PERGAMON

European Journal of Cancer 36 (2000) 423–427

European Journal of Cancer

www.elsevier.com/locate/ejconline

Effect of 1-γlinolenyl-3-eicosapentaenoyl propane diol on the growth of human pancreatic carcinoma *in vitro* and *in vivo*

D. Ravichandran*, A. Cooper, C.D. Johnson

University Surgical Unit, Southampton General Hospital, Southampton SO16 6YD, UK

Received 13 January 1999; received in revised form 16 August 1999; accepted 20 August 1999

Abstract

Essential fatty acids such as gammalinolenic (GLA) and eicosapentaenoic (EPA) acids have been proposed as anticancer drugs. The aim of this study was to test the effect of a lipid emulsion containing both GLA and EPA in a novel chemical formulation of 1-γlinolenyl-3-eicosapentaenoyl propane diol on the growth of human pancreatic carcinoma *in vitro* and in nude mice. This compound had a dose-dependent growth-inhibitory effect on human pancreatic cancer cell lines MIA PaCa-2 and Panc-1 *in vitro*. The concentration necessary for 50% growth inhibition was 25 μmol/l for MIA PaCa-2 and 68 μmol/l for Panc-1 (95% CI 20–29 and 59-77 μmol/l respectively). Nude mice bearing subcutaneous pancreatic tumours produced with the MIA PaCa-2 cell line were treated with the maximum tolerated dose (6.75 mg GLA and 7.3 mg EPA per g of body weight) administered over 10 days by daily intravenous (i.v.) bolus injections. No antitumour effect or major alteration in tumour lipid fatty acid composition was seen in comparison with control animals. Concurrent treatment with parenteral iron (iron saccharate, 5 μg/gram body weight daily) did not make a significant difference. Further improvements in fatty acid delivery mechanisms are necessary before they can become useful anticancer agents.

Keywords: Gamma linolenic acid; Eicosapentaenoic acid; Pancreatic neoplasms

1. Introduction

Pancreatic carcinoma is a common cause of cancer deaths and results of chemotherapy are disappointing. Essential fatty acids (EFAs) that belong to the n-6 and n-3 groups, such as gammalinolenic acid (GLA) and eicosapentaenoic acid (EPA) are growth inhibitory to pancreatic cancer cells *in vitro* [1,2]. The antitumour effect is considered to be due to increased tumour lipid peroxidation and free radical damage [3–5]. GLA, as a lithium salt, has been reported to prolong the survival of patients with irresectable pancreatic cancer in a dose dependent fashion [6] and fish oil containing EPA has a beneficial effect on the cachexia that accompanies pancreatic cancer [7].

Free fatty acids and their salts are irritant to the endothelial surface causing thrombophlebitis and also cause haemolysis by their detergent-like effect on the red cell membranes, limiting the amount of fatty acid that can be administered parenterally [6]. To administer larger doses of EFAs a non-toxic (or less toxic) formulation is needed. In addition, in view of the fact that both EPA and GLA have individually proved effective in pancreatic cancer both in *in vitro* and clinical studies, a combination of both fatty acids may prove more effective than either alone. Here we have studied the effect of a lipid emulsion containing both GLA and EPA in the form of a propane diol on the growth of human pancreatic carcinoma *in vitro* and in nude mice.

2. Materials and methods

2.1. In vitro *study*

The pancreatic ductal carcinoma cell lines MIA PaCa-2 and Panc-1 (European Collection of Animal Cell Cultures, Salisbury, UK) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% v/v L-glutamine-antibiotic

* Corresponding author at Department of Surgery, Box 201, Addenbrooke's Hospital, Cambridge CB2 2QQ, UK. Fax: +44-1223-586932.

0959-8049/00/$ - see front matter
PII: S0959-8049(99)00268-3

solution (G-1146) (all from Sigma, Poole, UK), at 37°C in the presence of humidified 5% CO_2 in air. Cells were harvested using trypsin-EDTA (Sigma), viability was established by trypan blue exclusion, cells were counted using a haemacytometer and seeded at 2500 cells in 100 μl of medium per well in 96-well flat bottom cell culture plates.

The EFA containing lipid emulsion (Efamol Research Inc., Kentville, Nova Scotia, Canada) consisted of 20 g 1-γlinolenyl-3-eicosapentaenoyl propane diol and 2 g oat galactolipid made up to 100 g with 2.3% (w/w) glycerol in water, homogenised in an Emulsiflex C-30 homogeniser for 5 min at 11×10^3 psi and stored between 0 and 5°C under nitrogen. The final gas chromatography analysis revealed a GLA concentration of 9 g/100 ml and an EPA concentration of 9.7 g/100 ml. 24 h after the seeding of cells, this lipid emulsion in 100 μl of medium was added to the wells to achieve final GLA concentrations varying from 2.5 to 640 μmol/l. The corresponding EPA concentrations were very similar (2.48–635 μmol/l).

Control experiments were carried out with a commercially available lipid emulsion used for parenteral nutrition (Ivelip 20%, Baxter, Norfolk, UK) to exclude a non-specific effect of lipid emulsion on the cells. Ivelip 20% consists of Soya oil 20 g and glycerol 2.5 g/100 ml. This contained no EPA or GLA and the predominant fatty acids were linoleic and oleic acids. There were also control wells containing culture medium only (no additives).

The cell culture plates were inspected daily under a microscope. After 7 days of incubation, the plates were removed and the number of live cells in each well was estimated using the microculture tetrazolium (MTT) assay [8]. This assay had been previously validated against these cell lines in our laboratory. Cytotoxicity or growth inhibition (cytostasis) was calculated as the percentage optical density of test wells relative to the control wells containing cells in culture medium only.

2.2. In vivo *study*

6 to 8-week-old BALB/c nude mice (Harlan UK Ltd, Bicester, Oxon, UK) were housed in groups of four to six in polycarbonate cages fitted with filter lids and sterilised wood chip bedding in a dedicated pathogen-free clean isolation unit with controlled temperature (19–21°C) and humidity (>50%) with 12 h light/dark cycles. Throughout the experiment they were allowed free access to sterilised standard mouse diet (Rat and mouse breeding diet no: 3, Special Diet Services Ltd, Essex, UK) with water '*ad libitum*'. All experiments were conducted in conformity with the laws and regulations controlling experiments on live animals by the Home Office, UK.

2.3. *Tumour transplantation*

A tumour was generated from MIA PaCa-2 cells by innoculation into the flank of a nude mouse. Small fragments of this tumour were placed subcutaneously over the left flank of nude mice. This tumour model was characterised by a progressive increase in tumour volume without local invasion, distant metastasis or any significant loss of host body weight. When two subsequent tumour volume measurements at weekly intervals showed progressive growth, the mice were randomly divided into treatment and control groups.

2.4. *Treatment*

One treatment group ($n=11$) received the diol at a total dose of 6.75 mg of GLA/g of body weight and 7.275 mg of EPA/g of body weight, administered over 10 days by daily intravenous (i.v.) bolus injections in the tail veins. This was found to be the maximum tolerated dose by the nude mice in preliminary experiments. Another group ($n=6$) received a similar dose of EFAs, but in addition received parenteral iron (as iron saccharate, Veno-Ferrum, Vifor (International) Ltd, Switzerland) at a dose of 5 μg/g of body weight daily, for 10 days, dissolved in 0.9% saline. The first day's dose of iron was given i.v. as a loading dose followed by 9 daily intraperitonial (i.p.) injections. The control group ($n=8$) received 10 daily injections of normal saline in the tail vein.

2.5. *Tumour response*

Tumour response was assessed from relative change in the tumour volume in relation to the volume at the beginning of therapy. The tumour volume was calculated from the greatest transverse (width) and longitudinal (length) diameters of the tumour using the formula: volume = length × width^2/2 [9]. Tumour volume was measured at the beginning of treatment, at the end of treatment and then weekly for 4 weeks.

2.6. *Adverse effects*

The mice were inspected daily during the period of experiment for any signs of disease or distress. Each mouse was weighed at the beginning of the treatment, at the end of treatment and then weekly for 4 weeks.

2.7. *Fatty acid analysis of tumours*

Six animals with tumours not taking part in the study were treated in a similar manner (diol $n=3$, control $n=3$). They were killed at the end of the treatment period. At the end of the 4-week follow-up period 3–4 mice from each group in the study were also killed. Tumours

were quickly removed, snap frozen in liquid nitrogen after discarding the central necrotic portion and stored at −80°C. These were shipped in dry ice to Efamol Research Laboratories in Nova Scotia, Canada where the analysis was performed without knowledge of the treatment groups [10]. Briefly, the total lipids were extracted from the tumours with chloroform–methanol [11], the phospholipid fraction was separated by thin layer chromatography, and fatty acids of the total lipid and phospholipid fractions were methylated and the resulting methyl esters of fatty acids were analysed at room temperature by reversed-phase high performance liquid chromatography (HPLC). The results are presented as the percentage of total fatty acids.

2.8. Statistical methods

The difference in tumour volumes and body weights between treatment and control groups was studied using the Mann–Whitney U test. The tumour fatty acid content was compared between groups using the *t*-test for two independent samples. $P < 0.05$ was considered significant.

3. Results

3.1. In vitro studies

GLA/EPA emulsion showed a dose-dependent growth-inhibitory effect on both human pancreatic cancer cell lines *in vitro* (Fig. 1). The concentration necessary for 50% growth inhibition was 25 μmol/l of GLA and EPA for MIA PaCa-2 cell line and 68 μmol/l for Panc-1 cell line (95% CI were 20–29 and 59–77 μmol/l respectively). In contrast, the control lipid emulsion with a similar lipid load did not have any effect on the growth of these cells (data not shown).

3.2. In vivo studies

In nude nice the tumour volumes and mouse body weights were not statistically different between treatment and control groups at the beginning of the treatment. Administration of EFA diol did not result in tumour growth inhibition (Fig. 2). Concurrent administration of parenteral iron also did not make a significant difference although the tumours were generally smaller than the other groups. No adverse effects or significant difference in the host body weight were seen between the treatment and control groups throughout the experiment (Fig. 3).

Fatty acid analysis revealed no significant differences in n-6 and most n-3 fatty acid contents of tumour phospholipids and total lipids between the treated and untreated groups, even at the end of the treatment period (Table 1) with the exception of 22:5n-3 which was high in the treated group at the end of the treatment period ($P = 0.03$, paired *t*-test).

4. Discussion

Administration of a large amount of free fatty acids (FFA) (or their salts) i.v. can cause both local (thrombophlebitis) and general (haemolysis) toxic effects [6,12], and the half-life of FFA is extremely short in the circulation [13]. Administration of EFAs in the form of a lipid emulsion can overcome these difficulties.

The emulsion, which contained both GLA and EPA in a novel chemical formulation of propane diol, was

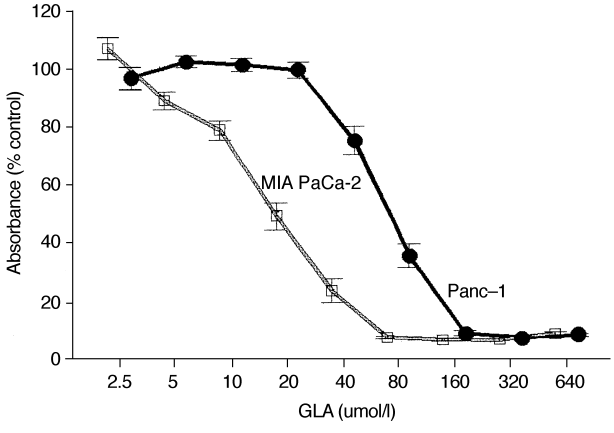

Fig. 1. The effect of 1-γlinolenyl-3-eicosapentaenoyl propane diol on the growth of MIA PaCa-2 and Panc-1 cell lines. The growth of cells exposed to the diol for 7 days (only the corresponding GLA concentrations are shown) is expressed as a percentage of the growth of cells exposed to culture medium only. The values are the means of three separate observations, each consisting of 6–12 test wells; with standard error of the mean (SEM).

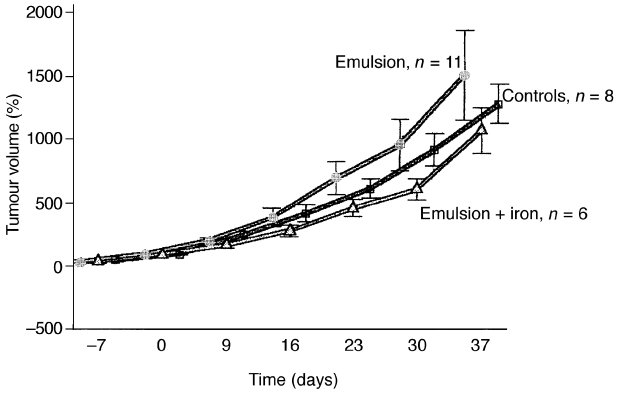

Fig. 2. The effect of intravenous (i.v.) 1-γlinolenyl-3-eicosapentaenoyl propane diol (total dose = 6.75 mg GLA and 7.3 mg EPA/g of body weight given over 10 days) with and without parenteral iron (iron saccharate 5 μg/g daily for 10 days) on tumour volumes in nude mice. The tumour volume is expressed as a percentage of the volume at the beginning of treatment (day = 0). The treatment period was from day 0 to 9. Means; with SEM

growth inhibitory to both human pancreatic cancer cell lines tested *in vitro* in a dose dependent fashion. Both EPA and GLA, on their own, also have a similar growth inhibitory effect on these cancer cell lines with the IC_{50} values in the low micromolar range [1,2]. Unlike many other fatty acid preparations used in previous *in vitro* studies, the diol allows the administration of a relatively large amount of EFAs parenterally. In a previous study using the same nude mouse–human pancreatic tumour model and the lithium salt of GLA, it was found that the maximum dose of GLA that could be administered over a 10-day period was 1 mg/g of body weight [12]. This did not influence the tumour growth or the tumour fatty acid composition. Using the diol a dose of approximately 7 mg of GLA and 7 mg of EPA/g of body weight was administered over a 10-day period in this study. Despite this higher dose and the fact that the MIA PaCa-2 cell line used in tumour production was highly sensitive to the diol *in vitro*, no tumour growth inhibition was seen in mice. This was consistent with the failure to influence tumour fatty acid composition even at the end of treatment. Modification of tumour membrane fatty acid composition appears to be necessary for the antitumour effect of EFAs [14]. This failure may be due to either an inadequate dose of EFAs or inadequate delivery of EFAs to tumours when administered in this chemical formulation. It may be feasible to increase the dose administered by continuous i.v. infusion, as there were no adverse effects and the dose tolerated was limited only by the volume that can be infused safely as a bolus. However, other factors such as albumin binding of fatty acids and antioxidants [1,15,16] may have interfered with the growth-inhibitory effect of EFAs *in vivo*.

Iron, a potentiator of lipid peroxidation both *in vitro* and *in vivo*, increases the cytotoxic effect of fatty acids on pancreatic and other cancer cells *in vitro* [2,3,17–20], but more physiologically relevant transferrin-bound iron is ineffective in this respect [1]. However, concurrent oral administration of iron has been reported to improve the antitumour effect of fish oil on human breast cancer in mice [20,21]. In this study, animals treated with both EFA emulsion and iron showed a similar increase in tumour volume to the other two groups supporting our *in vitro* findings [1]. Concurrent administration of iron to enhance the effect of fatty acids administered in this form does not appear to be of value.

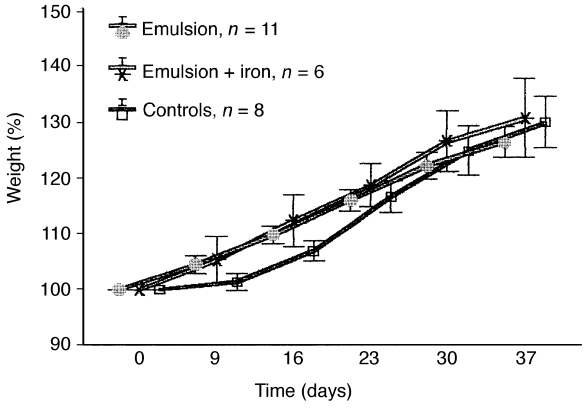

Fig. 3. The mean body weights of mice treated as in Fig. 2, presented as a percentage of the body weight at the beginning of therapy (day = 0); with SEM.

In summary, a propane diol containing both GLA and EPA had a dose-dependent growth-inhibitory effect on human pancreatic cancer cell lines *in vitro* but failed

Table 1
Tumour lipid fatty acid composition. Percentage of total fatty acids[a], mean of 3–4 tumours +/− SEM: (a) phospholipids; (b) total lipids

Fatty acid	End of treatment		End of follow-up	
	Treatment	Control	Treatment	Control
(a)				
14:0	0.6 ± 0.1	0.56 ± 0.02	0.63 ± 0.2	0.42 ± 0.3
16:0 (PA)	21.1 ± 0.6	19.0 ± 0.7	19.5 ± 0.7	19.4 ± 0.4
18:0 (SA)	15.4 ± 0.3	17.0 ± 0.1	16.1 ± 0.5	15.8 ± 0.4
18:1 n-9 (OA)	11.8 ± 0.5	13.0 ± 0.5	12.5 ± 0.6	13.7 ± 0.5
18:1 w-7	4.1 ± 0.1	4.4 ± 0.5	4.1 ± 0.4	4.4 ± 0.3
18:2 n-6 (LA)	10.7 ± 0.2	11.0 ± 0.9	11.3 ± 0.6	12.0 ± 0.8
18:3 n-6 (GLA)	ND	ND	ND	ND
20:2 n-6	1.0 ± 0.1	1.3 ± 0.2	1.2 ± 0.1	1.5 ± 0.2
20:3 n-6 (DGLA)	1.8 ± 0.15	1.9 ± 0.5	1.9 ± 0.4	1.3 ± 0.1
20:4 n-6 (AA)	15.9 ± 0.7	13.7 ± 0.9	14.2 ± 1.0	14.3 ± 1.2
22:4 n-6	1.5 ± 0.16	1.9 ± 0.4	1.6 ± 0.4	2.0 ± 0.2
22:5 n-6	ND	ND	ND	ND
18:3 n-3 (ALA)	ND	ND	ND	ND
20:5 n-3 (EPA)	0.72 ± 0.02	ND	ND	ND
22:5 n-3	2.4 ± 0.1[b]	1.7 ± 0.4	1.6 ± 0.1	1.5 ± 0.1
22:6 n-3 (DHA)	7.3 ± 0.6	8.2 ± 0.6	8.3 ± 0.1	7.9 ± 0.3
(b)				
14:0	0.7 ± 0.1	0.88 ± 0.1	1.1 ± 0.1	0.6 ± 0.1
16:0 (PA)	20.0 ± 0.4	19.0 ± 1.2	20.3 ± 0.4	20.0 ± 0.4
18:0 (SA)	12.2 ± 1.0	11.6 ± 2.5	10.0 ± 0.8	13.5 ± 0.4
18:1 n-9 (OA)	18.5 ± 1.8	18.5 ± 3.0	20.8 ± 3.0	18.2 ± 1.0
18:1 w-7	3.8 ± 0.3	3.75 ± 0.4	3.5 ± 0.3	4.2 ± 0.1
18:2 n-6 (LA)	13.7 ± 0.7	14.9 ± 2.4	14.4 ± 0.3	13.8 ± 0.4
18:3 n-6 (GLA)	ND	ND	ND	ND
20:2 n-6	1.25 ± 0.2	1.35 ± 0.3	0.85 ± 0.1	1.8 ± 0.2
20:3 n-6 (DGLA)	1.6 ± 0.15	1.8 ± 0.7	1.5 ± 0.6	1.2 ± 0.1
20:4 n-6 (AA)	11.0 ± 1.2	10.7 ± 1.2	9.3 ± 1.7	10.8 ± 1.0
22:4 n-6	1.8 ± 0.4	1.77 ± 0.2	1.2 ± 0.3	2.1 ± 0.3
22:5 n-6	ND	ND	ND	ND
18:3 n-3 (ALA)	ND	ND	0.3 ± 0.02	ND
20:5 n-3 (EPA)	0.65 ± 0.05	ND	0.25 ± 0.02	ND
22:5 n-3	1.9 ± 0.2	1.5 ± 0.2	1.2 ± 0.3	1.4 ± 0.6
22:6 n-3 (DHA)	5.9 ± 0.6	6.8 ± 1.2	5.5 ± 1.2	6.6 ± 0.4

ND, not detected; PA, palmitic acid; SA, stearic acid; OA, oleic acid; LA, linoleic acid; ALA, alpha linolenic acid; GLA, gamma linolenic acid; DGLA, dihomo gamma linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

[a] Total value for each group is less than 100% as irrelevant fatty acids and those present in small amounts are omitted.

[b] $P = 0.03$ compared with controls, paired t test. Due to the small numbers significance was also calculated using the ETA test. The results were the same.

to influence the growth and fatty acid composition of tumours in mice with i.v. bolus dosing. Further refinements of fatty acid delivery mechanisms to improve tumour uptake of EFAs *in vivo* are needed before EFAs can become useful in cancer therapy.

Acknowledgements

Financial support for the study was provided by Scotia Pharmaceuticals, Stirling, Scotland. The Efamol Research Inc., Nova Scotia, Canada provided the lipid emulsion containing 1-γlinolenyl-3-eicosapentaenoyl propane diol and also performed the tumour fatty acid analysis.

References

1. Ravichandran D, Cooper A, Johnson CD. Growth inhibitory effect of lithium gammalinolenate on pancreatic cancer cell lines: the influence of albumin and iron. *Eur J Cancer* 1998, **34**, 188–192.
2. Falconer JS, Ross JA, Fearon KCH, Hawkins RA, O'Riordain MG, Carter DC. Effect of eicosapentaenoic acid and other fatty acids on the growth *in vitro* of human pancreatic cancer cell lines. *Br J Cancer* 1994, **69**, 826–832.
3. Begin ME, Ells G, Horrobin DF. Polyunsaturated fatty acid-induced cytotoxicity against tumour cells and its relationship to lipid peroxidation. *J Natl Cancer Inst* 1988, **80**, 188–194.
4. Mengeaud V, Nano JL, Fournel S, Rampal P. Effects of eicosapentaenoic acid, gammalinolenic acid and prostaglandin E1 on three human colon carcinoma cell lines. *Prostaglandins Leukot Essent Fatty Acids* 1992, **47**, 313–319.
5. Madhavi N, Das UN. Effect of n-6 and n-3 fatty acids on the survival of vincristine sensitive and resistant human cervical carcinoma cells *in vitro*. *Cancer Lett* 1994, **84**, 31–41.
6. Fearon KCH, Falconer JS, Ross JA, *et al*. An open label phase I/II dose escalation study of the treatment of pancreatic cancer using lithium gammalinolenate. *Anticancer Res* 1996, **16**, 867–874.
7. Wigmore SJ, Ross JA, Falconer JS, *et al*. The effect of polyunsaturated fatty acids on the progress of cachexia in patients with pancreatic cancer. *Nutrition* 1996, **12**, S27–S30.
8. Alley MC, Scudiero DA, Monks A, *et al*. Feasibility of drug screening with panels of human tumour cell lines using a microculture tetrazolium assay. *Cancer Res* 1988, **48**, 589–601.
9. Euhus DM, Hudd C, LaRegina MC, Johnson FE. Tumour measurement in the nude mouse. *J Surg Oncol* 1986, **31**, 229–234.
10. de Antueno RJ, Cantrill RC, Huang YS, Ells GW, Elliot M, Horrobin DF. Metabolism of n-6 fatty acids by NIH-3T3 cells transfected with the *ras* oncogene. *Mol Cell Biochem* 1994, **139**, 71–81.
11. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 1957, **226**, 497–509.
12. Ravichandran D, Cooper A, Johnson CD. Effect of lithium gammalinolenate on the growth of experimental human pancreatic carcinoma. *Br J Surg* 1998, **85**, 1201–1205.
13. Heiling VJ, Miles JM, Jensen MD. How valid are isotopic measurements of fatty acid oxidation? *Am J Physiol (Endocrinol Metab 24)* 1991, **261**, E572–577.
14. Fujiwara F, Todo S, Imashuku S. Fatty acid modification of cultured neuroblastoma cells by gamma linolenic acid relevant to its antitumour effect. *Prostaglandins Leukotrienes Medicine* 1987, **30**, 37–49.
15. Hayashi Y, Fukushima S, Hirata T, Kishimoto S, Katsuki T, Nakano M. Anticancer activity of free gamma linolenic acid on AH-109A rat hepatoma cells and the effect of serum albumin on anticancer activity of gamma linolenic acid *in vitro*. *J Pharmacobio-Dyn* 1990, **13**, 705–711.
16. Hostmark AT, Lystad E. Growth inhibition of human hepatoma cells (HepG2) by polyunsaturated fatty acids. Protection by albumin and vitamin E. *Acta Physiol Scand* 1992, **144**, 83–88.
17. Das UN. Tumoricidal action of *cis*-unsaturated fatty acids and their relationship to free radicals and lipid peroxidation. *Cancer Lett* 1991, **56**, 235–243.
18. Cantrill RC, Ells G, Chisholm K, Horrobin DF. Concentration-dependent effect of iron on gamma-linolenic acid toxicity in ZR-75-1 human breast tumour cells in culture. *Cancer Lett* 1993, **72**, 99–102.
19. Takeda S, Horrobin DF, Manku M, Sim PG, Ells G, Simmons V. Lipid peroxidation in human breast cancer cells in response to gamma linolenic acid and iron. *Anticancer Res* 1992, **12**, 329–334.
20. Gonzalez MJ, Schemmel RA, Gray JI, Dugan L, Sheffield LG, Welsch CW. Effect of dietary fat on growth of MCF-7 and MDA-MB231 human breast carcinomas in athymic nude mice: relationship between carcinoma growth and lipid peroxidation product levels. *Carcinogenesis* 1991, **12**, 1231–1235.
21. Hardman WE, Cameron IL, Knight C, Barnes CJ, Grant W. Dietary n-3 polyunsaturated fatty acids as an effective adjuvant to ET-18-OCH3 (edelfosine) chemotherapy. *Am Assoc Cancer Res* 1996, **37**, 387 (abstract).