in SK-Br3, T47D and MCF-7 cells, suggesting that the synergism between GLA and paclitaxel is schedule-dependent. Since the concurrent administration of paclitaxel and GLA is the optimal schedule in terms of cytotoxic effect, this suggests that GLA modulates the sensitivity to paclitaxel by a mechanism other than permeabilising cell membranes. A recent report, demonstrating that the pretreatment of breast cancer cells with GLA increased their sensitivity to cytotoxic drugs without increasing the drug uptake due to GLA incorporation into the cells [19], provides further evidence to support our experiments. Furthermore, the fact that GLA can interact synergistically with a concurrent paclitaxel exposure has important mechanistic implications. Recent studies on the molecular mechanisms by which paclitaxel leads to cell cycle arrest and cell death indicate that the drug modulates key elements in signalling pathways governing cell cycle regulation and apoptosis [20]. Similarly, it has been reported that GLA inhibits cell cycle progression affecting cell cycle events and also inducing apoptosis [21]. Because apoptosis is activated through several mechanisms at different phases of the cell cycle, it is possible that the mechanism by which GLA enhances

paclitaxel-induced cytotoxicity may involve the upregulation of apoptotic signalling pathway(s).

Enhanced lipid peroxidation has been proposed as one of the main mechanisms by which PUFAs such as GLA inhibit tumour cell growth *in vitro* [18,22]. Moreover, the cytotoxic effects of PUFAs through a mechanism involving a generation of lipoperoxides may relate to the different modes of exposing breast cancer cells to PUFAs. Thus, the presence of albumin in the culture medium may decrease the inhibitory effects of free fatty acids *in vitro* [23]. Furthermore, a variety of antioxidants such as Vitamin E have been shown to decrease the growth-inhibitory effects of PUFAs *in vitro* [24]. In our experiments, we employed an ethanolic solution of GLA methyl ester in the presence of 10% serum, a form of delivery which may increase the lipid peroxidation process. In order to investigate whether the effects of GLA on paclitaxel cytotoxicity are dependent on the oxidative status of the ethanolic solution of the PUFA and also to study lipid peroxidation as a potential mechanism through which GLA may modulate the chemosensitivity of tumour cells, we examined paclitaxel activity in GLA-supplemented cells under conditions inhibiting lipid peroxidation. We found that Vitamin E, known as a radical scavenging antioxidant, significantly prevented the additive interaction observed in the sequential schedule at most concentrations of GLA described in the experiment. In contrast, addition of Vitamin E was ineffective in abolishing the synergistic effect of GLA on paclitaxel cytotoxicity observed in the simultaneous schedule except when GLA was used at low concentrations. This suggests that the enhanced cytotoxicity reported after the pretreatment of cancer cells with GLA could be due to the additive effects of the cytotoxicity of the fatty acid (involving lipid peroxidation) and the drug. There are, however, additional mechanism(s) probably not related to lipid peroxidation which may account for part of the synergistic cytotoxicity observed in the simultaneous schedule.

Our results show that the pre-exposure of MDA-MB-231 cells to OA enhances paclitaxel cytotoxicity efficiently in a dose-dependent manner. This did not occur when OA was simultaneously supplemented, which suggests that the enhancement of paclitaxel sensitivity by OA may involve perturbations in the fluidity and permeability of tumour cell membranes. However, this result was unique to MDA-MB-231 cells. Using the sequential schedule no consistent changes in paclitaxel-induced growth inhibition were noted in the other cell lines. Mechanistically, enhanced paclitaxel cytotoxicity induced by OA must be complex and not just related to membrane fluidity.

The effects of the concurrent exposure to a panel of PUFAs on paclitaxel-induced cytotoxicity was examined for comparison. It was found that GLA was the most potent of all tested PUFAs at enhancing paclitaxel efficacy. For most of the PUFAs, the effect on drug activity decreased with the double bond index of the PUFAs. The only exception was LA, which did not increase the sensitivity of MDA-MB-231 cells to paclitaxel. Because peroxidation of highly unsaturated fatty acids is a mechanism through which PUFAs modulate the chemosensitivity of tumor cells [1], this result, in conjunction with our observation employing the antioxidant Vitamin E, strongly suggests that the synergistic effect of GLA on paclitaxel activity could result from a mechanism unrelated to lipid peroxidation.

We are presently investigating whether our observations employing the dietary fatty acids GLA and OA might extend to other anticancer drugs. Using a panel of human breast cancer cell lines, we have found that GLA significantly potentiates the cytotoxic activity of the semisynthetic vinca alkaloid vinorelbine (Navelbine®), a lipophilic antimicrotubule agent. When these interactions were analysed through median-effect and isobologram techniques, a synergistic interaction between GLA and the chemotherapeutic drug was identified, which was independent of the treatment schedule. The interaction between GLA and the anticancer agent 2′,2′-difluorodeoxycytidine (Gemcitabine®) was modest, with only additive to slightly antagonistic effects. OA significantly increased the sensitivity to vinorelbine, which was dependent on the treatment schedule and the type of breast cancer cell line. In contrast, OA did not increase the antitumour activity of 2′, 2′-difluorodeoxycytidine (data not shown).

In conclusion, our study has shown that GLA potentiates the cytotoxicity of paclitaxel in human breast cancer cells, and that the nature of the interaction between GLA and paclitaxel is synergistic using both isobologram and median-effect analysis when the two compounds are administered concurrently. Since it has been reported that GLA can selectively kill malignant cell lines while causing little or no harm to normal cell lines [8], additive and supra-additive toxicities can be of great value in the clinic. Our study has also shown the first evidence that OA, a dietary fatty acid inversely correlated with breast cancer risk [14,15], modulates the sensitivity of breast cancer cells to paclitaxel. The present findings, using a panel of four human breast cancer cell lines, strongly suggest that GLA or OA supplementation during administration of paclitaxel could improve the effects of chemotherapy in breast cancer patients, although caution must be applied when extrapolating *in vitro* results into clinical practice.

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