Pergamon

European Journal of Cancer, Vol. 34, No. 1, pp. 188–192, 1998

Printed in Great Britain
0959-8049/98 $19.00+0.00

PII: S0959-8049(97)00369-9

Original Paper

Growth Inhibitory Effect of Lithium Gammalinolenate on Pancreatic Cancer Cell Lines: the Influence of Albumin and Iron

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

University Surgical Unit, Southampton General Hospital, Southampton SO16 6YD, U.K.

Essential fatty acids, especially gamma linolenic (GLA) and eicosapentaenoic acids, have been proposed as potential anticancer drugs. Our aim was to study the effect of the lithium salt of gamma linolenic acid (LiGLA) on the growth of two human pancreatic cancer cell lines (MIA PaCa2 and Panc 1) and primary human fibroblasts (HFF 5) *in vitro*. Cell growth was assessed by a microculture tetrazolium (MTT) assay. LiGLA had a selective growth inhibitory effect on pancreatic cancer cell lines with 50% growth inhibition (IC_{50}) at approximately 6–16 μmol/l compared with approximately 111 μmol/l for the fibroblasts. The degree of growth inhibition increased with the time of exposure to LiGLA. Special attention was paid to the influence of albumin and iron on LiGLA-mediated growth inhibition. Albumin incorporated into essentially serum-free culture medium inhibited the effect of LiGLA in a dose-dependent manner, associated with reduced GLA uptake by cancer cells. Ferric ions were confirmed as potentiators of the growth inhibitory effect of LiGLA but more physiologically relevant transferrin-bound iron was ineffective. With further improvements in the fatty acid delivery mechanism, LiGLA may become a useful adjunct in the management of pancreatic cancer patients.

Key words: gamma linolenic acid, pancreatic neoplasms, tumour cells, cultured
Eur J Cancer, Vol. 34, No. 1, pp. 188–192, 1998

INTRODUCTION

PANCREATIC CANCER is a common and lethal malignancy. The five-year survival is the worst of all common human cancers [1]. Most patients present late with advanced inoperable tumours and can only be offered palliative therapy. Currently available chemotherapy is mostly ineffective in prolonging survival. In addition, the side-effects of therapy impair the quality of life during the short period between diagnosis and death [2]. There is a need for effective antitumour therapy with minimal side-effects.

Polyunsaturated fatty acids, mainly those derived from the two essential fatty acids linoleic and alpha linolenic acids, may fulfil this role. Fatty acids such as gamma linolenic acid (GLA) and eicosapentaenoic acid (EPA) have direct growth inhibitory effects on malignant cells *in vitro* [3]. GLA, from the linoleic acid (*n*-6) family, has shown high selectivity for cancer cells with good cytotoxicity [4]. Until recently, GLA was available for clinical use only in the form of evening primrose oil (9% GLA) which is unsuitable for administering large therapeutic doses of GLA. A relatively stable and pure form of injectable GLA is now available as a lithium salt (LiGLA) (Scotia Pharmaceuticals, Stirling, U.K.) and this compound has been reported to prolong the survival of patients with inoperable pancreatic cancer [5]. In this study, we tested the effect of LiGLA *in vitro* on two human pancreatic ductal carcinoma cell lines and non-malignant fibroblasts. As binding to plasma albumin is reported to interfere with the cytotoxic effect of fatty acids *in vivo* [6, 7], we also studied the influence of human albumin on the action of LiGLA on pancreatic cancer cells. Although GLA is the precursor of biologically important eicosanoids, its antitumour activity is considered to be related to increased tumour cell lipid peroxidation rather than to its metabolites [8–10]. As iron is a promoter of lipid peroxidation, it may be feasible to increase the cytotoxic effect of LiGLA by concurrent administration of iron to patients. Thus, we studied the influence of both free and transferrin-bound iron on the effect of LiGLA.

Correspondence to C. D. Johnson.
Received 11 Dec. 1996; revised 1 Jul. 1997; accepted 15 Jul. 1997.

MATERIALS AND METHODS

Cytotoxicity studies

The pancreatic ductal carcinoma cell lines MIA PaCa2 and Panc1 (European Collection of Animal Cell Cultures, Salisbury, U.K.), and primary low-passage non-malignant human skin fibroblasts HFF 5 (provided by A. Richter, Medical Oncology, Southampton General Hospital) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and L-glutamine-antibiotic solution (G-1146) (all from Sigma, Poole, U.K.), at 37°C in the presence of humidified 5% CO_2 in air. Cells were harvested using trypsin–EDTA (Sigma), counted using a haemacytometer and seeded at 2500 cells in 100 µl of medium per well in 96-well flat-bottomed cell culture plates.

24 h later, LiGLA in 100 µl of medium was added to the wells to achieve final LiGLA concentrations varying from 5 to 490 µmol/l. Control experiments were run with lithium ions (0.625–2 mmol/l, as lithium chloride, Sigma), palmitic acid (up to 490 µmol/l, Sigma) and culture medium only (no additives). The plates were inspected daily under a microscope. After 7 days the plates were removed and a microculture tetrazolium (MTT) assay [11], which had been previously validated against these cell lines in our laboratory, was used to estimate the live cell number in the wells. 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.

For time course studies, multiple plates were seeded with MIA PaCa 2 cells and LiGLA (5–100 µmol/l) was added as outlined above. Plates were taken for MTT assay 24 h after the addition of LiGLA and then sequentially every 24 h until and including day 7.

Effect of albumin on LiGLA cytotoxicity

Panc 1 cells were grown in low serum culture medium (LSM) which consisted of equal volumes of Ham Nutrient Mixture (F-12) and DMEM, 0.5% FBS and L-glutamine-antibiotic solution (G-1146) (all from Sigma). The cells were seeded as described previously. 24 h after plating the cells, the medium in the wells was replaced with 200 µl of LSM containing 10 µmol/l of LiGLA and varying levels of albumin. Control experiments were run with LSM alone, albumin alone in LSM (levels corresponding the test wells) and 10 µmol/l LiGLA alone in LSM.

The albumin–LiGLA mixture was prepared as follows: fatty acid-free human albumin (Sigma) was added to warmed (37°C) LSM to achieve a concentration of 80 µmol/l. This was mixed well, filter sterilised and serially diluted to 2.5 µmol/l. The pH of this medium was 7.6 ± 0.2. LiGLA was added to each albumin concentration to achieve a level of 10 µmol/l and mixed. The mixture was gently agitated at 37°C for 2 h under nitrogen before being added to the cells. Plates were then handled as before and incubated for 7 days. Growth inhibition was expressed as the percentage growth in test wells containing LiGLA and albumin compared with control wells containing similar levels of albumin only.

Effect of albumin on GLA uptake by tumour cells

1×10^5 Panc 1 cells were seeded in to 12.5 cm^3 culture flasks in 2 ml of LSM and left to settle overnight. The next day the medium was removed and replaced with 2 ml of LiGLA–albumin mixture prepared as above. This contained 10 µmol/l of LiGLA spiked with ^{14}C LiGLA, giving a radioactivity of 0.5 µCi/ml, and varying levels (80, 40, 10, 5 and 0 µmol/l) of fatty acid free human albumin. After 24 h of incubation, the medium was removed and centrifuged to recover any non-adherent cells. The cells in the flask were then harvested. All cells were washed in saline three times to remove unbound radioactive material, solubilised by the addition of a drop of solvable™ solution (Canberra-Packard, U.K.), 15 ml of scintillation fluid (Ultima Gold, Canberra-Packard, U.K.) was added and radioactivity was determined in a liquid scintillation counter. The results were expressed as a percentage of radioactivity incorporated at a particular albumin concentration in relation to the radioactivity incorporated in the absence of added albumin.

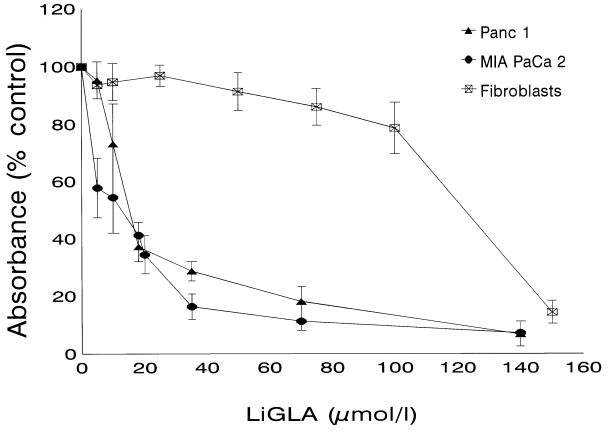

Figure 1. The effect of increasing concentration of LiGLA on the growth of MIA PaCa2 and Panc 1 cell lines and fibroblasts. The optical density of the wells containing cells exposed to LiGLA for 7 days is expressed as a percentage of the medium-only control wells. The values at each concentration are the means of 3–8 separate observations, each consisting of 4–10 test wells; vertical bars represent standard error of the mean (SEM).

Effect of iron on LiGLA cytotoxicity

MIA PaCa2 cells were seeded in 96-well cell culture plates. The following day iron, as ferric chloride (Sigma) or as

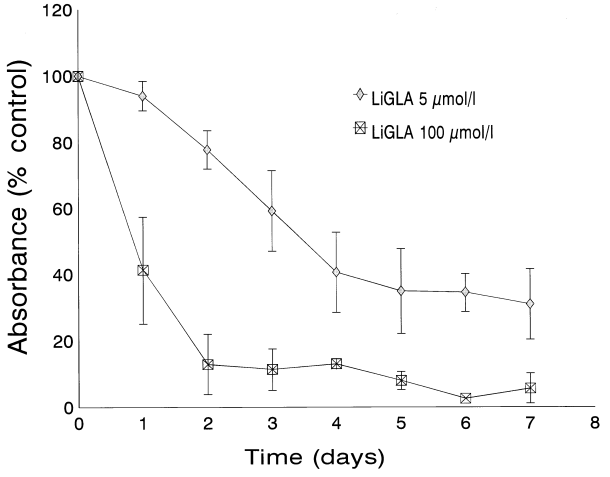

Figure 2. Time course of the effect of LiGLA (5 µmol/l and 100 µmol/l) on MIA PaCa2 cells. The optical density of the wells containing cells exposed to LiGLA for 7 days is expressed as a percentage of the medium-only control wells. The values are the means of three experiments, each consisting of six test wells; with SEM.

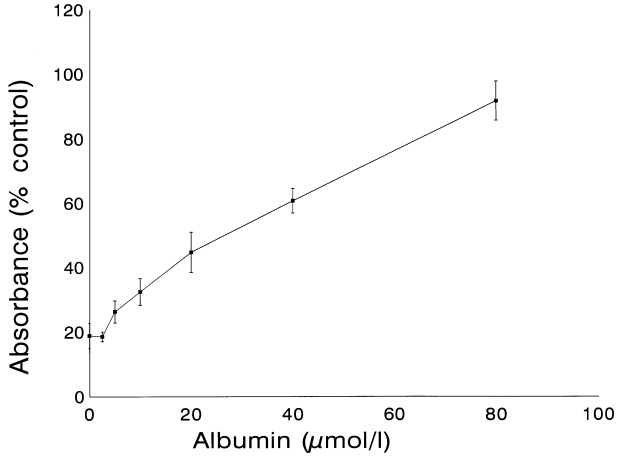

Figure 3. The effect of increasing albumin concentration on the cytotoxicity of 10 μmol/l of LiGLA on Panc 1 cell line. The optical density of the wells containing cells exposed to LiGLA for 7 days is expressed as a percentage of the optical density of the control wells containing the same level of albumin but no fatty acid. The values are the means of three separate experiments, each consisting of 12 test wells; vertical bars represent SEM.

human ferro-transferrin (holo-Transferrin, Sigma) was added to the wells (12.5 and 100 μmol/l) dissolved in standard culture medium followed by LiGLA (0–150 μmol/l). Subsequently, the plates were handled as for the cytotoxicity assay. The cell growth at each iron concentration is calculated as the percentage optical density of wells containing LiGLA and iron in relation to wells containing culture medium only.

RESULTS

Cytotoxicity and time course studies

LiGLA had a dose-dependent growth inhibitory effect on both cancer cell lines (Figure 1). The cytotoxic effect of LiGLA at high concentrations was visible under the microscope within 24 h of addition and was complete by 72 h. At low concentrations (< 20 μmol/l), cytotoxicity was not observed but significant growth inhibition was seen. Fifty percent growth inhibition (IC_{50}) of MIA PaCa 2 cell line was seen at 10.7 μmol/l (95% confidence interval (CI) 6.3–15.2 μmol/l) and that of Panc 1 at 12.6 μmol/l (95% CI 9.3–15.8). By contrast, the IC_{50} of benign fibroblasts was 110.8 μmol/l (95% CI 105.9–115.8). Very high concentrations were cytotoxic to both benign and malignant cells. Time course studies (Figure 2) showed that the degree of growth inhibition increased with the time of exposure to LiGLA, reaching a maximum after 5–7 days.

Lithium chloride, up to a concentration of 2 mmol/l and palmitic acid up to a concentration of 490 μmol/l, had no significant effect on the growth of any of the cell lines (data not shown).

Effect of albumin on LiGLA-induced growth inhibition

Human albumin reduced the growth inhibitory effect of 10 μmol/l of LiGLA on Panc 1 cell line in a dose-dependent manner (Figure 3). At a LiGLA:albumin molar ratio of 1:8 (albumin level of 80 μmol/l), nearly all growth inhibition was

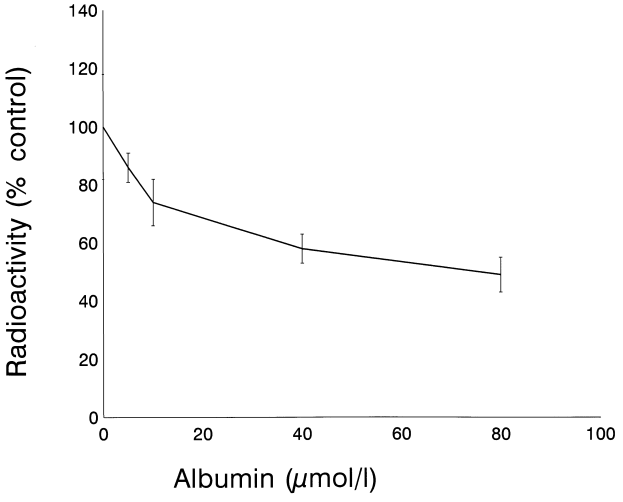

Figure 4. The effect of increasing albumin concentration on the uptake of radiolabelled GLA (10 μmol/l of LiGLA spiked with ^{14}C LiGLA) by Panc 1 cells over 24 h. The radioactivity incorporated at different albumin concentrations is expressed as a percentage of the radioactivity incorporated in the absence of added albumin. The values are the means of five separate experiments; vertical bars represent SEM.

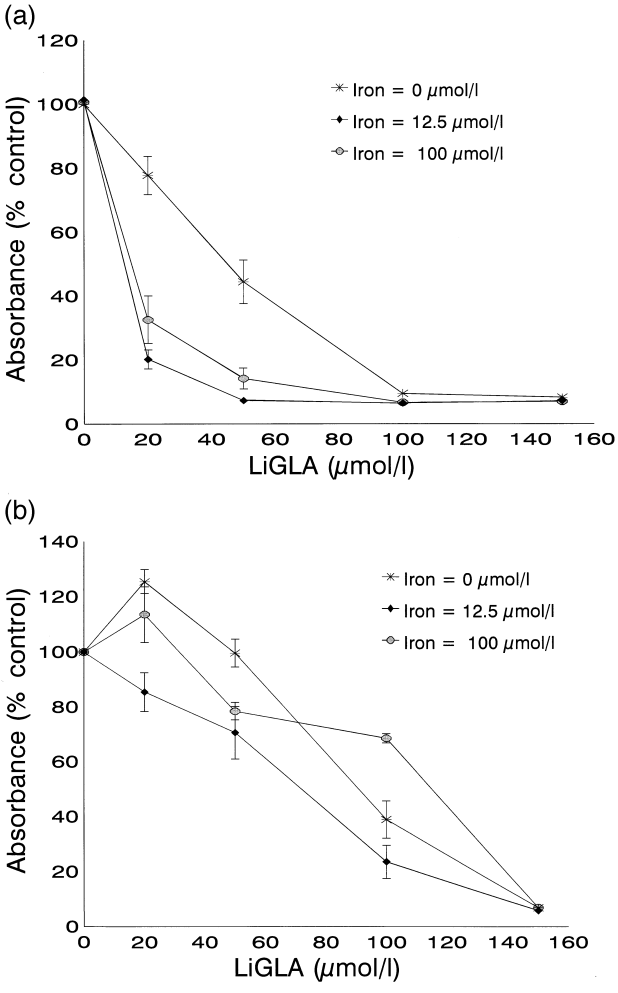

Figure 5. The effect of iron at 0, 12.5 and 100 μmol/l on the cytotoxicity of LiGLA on MIA PaCa2 cell line. The optical density of the wells containing cells exposed to LiGLA and iron for 7 days is expressed as a percentage of the medium-only control wells. (a) Free ferric ions as ferric chloride. (b) Transferrin-bound iron. The values are the means of three separate experiments; vertical bars represent SEM.

lost and in the complete absence of albumin the growth inhibition was maximal at over 80%. Fifty percent growth inhibition of Panc 1 cells was achieved at a molar ratio of 0.48 (95% CI, 0.26–0.7).

Effect of human albumin on the uptake of LiGLA by MIA PaCa2 cells

Albumin significantly ($P < 0.00005$, Student's t-test) inhibited the uptake of LiGLA by cancer cells over 24 h in a dose-dependent manner (Figure 4).

Effect of iron on LiGLA-induced growth inhibition

Ferric chloride increased the growth inhibitory effect of LiGLA at both 12.5 and 100 μmol/l (Figure 5(a)). In contrast, transferrin-bound iron had no influence on the effect of LiGLA (Figure 5(b)). While ferric chloride on its own had no effect on cell growth, transferrin-bound iron added to culture medium promoted the growth of MIA PaCa2 cells (data not shown).

DISCUSSION

Gamma linolenic acid (GLA) is an attractive concept as an anticancer agent in pancreatic carcinoma. It does not have the side-effects of conventional chemotherapy and does not impair the quality of life of cancer patients. This is the first study of the lithium salt of GLA (LiGLA) on pancreatic cancer cells and has shown that LiGLA has a significant growth inhibitory effect on these cells at relatively low concentrations of 9–16 μmol/l. These concentrations are much lower than those reported for other cell lines, thus pancreatic cancer cell lines appear to be more susceptible to the cytotoxic effect of fatty acids [12]. The effect of LiGLA is very selective towards malignant cells as the concentration necessary for 50% growth reduction of non-malignant fibroblasts was up to 10 times greater than that needed for the cancer cells. Although it would have been ideal to demonstrate this selectivity with a non-malignant pancreatic epithelial cell line, such a cell line is not available. The effect was due to the presence of GLA as control experiments excluded a lithium effect and a non-specific fatty acid effect.

Although GLA is uniformly growth inhibitory when added to malignant cells of both epithelial and non-epithelial origin in culture, the results of *in vivo* studies have been variable, even when the corresponding tumour cell lines were susceptible *in vitro* [9, 13–19]. One possible reason for this discrepancy was the lack of a suitable, stable preparation of GLA. Most animal and clinical studies have used the oil of evening primrose (EPO) [14, 16–19]. As EPO contains only 9% GLA, patients had to take up to 36 capsules per day [14, 19], resulting in problems of compliance. In our experience, up to 10 g of LiGLA may be infused i.v. daily in patients for a period of up to 2 weeks without any significant adverse effects.

Another reason suggested for the possible lack of efficacy of GLA *in vivo* is albumin binding [6, 7], as, like all long-chain fatty acids, GLA is transported bound to albumin in the circulation. However, in other studies, albumin-bound fatty acids have been equally cytotoxic *in vitro* against pancreatic and other cancer cells [12, 20]. In order to elucidate these problems, we studied the effect of albumin on the cytotoxicity of LiGLA *in vitro*. The most important factor regulating the availability of fatty acid to tissues is the molar ratio of fatty acids to albumin [21]. Our results show that albumin interferes with the growth inhibitory effect of LiGLA on malignant cells in a dose-dependent manner. This is associated with a significant dose-dependent reduction in the uptake of the fatty acid by the tumour cells. Fifty percent growth inhibition of MIA PaCa2 cells was achieved at an LiGLA:albumin molar ratio of 1:2.1. As the plasma albumin levels vary between 450 and 750 μmol/l (30–50 g/l) and the interstitial fluid albumin levels can be up to 450 μmol/l (30 g/l) [22], it may be assumed that a much higher concentration of GLA than the concentration which causes growth inhibition *in vitro* is necessary for significant antitumour activity *in vivo*. It is likely that local or regional administration of LiGLA will be necessary to achieve these high concentrations.

Metals such as iron and copper promote lipid peroxidation and GLA-induced cytotoxicity *in vitro*. Ferrous chloride has a dose-dependent promoting effect on the cytotoxicity of GLA against a breast cancer cell line [23] associated with an increase in lipid peroxidation [24]. In human breast cancer grown in nude mice, concurrent administration of oral iron with fish oil resulted in improved tumour regression [25]. However, iron can bind to and influence the structure and function of a variety of macromolecules with deleterious effect to the organism. To protect against this, a number of iron binding proteins exist *in vivo* to store and transport iron. The ferric ions are transported bound to transferrin, a B1 glycoprotein in plasma with a high affinity for iron. Each molecule binds two ferric ions to form ferrotransferrin. In the interstitial fluid the iron–transferrin complex is avidly taken up by the cells by receptor-mediated endocytosis. Under physiological conditions there is always an excess of transferrin in plasma, thus free iron will not be available to cancer cells [26]. This study has shown that transferrin-bound iron (TBI) has no effect on GLA-mediated cytotoxicity *in vitro*. Thus, concurrent parenteral administration of iron with GLA is unlikely to improve its antitumour effect *in vivo*.

In conclusion, LiGLA has a selective, dose- and time-dependent growth inhibitory effect at relatively low concentrations on human pancreatic cancer cell lines. Albumin, in a dose-dependent manner, reduces this growth inhibitory effect which appears to be due to a reduction in the uptake of the GLA by cancer cells. Significant cytotoxicity may still be achieved in the presence of albumin, but requires a much higher concentration of GLA. This may be feasible with the use of LiGLA as large amounts of fatty acid may be infused intravenously or locoregionally in this form. Concurrent administration of iron is unlikely to improve the antitumour effect of LiGLA in patients as in the physiological (transferrin bound) form it is ineffective *in vitro*. Results of early clinical studies are promising [5] and with further improvements in the fatty acid delivery mechanism, LiGLA may become a useful adjunct in the management of pancreatic cancer patients.

1. Coeberg JWW. Summary and discussion of results. In Berrino F, Sant M, Verdecchia A, Capocaccia R, Hakulinen T, Esteve J, eds. *Survival of Cancer Patients in Europe*. The Eurocare study. Lyon, IARC Scientific Publications No. 132, 1995, 447–463.
2. Cullinan S, Moertel CG, Wieand HS, *et al.* A phase 3 trial on the therapy of advanced pancreatic carcinoma. Evaluations of the Mallinson regimen and combined 5-fluorouracil, doxorubicin and cisplatin. *Cancer* 1990, **65**, 2207–2212.
3. Begin ME, Das UN, Ells G. Cytotoxic effects of essential fatty acids (EFA) in mixed cultures of normal and malignant human cells. *Prog Lipid Res* 1986, **25**, 573–576.

4. Begin ME, Ells G, Das UN, Horrobin DF. Differential killing of human carcinoma cells supplemented with *n*-3 and *n*-6 polyunsaturated fatty acids. *J Natl Cancer Inst* 1986, **77**, 1053–1062.
5. 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 gammalinoleate. *Anticancer Res* 1996, **16**, 867–874.
6. 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.
7. 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.
8. 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.
9. 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 Leuk Essent Fatty Acids* 1992, **47**, 313–319.
10. 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.
11. 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.
12. 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.
13. Pritchard GA, Jones DL, Mansel RE. Lipids in breast carcinogenesis. *Br J Surg* 1989, **76**, 1069–1073.
14. van der Merve CF, Booyens J. Oral gamma linolenic acid in 21 patients with untreatable malignancy. An ongoing pilot open clinical trial. *Br J Clin Prac* 1987, **41**, 907–915.
15. Botha JH, Robinson KM, Leary WP. Parenteral gamma linolenic acid administration in nude mice bearing a range of human tumour xenografts. *S Afr Med J* 1983, **64**, 11–12.
16. Ramchurren N, Botha JH, Robinson KM, Leary WP. Effects of gamma linolenic acid on murine cells *in vitro* and *in vivo*. *S Afr Med J* 1985, **68**, 795–798.
17. Booyens J, Koenig L. The effect of dietary supplementation with gamma-linolenic acid on the growth of induced tumours in rats. *S Afr Med J* 1984, **65**, 660–661.
18. McIllmurray MB, Turkie W. Controlled trial of gamma linolenic acid in Duke's C colorectal cancer. *BMJ* 1987, **294**, 1260.
19. van der Merwe CF, Booyens J, Joubert HF, van der Merwe CA. The effect of gamma-linolenic acid, an *in vitro* cytostatic substance contained in evening primrose oil, on primary liver cancer. A double-blind placebo controlled trial. *Prostaglandins Leuk Essent Fatty Acids* 1990, **40**, 199–202.
20. Anel A, Naval J, Desportes P, Gonzalez B, Uriel J, Pineiro A. Increased cytotoxicity of polyunsaturated fatty acids on human tumoral B and T-cell lines compared with normal lymphocytes. *Leukaemia* 1992, **6**, 680–688.
21. Spector AA. Plasma albumin as a lipoprotein. In Scanu AM, Spector AA, eds. *Biochemistry and Biology of Plasma Lipoproteins*. New York, Marcel Dekker, 1986, 247–279.
22. Peters T, Reed RG. Serum albumin as a transport protein. In Blauer G, Sund H, eds. *Transport by Proteins*. Berlin, Walter de Gruyter, 1978, 57–78.
23. 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.
24. 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.
25. 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.
26. Wells MS, Awad WM. Iron and heme metabolism. In Devlin TM, ed. *Text Book of Biochemistry with Clinical Correlations*. New York, John Wiley & Sons, 1986, 855–874.