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Preclinical report

Cytotoxic effects of two gamma linoleic salts (lithium gammalinolenate or meglumine gammalinolenate) alone or associated with a nitrosourea: an experimental study on human glioblastoma cell lines

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Gamma linoleic acid (GLA) salts may exert a direct antiproliferative activity on tumor cells. The cytotoxicity is linked to the generation of conjugated dienes, peroxyl radicals and superoxide radicals. Lithium gammalinolenate (LiGLA) and meglumine gammalinolenate (MeGLA) have been recently developed for enhancing the water solubility of these compounds. MeGLA or LiGLA (10^{-5} to 10^{-4} mol/l) and fotemustine (Fote) (2×10^{-6} to 2×10^{-4} mol/l) were applied, alone or in combination, for up to 9 days to two human glioblastoma cell lines A172 and U373MG. Fote was applied first followed by LiGLA and/or MeGLA. Cytotoxicity was evaluated by the MTT test, and the effects of drug combinations were analyzed by the isobolographic representation according to the Chou and Talalay method (combination indexes). For both GLA salts, cytotoxicity was manifested after 4 days of cell exposure and with very sharp dose–response curves. Comparison of IC_{50} values indicated that MeGLA was more active than LiGLA. There was a constant reduction in IC_{50} values following an increase in exposure time for A172 cells: between 4 and 9 days of cell exposure, IC_{50} changed from 73 to 46 μM for LiGLA and from 49 to 31 μM for MeGLA ($p<0.05$). With U373MG cells, there was no influence of exposure duration on IC_{50} values. Combination index values indicated that association between Fote and GLA salts globally resulted in slightly antagonistic effects. These results may be useful for further development of GLA salts at the clinical level. [© 1999 Lippincott Williams & Wilkins.]

Key words: Fotemustine, gamma linoleic acids, glioblastoma, *in vitro* cytotoxicity.

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Introduction

Gamma linoleic acid (GLA) is a member of the large family of essential fatty acids (EFAs) which are those polyunsaturated fatty acids of the $n-6$ and $n-3$ series containing two or more double bonds. EFAs cannot be synthesized *de novo* by mammals, and are essential for the maintenance of normal mammalian health and function. Much evidence now exists on the anti-tumor and antiproliferative effect of EFAs.[1,2] GLA in particular has been shown to be selectively cytotoxic to more than 30 human cancer cells lines at concentrations that do not harm normal cells.[2–5] Clinical trials with GLA in liver, lung, esophageal, gastric cancers,[6,7] glioma[8] and pancreatic cancer[9] report encouraging results by slowing down tumor growth and prolonging survival with minimal associated toxicity.

Lithium gammalinolenate (LiGLA) and meglumine gammalinolenate (MeGLA) are GLA salts which have been recently developed to allow i.v. administration of GLA because of the enhanced water solubility of these saline forms. Glioblastomas appear to be suitable targets for testing LiGLA and MeGLA because of the relatively high liposolubility of these compounds, allowing their diffusion throughout the CNS barrier. We investigated the cytotoxic effects of LiGLA and MeGLA on two human glioblastoma cell lines. The drugs were tested alone or combined with a reference anti-cancer drug for the treatment of glioblastoma, i.e. the nitrosourea fotemustine (Fote).

Materials and methods

Chemicals

Fote (Servier, Courbevoie, France), commercial source, was obtained from the Pharmacy of our institute. LiGLA and MeGLA were kindly provided by Scotia Pharmaceuticals (Stirling, UK). Dulbecco's modified Eagle's medium (DMEM) and glutamine were from Gibco (Paisley, UK), and fetal bovine serum (FBS) was from Dutscher (Brumath, France). Penicillin and streptomycin were from Merieux (Lyons, France). All other chemicals including MTT [3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide] were obtained from Sigma (St Quentin Fallavier, France).

Culture conditions

Two human cancer cell lines of glial origin were investigated: A172 (ATCC CRL 1620) and U373MG (ATCC HTB 17). Cell doubling times were between 1.26 and 2 days. Cells were grown in a humidified incubator (Sanyo, Tokyo, Japan) at 37°C with an atmosphere containing 8% CO_2. Cells were routinely cultured in a regular DMEM medium supplemented with 10% FBS, 2 mM glutamine, 50 000 U/l penicillin and 80 μM streptomycin. For cytotoxicity experiments, cells were grown in 96-well microtitration plates (0.32 cm^2/well) with the same culture medium. In a preliminary experiment, different cell concentrations were tested and the cell concentration of the inoculate giving the longest exponential growth was retained. In all cases, confluence was not reached after 9 days (70–80% of confluence). All investigations were performed in three independent experiments.

Evaluation of cytotoxicity

Cells were plated in 96-well microtiter plates in order to obtain exponential growth for the whole duration of the experiment (initial cell density 2500 cells/well for A172 and 1700 cells/well for U373MG). Twenty-four hours later, cells were exposed to drugs: LiGLA, MeGLA, Fote and combinations of Fote-GLA. The GLA salts were added as pure compounds (both LiGLA and MeGLA) and Fote as an ethanolic solution; the ethanol solution had no noticeable influence on the culture conditions since at the highest Fote concentration the ethanol concentration was below 1/1000 in the culture medium. The duration of cell exposure to individual drugs was tested from 1 to 9 days. For drug combinations, the duration of cell exposure was from 4 to 9 days.

For drug combinations, Fote was always applied first. Drugs were not renewed during the entire period of cell exposure. Fote concentrations ranged between 10^{-5} and 2×10^{-4} M (11 concentrations). LiGLA and MeGLA concentrations ranged between 10^{-5} and 2×10^{-4} M (11 concentrations). Experimental conditions were tested in quintuplicate. Growth inhibition was assessed by the MTT test 112 h after the end of drug exposure.[10] Results were expressed as the percentage of absorbance compared to controls without drug. Concentration ratios between drugs were chosen following the examination of the respective dose-response curves for GLA and Fote. According to this, the ratio concentration Fote:concentration GLA was 1.

Analysis of data

For *in vitro* investigations, curve fittings were done on Graph Pad Software (ISI, Philadelphia, PA). For each parameter studied, the mean values of three separate experiments were calculated with SD. Drug combinations were analyzed using the method developed by Chou and Talalay:[11] a combination index smaller than 1 or higher than 1 indicates synergism or antagonism, respectively. The effects of cell exposure duration on IC_{50} values were tested by the Spearman rank test and paired compari-sons were performed according to the Wilcoxon paired test.

Results

Cytotoxicity was well established for GLA salts after a minimal exposure duration of 4 days and, in this case, the concentration-effect curves were very sharp (Figure 1). When compared at equimolarity and in terms of IC_{50} values, it appears that for both tested cell lines MeGLA was more active than LiGLA, $p < 0.05$ (Table 1). When comparing cell lines, U373MG cells were relatively more resistant to LiGLA ($p < 0.01$) and MeGLA ($p < 0.05$). For A172 cells there was a constant reduction in IC_{50} values following the increase in exposure time: between 4 and 9 days of cell exposure, IC_{50} (μM) values changed from 73 to 46 for LiGLA and from 49 to 31 for MeGLA (Table 1, $p < 0.05$). However, this decrease in IC_{50} values was not directly related to this increase in time exposure (2.25-fold increase in time and in comparison only 1.58-fold decrease in IC_{50} values for both LiGLA and MeGLA). With U373MG cells there was a non-significant influence of exposure duration on IC_{50} values either for LiGLA or for MeGLA

(Table 1). For Fote, IC_{50} values differed between cell lines, with U373MG cells being relatively more resistant to the effects of the nitrosourea. Combination indexes indicated that the association between Fote and GLA salts globally resulted in values between 1.0 and 2.0. This suggests a slightly antagonistic effect between GLA salts and Fote (Table 2 and Figure 2).

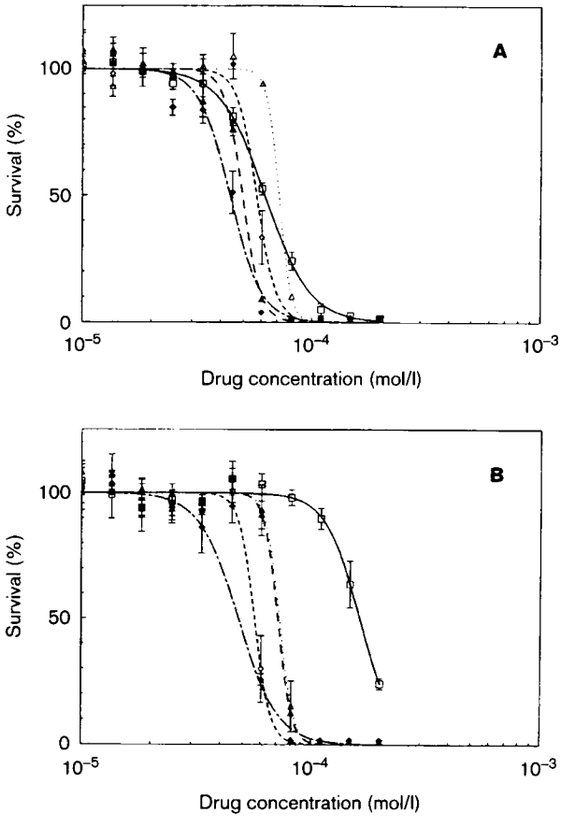

Figure 1. Dose–response curves following a 4 day exposure to Fote alone (□), LiGla alone (△), MeGla alone (◇), Fote+LiGla (▲) or Fote+MeGla (◆). When tested in combination the drug concentration ratio was 1. (A) A172 cells. (B) U373 MG cells.

Discussion

Pilot clinical studies of i.v. and oral treatment with GLA have already been carried out, and tumor regression was noted in patients with mesothelioma, bronchial carcinoma, squamous cell carcinoma, hepatoma and melanoma.[6,7] Treatment of these patients with GLA in clinical trials has shown GLA to be well tolerated in the majority of cases with a low toxicity profile compared to conventional cytotoxic agents. The high liposolubility intrinsic to LiGLA and MeGLA led us to question their use for the treatment of brain tumors. One of the aims of the present study was to test the cytotoxic effects of LiGLA and MeGLA against glioblastoma cells of human origin. Several pieces of clinically useful information are obtained from our study. First, it appears that a critical duration of cell exposure, i.e. 4 days, is needed to achieve a well-established cytotoxicity. It is not certain that prolonging cell exposure beyond this period may result in further cytotoxicity; for one cell line (U373MG), IC_{50} values were not significantly changed from 4 to 9 day exposure, and for the other (A172) there was a significant but nevertheless slight decrease in IC_{50} values which was not

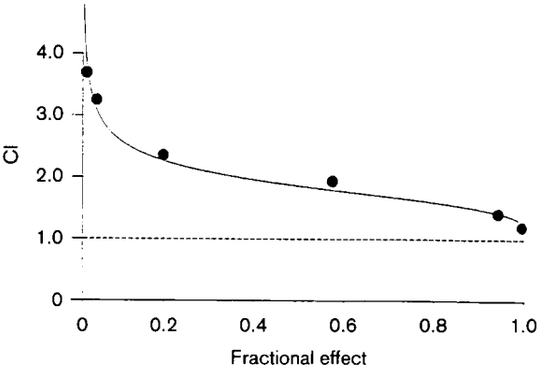

Figure 2. Combination index values of the association of Fote plus MeGla on A172 cells computed according to the mutually non-exclusive Chou and Talalay model.[11]

Table 1. IC_{50} values for A172 and U373MG cell lines (μM, mean ± SD)

Exposure (days)	A172			U373MG		
	Fote	LiGLA	MeGLA	Fote	LiGLA	MeGLA
4	50±10	73±26	47±3	133±30	86±38	52±4
5	50±10	58±7	48±7	140±26	91±34	50±6
6	54±3	60±25	44±2	167±15	80±36	53±6
7	55±4	57±18	34±7	150±44	80±37	51±6
8	60±10	49±7	33±7	150±26	84±33	50±3
9	61±5	46±13	31±5	167±25	93±42	52±6

Table 2. Combination index values for A172 and U373MG cell lines (mean ± SD)

Exposure (days)	A172				U373MG			
	LiGLA + Fote		MeGLA + Fote		LiGLA + Fote		MeGLA + Fote	
	ME	MNE	ME	MNE	ME	MNE	ME	MNE
4	1.4±0.1	1.9±0.2	1.4±0.1	1.9±0.1	1.4±0.1	1.9±0.2	1.3±0.1	1.7±0.3
5	1.4±0.2	1.9±0.3	1.3±0.1	1.7±0.1	1.5±0.4	2±0.7	1.2±0.1	1.5±0.3
6	1.4±0.2	1.9±0.3	1.4±0.1	1.8±0.2	1.4±0.2	1.9±0.2	1.2±0.1	1.4±0.2
7	1.3±0.1	1.7±0.2	1.3±0	1.8±0.1	1.6±0.4	2.2±0.7	1.3±0.1	1.6±0.2
8	1.4±0.2	1.7±0.1	1.3±0	1.7±0	1.6±0.3	1.9±0.5	1.4±0.3	1.6±0.1
9	1.4±0.2	1.7±0.2	1.5±0.3	1.8±0.2	1.4±0.2	1.8±0.4	1.3±0.1	1.7±0.2

According to the Chou and Talalay model,[11] ME=mutually exclusive drugs and MNE=mutually non-exclusive drugs.

proportional to the increase in exposure duration (Table 1).

As nitrosoureas are the reference anticancer agents for the treatment of glioblastoma, we investigated the combination of Fote with LiGLA or with MeGLA. This investigation was also motivated by previous experimental work showing the beneficial effects of the use of chemotherapy (cisplatin or doxorubicin) in combination with EFAs in ovarian and breast tumor cell lines.[12] The present study indicates that the combination Fote plus LiGla or Fote plus MeGLA leads to antagonistic interaction on tumor cells. There are several lines of evidence indicating that O^6-alkylguanine-DNA alkyltransferase (AT) plays an important role in the sensitivity to nitrosoureas. Cells with reduced AT activity are more sensitive to the cytotoxic effects of alkylating agents than cells which express the protein.[13] In addition, transfection experiments have shown that expression of AT cDNA in mammalian cells protects them against the cytotoxicity generated by nitrosoureas.[14] One of the mechanisms by which EFAs kill malignant cells and inhibit their growth is likely to be related to changes in the tumor cells' capacity to generate free radicals.[15,16] On exposure to GLA and other EFAs, cancer cells generate large amounts of conjugated dienes, peroxyl radicals and, in some cell lines, superoxide radicals, all of which are highly lethal to the cell.[17] It has been shown that radiation, which acts by forming free radicals, can induce AT.[18] It is therefore possible that LiGLA and MeGLA may induce AT activity; this induction of AT could explain why combined exposure to the nitrosourea Fote with LiGLA or MeGLA results in antagonistic effects (Table 2 and Figure 2).

The prognosis of glioblastoma remains very poor. Facing this reality there is a need for innovative treatments. The administration of GLA has been shown to produce minimal toxicity in cancer patients. Taking into account the preclinical data herein presented, it would be justified to evaluate the efficacy of LiGLA and MeGLA in patients with glioblastomas.

Conclusion

A least 4-5 days exposure to LiGla or MeGLA causes a marked cytotoxicity in glioblastoma cells. The combination of these drugs with nitrosourea should be avoided since antagonistic effects are observed.

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