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γ-linolenic acid therapy of human glioma-a review of *in vitro*, *in vivo*, and clinical studies

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Summary

γ-linolenic acid (GLA) induced apoptosis of tumor cells without harming normal cells. Both cyclo-oxygenase (COX) and lipoxygenase (LO) inhibitors did not inhibit the selective tumoricidal action of GLA in some, but not all, tumor cells suggesting that GLA by itself is active. In contrast, anti-oxidants such as vitamin E blocked the tumoricidal action of GLA. GLA-treated tumor but not normal cells produced a 2–3-fold increase in free radicals and lipid peroxides. GLA decreased the anti-oxidant content of tumor cells, expression of oncogenes *ras*, and *Bcl-2*, enhanced the activity of p53, protected normal cells and tissues from the toxic actions of radiation and anti-cancer drugs, enhanced the cytotoxic action of anti-cancer drugs and reversed tumor cell drug resistance. In the animal glioma model, GLA induced tumor regression and preserved the surrounding normal brain tissue. In three open-label clinical studies, intra-tumoral injection of GLA induced significant reduction of glioma without any significant side effects. The low neurotoxicity of GLA to normal brain neurons and selective activity against tumor cells suggests that it could be an effective anti-glioma molecule.

key words: **gamma-linolenic acid • glioma • free radicals • lipid peroxidation • apoptosis • oncogenes • neuronal cells • polyunsaturated fatty acids • anti-cancer drugs**

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BACKGROUND

During the therapy of cancer it is desired that agents preferentially kill tumor cells without exerting adverse effects on normal cells. But, this is rarely achieved with the currently available drugs and radiation.

Epidemiological studies revealed that Japanese and Eskimos who consume large amounts of marine fish have low incidence of cancer. Although, this has been attributed to the presence of high amounts of ω-3 fatty acids: eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the exact reason is unclear. Hence, several studies have been initiated to identify the mechanism(s) responsible for this beneficial action of ω-3 and other fatty acids in cancer. The low incidence of cancer among Eskimos is particularly intriguing since polyunsaturated fatty acids (PUFAs) such as EPA an DHA are particular targets for peroxidation. In this context, it is interesting to note that an inverse relationship exists between the concentrations of lipid peroxides and the rate of cell proliferation. Tumor cells have low concentrations of lipid peroxides and are resistant to lipid peroxidation compared to normal cells [1–3]. This low rate of lipid peroxidation and levels of lipid peroxides in the tumor cells could be attributed to their low content of polyunsaturated fatty acids (PUFAs).

The low content of PUFAs in the tumor cells is due to the loss or decreased activity of Δ^6 and Δ^5 desaturases [3], enzymes that are essential for the formation of their metabolites such as GLA, arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from their precursor essential fatty acids EFAs): linoleic acid (LA) and α-linolenic acid (ALA) (see Figure 1 for metabolism of EFAs). Hence, it has been suggested that consumption of large amounts of marine fish that are rich in EPA and DHA will lead to increase in lipid peroxidation, and these lipid peroxides, in turn, are believed to induce tumor cell death, ultimately contributing to low incidence of cancer among Japanese and Eskimos.

Tumor cells also have elevated levels of lipid-soluble antioxidant vitamin E. The higher the growth rate of the tumor cells the higher the vitamin E to PUFA ratio. This higher vitamin E to PUFA ratio in tumor cells is due to their decreased PUFA content, while the vitamin E content is relatively higher [4,5]. Tumor cells have low or almost no superoxide dismutase (SOD), glutathione peroxidase and catalase enzymes that quench free radicals [5–7]. This renders tumor cells highly susceptible to free radical induced toxicity.

Based on this background data, it was thought that an in depth study on the effect(s) of various fatty acids on the survival of normal and tumor cells and the mechanism(s) of their actions is needed. To explore these ideas, *in vitro*, *in vivo*, ad limited clinical studies were performed with various saturated and unsaturated fatty acids.

TUMORICIDAL ACTION OF GLA

In vitro studies

Studies revealed that GLA (18: 3 ω-6), AA (20: 4 ω -6), EPA (20: 5 ω -3) and DHA (22: 6 ω -3) selectively kill tumor cells without harming the normal cells [3,5–15]. My colleagues and I observed that when normal monkey kidney (CV-1), human skin fibroblasts (CCD-41-SK), murine fibroblast transformed by Abelson leukemia virus (BALB), human breast cancer (ZR-75-1), and human promyelocytic leukemia (HL-60) cells were supplemented with LA, ALA, GLA, DGLA (dihomo-gamma-linolenic acid, 20: 3 ω -6), arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) in culture, of all the fatty acids tested, GLA selectively killed tumor cells without any cytotoxic action on normal cells (Figure 2). Although, both GLA and AA were equipotent in their tumoricidal action, GLA was not toxic to normal cells whereas both AA and EPA were toxic to normal cells. Thus, GLA=AA>EPA>DHA in terms of their action on tumor cells, whereas GLA showed a much more selective tumoricidal action compared to AA and other fatty acids (Figure 3). This tumoricidal action of GLA was further confirmed by extending these studies to several other tumor and normal cells such as human lung cancer, A-549; human breast cancer, ZR-75-1; and human prostatic, PC-3, adenocarcinoma cells; and normal cell lines normal simian, CV-1 and BSC-1, canine, MDCK, kidney cells, and normal human fibroblasts CCD-41-SK [3,5,9,11]. The cytopathic effects appeared from the 4th day of incubation and reached maximum on day 7 after supplementation of GLA. The irreversible cytotoxic action of GLA was confirmed by the parallel release of lactate dehydrogenase and by the absence of colony formation after replating the GLA treated cells. If one combines potency at killing tumor cells with lack of effect on normal cells, GLA appears to be the most effective. On the other hand, saturated fatty acids were found to be ineffective in killing tumor cells.

Selective tumoricidal action of GLA was also confirmed by challenging tumor cells in the presence of normal cells, wherein human breast cancer (ZR-75-1) cells and human skin fibroblasts (CCD-41-SK) were co-seeded in 35-mm petri dishes with a tumor: normal cell ratio of 1: 80 giving a monolayer of normal fibroblastic cells scattered with growing tumor cell clones. Under these culture conditions, the malignant cells overgrew the normal fibroblasts. However, when mixed cultures (containing both normal skin fibroblasts and human breast cancer cells) were supplemented with GLA, the tumor cells were inhibited and/or killed. GLA could eliminate human breast cancer (ZR-75-1) cells completely leaving a clean fibroblast layer. GLA did not affect the normal skin fibroblasts (CCD-41-SK) at the time of differential effect was maximal. The same pattern was observed with lung and prostate tumor cell lines (A-549 and PC-3 cells respectively) [15]. These results clearly established that GLA has direct anti-neoplastic effects without any significant actions on normal cells. The absence of adverse effects on normal cells and the highly selective tumoricidal action of GLA on various tumor cells tested suggests that GLA could be an effective anti-cancer molecule without any significant side effects. No tumor cells grew back when the cultures demonstrated maximal cytopathic effects [9,11,15].

MODULATION OF TUMORICIDAL ACTION OF GLA

The degree of inhibition of growth and loss of cell viability was determined by the source and concentration of serum and depended on cell density, fatty acid concentration, and

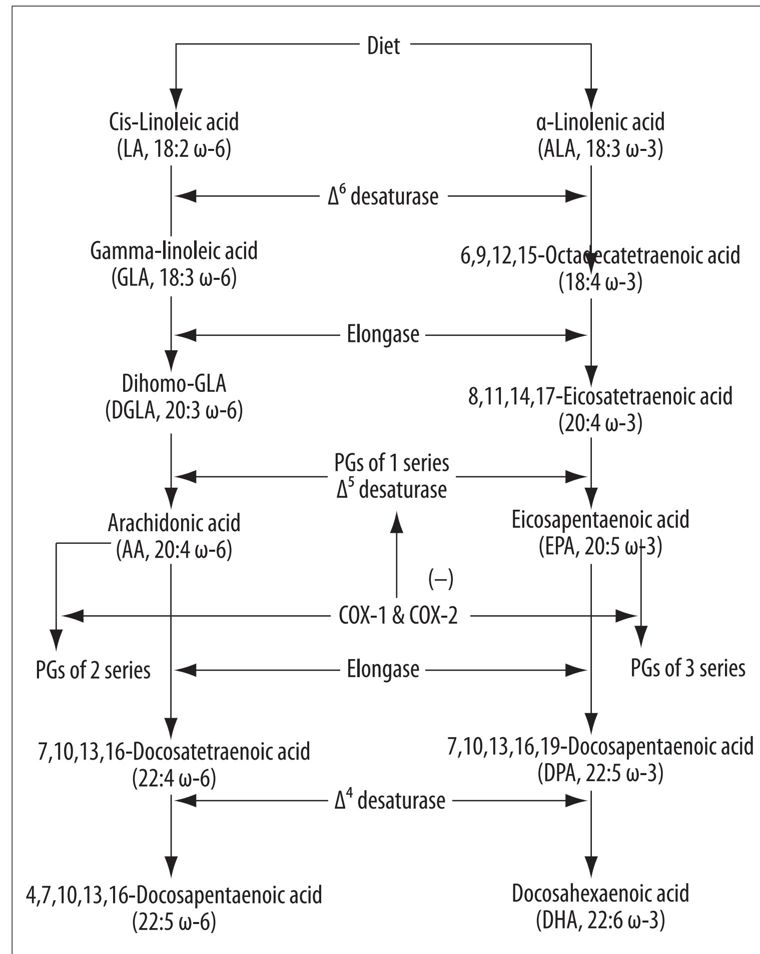

Figure 1. Metabolism of essential fatty acids.

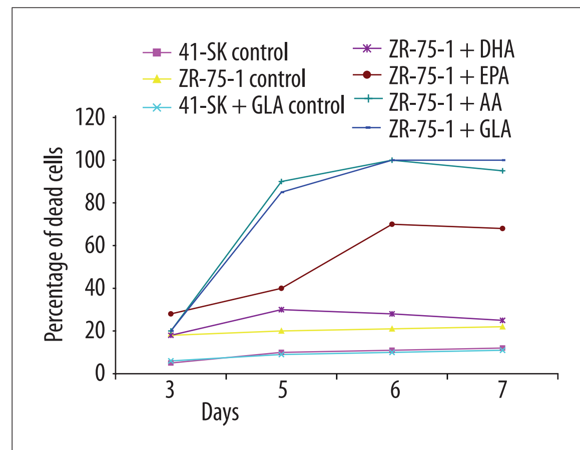

Figure 2. Effect of various PUFAs on the survival of normal and tumor cells *in vitro*.

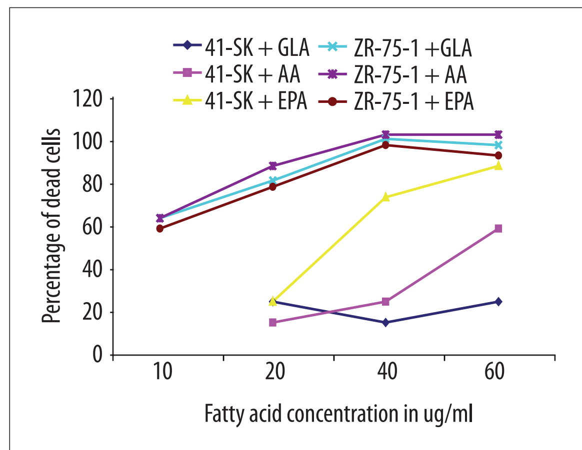

Figure 3. Effect of various PUFAs on the survival of normal and tumor cells *in vitro*. This data is modified from ref. 11.

the type of tumor cell. The percentage of dead cells was maximal when the cultures were challenged in the presence of 10% fetal calf serum. The use of serum concentration lower than 10% accelerated the rate of tumor cell killing. The extent of cytotoxic effects decreased with increasing serum concentration. The extent of killing varied from less than 20% to over 90% with the use of different concentrations of serum in the medium. These results suggest that use of higher concentrations of serum has the ability to inhibit the tumor cell killing action of GLA [9,11,15]. Subsequent studies showed that this is due to the presence of anti-oxidants in the serum and also the ability of proteins present in the serum to quench GLA. The practical implication of this observation is the fact that in patients with cancer, tumor is surrounded by inflammatory exudates that is rich in protein/serum that may inhibit the tumoricidal action of

Table 1. LD_{50} values of GLA on ZR-75-1 and PC-3 cells.

Cell line	LD_{50} value of GLA in µg
ZR-75-1	8
PC-3	16

Table 2. Levels of hydroxides formed in HeLa cells on treatment with GLA levels of hydroxides formed in HeLa cells treated with GLA. Values are expressed as µg/1×10^{4} cells.

Treatment	HODEs
Untreated HeLa cells	2.4
GLA-Treated HeLa cells	19.8

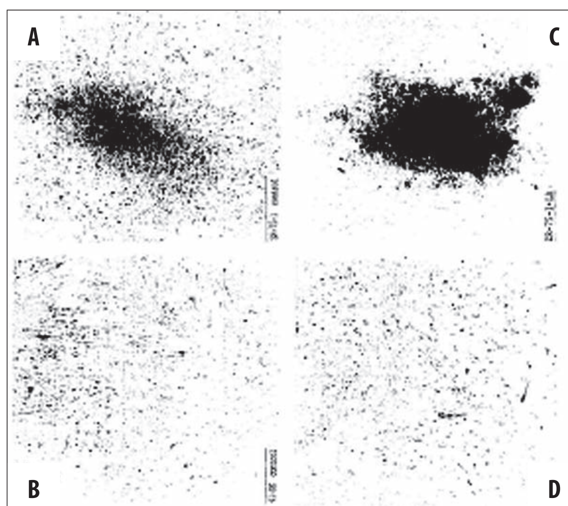

Figure 4. Photographs showing reduction of NBT to insoluble formazan within the normal human skin fibroblasts (41-SK) and human breast cancer (ZR-75-1) cells *in vitro* with and without GLA (see ref. 16 for further details). A = ZR-75-1 cells treated with vehicle; B = 41-SK cells treated with vehicle. C = ZR-75-1 cells treated with GLA; D = 41-SK cells treated with GLA. This data is modified from ref. 22.

GLA. It was observed that, in general, the higher number of tumor cells needed higher concentrations of GLA indicating that a minimum amount of GLA is needed to kill tumor cells, a concentration at which normal cells were not affected. The LD_{50} values of GLA for the 2 tumor cells lines are given in Table 1. Pro-oxidant such as copper and iron salts enhanced the cytotoxic action of GLA [9,11,15]

Mechanism(s) of tumoricidal action of GLA

Involvement of eicosanoids

GLA being an unsaturated fatty acid, one mechanism by which it produces its tumoricidal action is by enhancing the formation of lipid peroxides, and the other possibility is that eicosanoids that have cytotoxic actions are being generated from the supplemented GLA. Studies revealed that both cyclo-oxygenase (CO) and lipoxygenase (LO) inhibitors blocked the tumoricidal action of GLA on some, but not all, types of tumor cells tested. For instance, the cytotoxic action of GLA on human breast cancer (ZR-75-1) cells was not inhibited by both CO and LO inhibitors whereas partial to complete inhibition was noted in human cervical carcinoma (HeLa) cells [9,11,14,15]. All the types of tumor cells tested were found to be sensitive to the cytotoxic action of GLA and other fatty acids, though different cells showed varied degrees of sensitivity depending on their tissue of origin [7–9,11,14,15–18].

Studies on HeLa cells

The cytotoxic action of GLA on HeLa cells was inhibited by indomethacin and nordihydroguaiaretic acid (NDGA), a CO and LO inhibitors respectively, suggesting that prostaglandins (PGs) and leukotrienes (LTs) play a significant role in the cytotoxic action of GLA. This is supported by the observation that various prostaglandins (PGE_1, PGE_2, $PGF_{2\alpha}$, PGI_2) do inhibit the growth of HeLa cells at the doses tested (3 to 10 µg/ml/0.5×10^{4} cells) [14,17].

Lipoxygenase products of GLA synthesized using soybean and potato LO enzymes, 13-HPODE and 6-HPODE, were found to be inhibitors of HeLa cell growth. It was observed that LO products are more potent than PGs in inhibiting the growth of HeLa cells [14,17]. These results are supported by the observation that almost a 9-fold increased in the formation of hydroxides occurred in HeLa cells treated with GLA (Table 2).

In an extension of these studies, it was noted that superoxide dismutase (SOD), a superoxide anion quencher; catalase, an inhibitor of H_2O_2; mannitol, a hydroxyl radical quencher; and vitamin E, a potent inhibitor of lipid peroxidation process, have inhibitory action on the cytotoxic action of GLA on HeLa cells [14,17]. In addition, BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), and reduced glutathione also inhibited the tumoricidal action of GLA, suggesting that free radicals and lipid peroxidation products are involved in the tumoricidal action of GLA on HeLa cells.

Involvement of free radicals and lipid peroxides in the tumoricidal action of GLA

Involvement of free radicals and lipid peroxides in the cytotoxic action of GLA on HeLa cells is supported by the observation that GLA augmented superoxide, H_2O_2 generation and formation of lipid peroxides in HeLa and ZR-75-1 cells [5,9,11,14,15,17–23]. Although, EPA and DHA also enhanced superoxide and H_2O_2 generation and the formation of lipid peroxides in HeLa cells and this corresponded with their tumoricidal action; results from studies done with other tumor cells such as human breast cancer (ZR-75-1) cells revealed that GLA is more selective in killing tumor cells and less toxic to normal (human skin fibroblasts, CCD-41-SK) cells [9,11,15].

Unequivocal evidence that GLA and other PUFAs enhance free radical generation in the tumor cells but not normal cells is revealed the observation that a significant increase in the formation of the insoluble blue formazan (superox-

ide anion reduces NBT to the insoluble blue formazan) was noted only in human breast cancer (ZR-75-1) cells treated with GLA (GLA > AA > EPA > LA) compared to untreated ZR-75-1 cells or GLA-treated normal skin fibroblasts (41-SK) (see Figure 4) [22,23].

GLA induces apoptosis of tumor cells

GLA induced apoptosis and decreased the anti-oxidant content of tumor cells [7]. GLA is not only cytotoxic to tumor cells *in vitro* and *in vivo* but also inhibited Walker 256 tumor metabolism and altered mitochondrial ultrastructure [24]. GLA induced apoptosis of chronic B-lymphocytic leukemia, glioma, and LLC-WRC256 rat carcinosarcoma cells [24–28]. GLA suppressed the expression of oncogenes *ras* and *Bcl-2* and enhanced the activity of p53 [25].

GLA kills drug-resistant tumor cells and reverses tumor cell drug-resistance

Drug resistance is a major obstacle in the treatment of cancer. KB-Ch^R-8-5 cells are resistant to the cytotoxic action of vincristine whereas KB-3-1 cells are sensitive to its action, whereas GLA could kill both vincristine-resistant and sensitive human cervical carcinoma cells (KB-3-1- and KB-Ch^R-8-5) *in vitro* [20,29]. Further studies showed that indomethacin, a CO inhibitor, and NDGA, a specific leukotriene synthesis inhibitor, were ineffective in blocking the cytotoxic action of GLA and other fatty acids, suggesting that both prostaglandins and leukotrienes are not involved in the cytotoxic action of GLA and other fatty acids. Supplementation of GLA and other PUFAs to KB-3-1 and KB-Ch^R-8-5 cells enhanced the generation of free radicals and formation of lipid peroxides whereas anti-oxidants SOD (superoxide dismutase) and vitamin E not only blocked their cytotoxic action but also inhibited the generation of superoxide anion and lipid peroxides [20,29]. These studies suggest that GLA has the ability to kill even drug-resistant tumor cells through a free radical dependent process.

GLA augments the cytotoxic action of anti-cancer drugs and enhances the uptake of anti-cancer drugs by tumor cells

Several anti-cancer drugs such as cis-platinum and radiation are known to kill tumor cells by enhancing free radical generation. Studies revealed that GLA augmented the cytotoxic action of anti-cancer drugs by augmenting the uptake and inhibiting the efflux of anti-cancer drugs both by drug-sensitive and drug-resistant tumor cells [29,30] (Table 3). Menendez et al. [31], Hernandez et al. [32], Rudra et al. [33], and Guffy et al. [34] confirmed that GLA and other fatty acids do indeed augment the tumoricidal actions of various anti-cancer drugs and that a synergistic interaction exists between conventional anti-cancer compounds including radiation and GLA [35].

Anti-cancer action of GLA with particular reference to glioma

It is evident from the preceding discussion that GLA has potent ant-cancer actions. Furthermore, GLA showed selective tumoricidal action on glioma cells without harming the normal neuronal cells with little or no neurotoxicity. GLA induced apoptosis of glioma cells by enhancing free radical generation [27–29].

Table 3. Effect of GLA on the uptake of (^{3}H) vincristine by HeLa cells *in vitro*. For further details see ref. 24.

Group	CPM/l × 10^6 cells
Vincristine control	752±136
Vincristine + 20 µg/ml of GLA	1,617±210*

In vitro studies: Effect of GLA on neuroblastoma and glioma cells

Studies by Fujiwara et al. [36] revealed that GLA suppressed the proliferation of four neuroblastoma cell lines: NCG, GOTO, SK-NDZ, and NKP cells *in vitro*, though their sensitivity to GLA differed: GOTO was the most sensitive with an IC_{50} of 10 µg/ml, while NCG was less sensitive with an IC_{50} of 40 µg/ml. The cytotoxicity of GLA was inhibited by acetylsalicylic acid (ASA) and indomethacin, which are cyclo-oxygenase inhibitors; and caffeic acid (CA), a lipoxygenase inhibitor. Anti-oxidants vitamin E (α-tocopherol) and BHT reduced the cytotoxicity of GLA, suggesting a role for free radicals and lipid peroxides. These results are similar to those reported previously with human breast (ZR-75-1) cancer cells [9,11,15]. Fujiwara et al. [36,37] also observed that GLA-treated cells produced about 6-fold more lipid peroxides compared to the controls. Furthermore, neuroblastoma cells supplemented with GLA showed augmented levels of lipids peroxides, while indomethacin or caffeic acid, which are CO and LO inhibitors respectively, had no effect.

Neuroblastoma and other tumor cells incubated with GLA showed striking cell membrane modification in the form of a remarkable increase of GLA, DGLA, and AA and a decrease of OA (oleic acid, 18: 1) content [36], in parallel with cell growth inhibition, suggesting that the anti-tumor effect of GLA is due to cellular dysfunction caused by fatty acid incorporation. It was also noted that the cytotoxic effect of vinca alkaloids on neuroblastoma cells was enhanced by ~2-fold in the presence of GLA [37,38] due to increased influx of anti-cancer drugs, results that are similar to those reported previously [29,30].

GLA is cytotoxic to malignant astrocytoma cells but not to 'normal' astrocytes and enhances radiation-induced cytotoxicity to cancer cells

Both radiation and conventional anti-cancer drugs produce profound side effects due to their toxic actions on normal cells and tissues. Since GLA is selectively toxic to tumor cells with little or no actions on normal cells, Vartak et al. [39] studied the cytotoxic action of GLA on malignant rat astrocytoma cells and compared its action on normal astrocytes. GLA decreased the survival of 36B10 malignant astrocytoma cells compared to control that did not receive the fatty acid, confirming that GLA selectively kills malignant astrocytoma cells. In addition, Vartak et al. [39] observed that GLA is not only toxic to 36B10 malignant astrocytoma cells but is also able to enhance the cytotoxic action of gamma-

radiation on malignant astrocytoma cells but not of 'normal' astrocytes due the incorporation of GLA into the cell membrane of astrocytoma cells indicating its increased oxidative potential since the cytotoxic action of GLA was prevented by trolox, a potent anti-oxidant.

GLA is cytotoxic to malignant glioma but not to normal brain

Bell et al. [27] studied the effect of GLA on the survival and invasive capacity of glioma cells using glioma spheroids grown in collagen gel. The advantages of the use of collagen gel matrix compared to monolayer culture system to assess tumor growth is that individual cell migration from the spheroid into the gel matrix, and cellular proliferation can easily be assessed, and spheroids offer the advantage of allowing to observe the tumor mass in a three-dimensional way, the existence of cell-cell interactions in the biomass as would occur in an *in vivo* situation, and the presence of diffusion gradients within the tumor cell mass. Furthermore, culture of glioma cell lines in spheroid suspensions in collagen gels results in a tissue mass that can be fixed and neuropathologically evaluated using conventional and immunohistochemical techniques. Bell et al. [27] used C6 rodent glioma cell line, and the human cell lines MOG-G-CCM (anaplastic astrocytoma), U373 (glioblastoma), and U87 (glioblastoma) cell lines, and reported that GLA dose response patterns were found to be similar for all the four-glioma spheroids studied. GLA increased apoptosis, decreased proliferation in tumor growth and invasion, and impaired spheroid cell growth, suggesting that local delivery of GLA could significantly reduce tumor size and growth. In addition, GLA augmented free radical generation in glioma cells indicating that enhanced generation of free radicals could be responsible for the apoptosis of tumor cells [27,28].

GLA regresses glioma growth *in vivo* when locally given

In an extension of these studies [27,28] it was observed that administration of GLA to five glioma models, including primary cell suspensions and an *in vivo* rat C6 glioma cells or in 16 preparations of multicellular spheroids derived from human and rodent cell lines (C6, MOG, U87, U373) or administered intra-tumorally by infusion using osmotic minipumps in 48 rats, regressed glioma by inducing apoptosis. In the glioma infusion model, in addition to the apoptosis detected in glioma tissue infused with GLA for 3–7 days, preservation of normal neural tissue and vasculature in adjacent brain with little evidence of inflammation in regressing tumors was noted. These results imply that intraparenchymal infusion of GLA is effective in stimulating glioma regression [28].

GLA infused at a concentration of 40 µM/l for 7 days into rat brain parenchyma of 15 normal rats, revealed only small areas of brain tissue that showed histological evidence of tissue damage. Overall, the tissue area affected by these histological changes represented an average of <0.5% of the total brain area in both saline and GLA-infused sections, suggesting that the reactive damage associated with surgery and GLA infusion was small relative to abnormal areas associated with tumor infiltration. The lack of acute inflammatory response in saline control and GLA infusions into normal brain, suggested that the damage observed was solely related to the infusion procedure *per se* [28].

The balance between cell death and cell proliferation controls the rate of tumour growth. As a therapeutic target, stimulation of the process of apoptosis has the advantage of avoiding acute inflammatory responses, which may trigger neural damage in tumours of the CNS. Studies done with GLA clearly showed that it kills very selectively only the glioma cells (tumor cells) but not normal neuronal cells, suggesting that GLA is very unlikely to have any significant side effects when administered in to the tumor bed in patients with malignant glioma.

Results of studies done in normal dogs

Studies done in normal dogs (numbering two and three on two separate occasions) also showed that injection of GLA into the brain parenchyma is safe [40,41]. In these normal dogs, a twist drill was placed in the right frontal bone under anesthesia. After puncturing dura, a number 18 Teflon venous catheter was introduced into the frontal lobe, and the butterfly of the cannula was sutured to the scalp. GLA (0.25 mg in saline) was injected into the dog's brain tissue through the cannula everyday for 6 days under aseptic conditions and animals were sacrificed and their brains were removed on the seventh day, i.e. 24 hours after the last injection. The left cerebral hemisphere served as the control. None of the animals developed any side effects or complications due to the procedure or GLA injection, and CT scans, gross examination of the meninges and subarachnoid space, and histopathological examination of the brain did not show any abnormality. In the tissues studied even oil red 'O' staining for lipid was essentially normal except for an occasional positive stain only in the blood vessels at the site of GLA injection. Neither evidence of systemic toxicity nor any adverse effects on CNS function or motor activity of the treated animals was noticed. The low neurotoxicity of GLA to normal brain neurons and the selective activity against tumor tissue as indicated by the preservation of neuronal tissue at the tumor/neuronal tissue interface, suggests that GLA is safe and not toxic to normal tissues.

GLA protects against benzo(a)pyrene and radiation-induced genetic damage

Previously, we reported that oral BP (75 mg/kg of body weight) and whole body gamma-radiation (250 rads, 2.5 Gy/minute) induced DNA damage to the bone marrow cells of mice in the form of an increase in the number of polychromatic RBC with micronuclei is completely prevented by prior treatment with GLA [42–45]. Dominant lethal test (DLT) that evaluates the post-meiotic, meiotic, and pre-meiotic phases of spermatogenesis and their effect on the pregnancy rates and pre- and post-implantation lethality on the fetus also showed that prostaglandin E_1 (PGE_1), which is derived from GLA, reduced the incidence of dead implants induced by radiation and completely protected the implants [43].

Micronucleus (MN) test is a common test that is used to test the genotoxic actions of chemicals and drugs both *in vitro* and *in vivo*. In the *in vivo* test, the bone marrow cells of mice are studied for damage to DNA in the form of presence

Table 4. Effect of GLA against BP-induced genetic damage to the bone marrow cells of mice. Dose response study with GLA.

Group control	PCE/MN	NCE/MN	P/N ratio
Control	6090/24 (0.39)	4929/7 (0.14)	1.2
Corn oil control	6121/18 (0.29)	6013/2 (0.39)	1.01
BP (75 mg/Kg)	6102/97* (1.59)	5974/21 (0.35)	1.02
BP + GLA 10^{-4} M	5832/30** (0.51)	4695/9 (0.19)	1.2
BP + GLA 10^{-5} M	6088/41** (0.66)	5337/11 (0.20)	1.14
BP + GLA 10^{-6} M	6000/27** (0.45)	4582/12 (0.28)	1.3
GLA control 10^{-5} M	6339/25 (0.38)	6352/6 (0.09)	0.99

* P<0.05 compared to control. ** P<0.05 compared to control.
BP was given orally whereas and GLA was given intraperitoneally one hour after BP.
These results showed that GLA produced a significant reduction in the number of MN containing PCE suggesting that GLA prevents/reverses DNA damaging action of BP to the bone marrow cells of mice.

Table 5. Effect of GLA against BP-induced genetic damage to the bone marrow cells of mice. Time course study with GLA.

Group	PCE/MN	NCE/MN	P/N ratio
Control	4020/13 (0.03)	4012/4 (0.1)	1.00
Corn oil control	4015/12 (0.30)	4013/2 (0.05)	1.00
BP (24 hrs)	4090/42* (1.03)	4015/20 (0.50)	1.01
BP + GLA 10^{-6} M (24 hrs)	4092/21** (0.51)	4077/0	1.00
BP (48 hrs)	4053/48* (1.18)	4095/5 (0.12)	1.00
BP + GLA 10^{-6} M (48 hrs)	4095/14** (0.34)	4043/0	0.99
GLA control 10^{-6} M (24 hrs)	4012/13 (0.32)	4048/0	1.00
GLA control 10^{-6} M (48 hrs)	4029/15 (0.37)	4057/0	1.00

The dose of BP used was 75 mg/kg body weight. GLA was administered 24 hours and 48 hours after BP. BP was given orally whereas GLA was given intraperitoneally after BP. * P<0.05 and ** P<0.05 compared to controls.

of micronucleus in the polychromatic erythrocytes (PCE). The results of these studies given in tables 4 and 5 suggests that GLA has the ability to prevent/reverse BP and radiation-induced genetic damage to the bone marrow cells of mice. Thus, GLA not only has tumoricidal action but also protects normal cells from the cytotoxic and genotoxic actions of various chemicals and radiation [43].

Local infusion of GLA regressed malignant glioma tumors in humans

Based on the results of various *in vitro* and *in vivo* studies that have been enumerated above, limited open-label clinical studies were done in 30 patients with the permission of the local ethics committee that qualify to be phase I and II studies.

Data from these three open label clinical studies [41,46,47] are given here. Since it is not appropriate to include a placebo control group due to ethical reasons, comparison in the size of the glioma tumor based on computerized axi-

Table 6. Details of patients treated with intra-tumoral GLA (clinical study 1).

Case no.	Age/sex	Diagnosis	Prior therapy	No. of injections	Total dose	Response to therapy	Comments
1	43, M	GBM	S	7	4.5 mg	40–60%	Lost for follow-up; stable at discharge
2	13, M	ODG	S, R	8	8 mg	40%	Tumor grew and died
3	40, M	MG	S	10	10 mg	60%	Alive and Well till follow-up period – 2 years and 8 months
4	15, M	AAC	S	10	10 mg	60%	Alive and Well till follow-up period – 2 years and 4 months
5	46, M	MG	S	10	10 mg	60%	Alive and Well till follow-up period – 2 years and 4 months
6	43, M	GBM	S, R, C	20	20 mg	40-60%	Alive and Well at discharge, lost for follow-up

GBM – Glioblastoma Multiforme; AA – Anaplastic Astrocytoma; MMG – Malignant Mixed Glioma; MA – Malignant Astrocytoma; S – Surgery; R – Radiation Therapy; C – Chemotherapy.

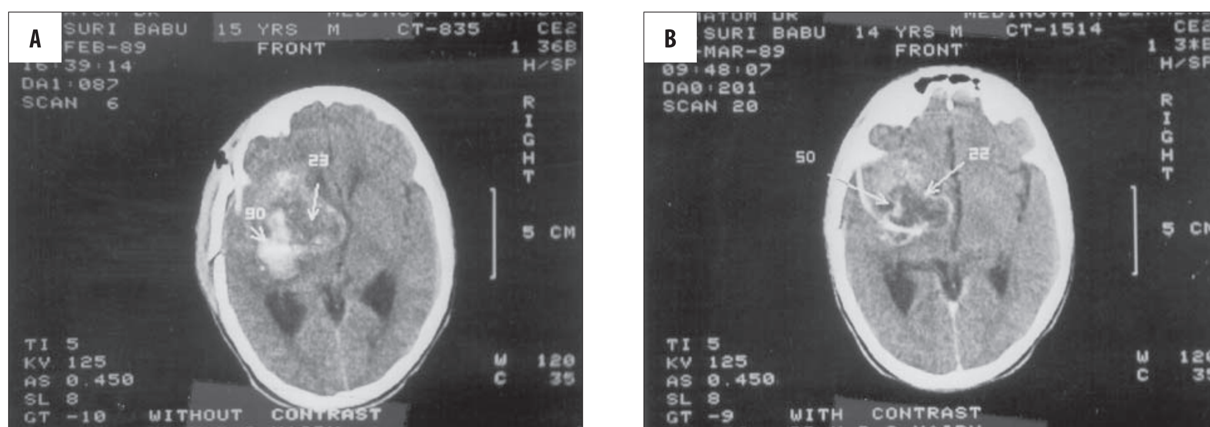

Figure 5. CT scans of a patient with anaplastic astrocytoma (malignant glioma) before and after treatment with GLA. This patient had a recurrent glioma for which he underwent a cytoreductive neurosurgical procedure (debulking operation). Ten days after surgery, following the healing of the surgical wound, a contrast CT scan was done to know the size of the residual tumor (Figure 5A). The residual tumor appears as a white mass due to contrast uptake. This patient received 10 injections of GLA at the rate of 1 mg/day for 10 days. Twenty-eight days after the last injection of GLA, a repeat follow up contrast CT scan was done to know the size of the tumor and its response to treatment. It is seen from Figure 5B that the size of the tumor is much less compared to the size of the tumor in Figure 5A. Repeat CT scan was done 28 days after stopping GLA injections. It is obvious from the comparison of pre- and post-treatment CT scans that despite discontinuation of GLA after 10 injections there is no regrowth of the glioma tumor mass. This patient did not have any side effects during the administration of GLA or during the follow up period. These CT scans belong to the patient no. 4 in Table 5.

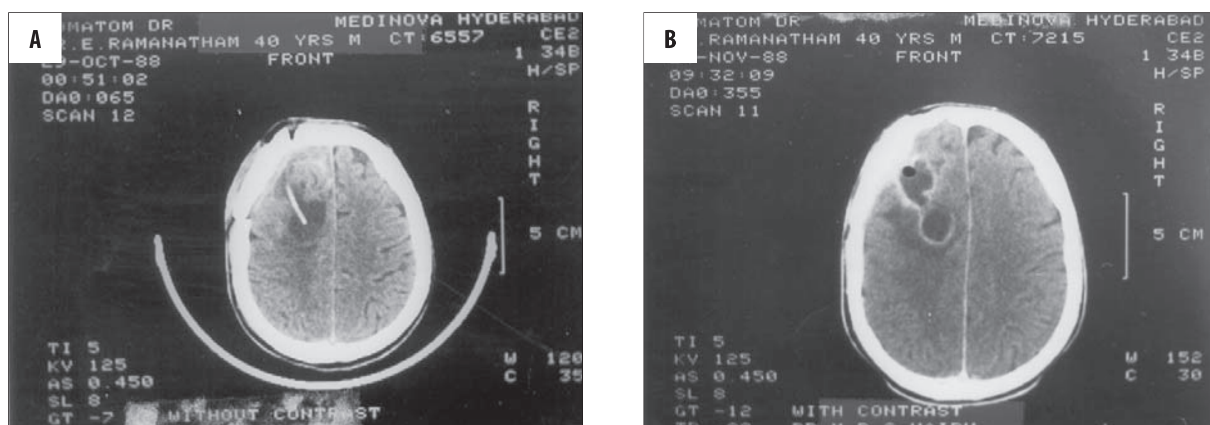

Figure 6. Shows the CT scans of the patient with malignant mixed glioma (patient no. 3 in Table 5) before and after treatment with GLA. This patient had a recurrent glioma for which he underwent a cytoreductive neurosurgical procedure. Ten days after surgery, following healing of the surgical wound, a contrast CT scan was done to record size of the residual tumor (Figure 6A). Residual tumor appears as white mass due to the uptake of contrast by cancer cells. This patient received 10 injections of GLA at the rate of 1 mg/day for 10 days. Two weeks after the last injection of GLA, a repeat follow up contrast CT scan was done. It is evident from Figure 6B that the tumor has regressed to a significant degree compared to its size in Figure 6A. Tumor that underwent necrosis looks dark on CT scan. The site of GLA injection is seen as a dark spot. This is so since; lipid has low attenuation value in CT scans. Figure 6A also shows the tip of the catheter *in situ*. Post-treatment CT scan was done 2 weeks after stopping GLA injections. Comparison of pre- and post-treatment CT scans that despite discontinuation of GLA after 10 injections there is no recurrence of the tumor. This patient did not have any side effects during the administration of GLA or during the follow up period. Both these two patients (Figures 5 and 6) survived for 2 years 4 months and 2 years 8 months respectively in contrast to the expectation that they would not live for more than 3 to 4 months due to the recurrence of glioma. This is a significant improvement in their survival.

al tomography (CT) between pre- and post-GLA treatment schedules was done in these studies. Since it is inappropriate to inject GLA into brain parenchyma of healthy subjects, these 3 studies [41,46,47] could be considered as representative of combined phase I and phase II studies. In these 3 studies, in which only those patients who had advanced disease (grade III or IV glioma) were included, and possible side effects and/or toxicity and efficacy of GLA in glioma were studied.

In the preliminary open label clinical study (see Table 6) done in 6 patients with recurrent glioma or anaplastic astrocytoma, intra-tumoral injection of GLA given at the rate of 0.5 mg to 1 mg/day for 7 to 10 days produced significant reduction in the tumor size without any acute side-effects [46]. For a better visualization of the efficacy of GLA in malignant glioma, a representative pre- and post-treatment CT scan pictures are given in Figures 5 and 6.

Table 7. Descriptive statistics of CT parameters before and after GLA therapy for cerebral malignant gliomas Clinical study 2).

		Median	IQR	95% CI for median
1	Cyst Size (cm) Pretreatment	0.7	0–1	0.15–1.0
2	Cyst Size (cm) Post treatment	1.4	1.2–1.6	1.2–1.6
3	Mass Effect (mm) Pretreatment	2.0	1–2.5	1.2–2.3
4	Mass Effect (mm) Post treatment	0.5	0.5–1.0	0.5–1.0

Table 8. Inferential statistics of CT parameters before and after GLA therapy for cerebral malignant glioma (clinical study 2).

	Median difference	95% CI for median difference	P value (based on Wilcoxo test for paired data)
Cyst size (cm) (After minus before)	0.60	0.3–1.2	0.001
Mass effect (mm) (Before minus after)	1.0	1–1.3	0.001

CI – Confidence interval; IQR – Inter-quartile range.

Table 9. Details of patients treated with intra-tumoral injection of GLA (study 2).

Case no.	Age/sex	Site of tumor	Diagnosis	Follow-up
1	60, M	Left Temporal	GBM, grade IV	Well till 2 years 10 months
2	55, M	Left Parietal	GBM, grade IV	Well till 2 years 10 months
3	46, M	Left Parietal	MMG	Well till 2 years 10 months
4	55, F	Bi-Frontal	MMG	Well till 2 years 10 months
5	55, M	Left Temporal	GBM, grade IV	Well till 2 years 10 months
6	74, M	Right Parietal	AA, grade III	Died
7	85, M	Left Parieto-Occipital	GBM, grade IV	Died
8	45, M	Left Fronto Temporo Parietal	AA, grade III	Well till 2 years 10 months
9	60, M	Right Temporo Parietal	MA, grade III	Well till 2 years 10 months
10	43, F	Right Frontal	MMG	Well till 2 years 10 months
11	40, M	Right Temporal	GBM, grade IV	Well till 2 years 10 month
12	30, F	Left Temporo Parietal	GBM, grade IV	Well till 2 years 10 months
13	45, M	Left Temporal	AA, grade III	Well till 2 years 10 months
14	65, F	Left Temporal	AA, grade III	Died
15	40, M	Bi-Frontal	AA, grade III	Well till 2 years 10 months

All the six patients studied in this first clinical study [46] not only showed good response to GLA administration but also did not show any side effects. It is pertinent to note, as shown in Table 6, that the first two patients received only 4.5 and 8 mg of GLA. This is so since, when the first patient was recruited into the study, it was not evident how much of GLA could be administered. Hence, the first patient was given 0.5 mg on the first day and was carefully observed for any possible local or systemic side effects, was monitored closely for any possible side effects such as headache, convulsions, sensory and motor symptoms and signs, and was on 24-hour ECG monitor to record any cardiac abnormalities. All routine biochemical parameters were also studied. Since in the first 48 hours of observation no side effects were noted following the intra-tumoral administration of GLA, the patient received a second dose of 0.5 mg, and was again observed for the next 48 hours and after confirming that there were no side effects he was given an additional 0.5 mg of GLA and once again observed for the next 48 hours. In this manner, this patient received 0.5mg of GLA once every 48 hours. Thus, this patient received 5 injections of GLA at the rate of 0.5 mg each time totaling 2.5 mg. Once it became clear that there were no side effects due to the intra-tumoral injection of GLA, he was given 1 mg of GLA once in every 24 hours for the next 2 days. Thus, this first patient received a total of 4.5 mg of GLA. In view of the fact that this first patient received in a graded fashion first 0.5 mg and later 1 mg, this could be considered as a dose escalation study that is typical of Phase I clinical study. Based on our experience with the first patient, the second patient included in the study, received 1 mg of GLA every 24 hours for 8 days and thus, he received a total dose of 8 mg.

The 3rd, 4th and 5th patients were given 1 mg of GLA once every 24 hours for 10 days and thus, these three patients re-

Table 10. Details of patients treated with intra-tumoral injection of GLA (clinical study 3).

Case no.	Age/sex	Diagnosis	Prior therapy	Response	Side-effects	GLA effects	Follow up
1	46/M	Left temporo-parietal anaplastic astrocytoma	Subtotal cytoreductive surgery; 60 Gy cranial RT; Large tumor recurrence-GLA given 8 months after surgery	<40%	↑ ICP Mild neurological deterioration	No significant change	Died 1 week after completion of therapy
2	44/F	Left temporo-parietal GBM	3 cytoreductive surgeries; 60 Gy cranial RT; Recd. Temozolomide 4 cycles; GLA given 11 months after 1st surgery; 4 months after 3rd surgery	<40%	None	Reduction of contrast enhancement	Died 4 weeks after completion of therapy
3	60/M	Right frontal GBM	Reservoir placed during first cytoreductive surgery; GLA given 8 days after surgery; 60 Gy RT given subsequently	<40%	None	Considerable reduction in contrast enhancement	Last follow up 11 months after GLA therapy. Had subdural effusion & recurrence
4	54/M	Left temporo-parietal GBM	Near total cytoreductive surgery; 60 Gy postop RT; Procarbazine; GLA given 5 months after first surgery	<40%	None	Reduction of contrast enhancement lining the cyst cavity	Died 10 months after GLA therapy
5	48/M	Left occipital GBM	Cytoreductive surgery; Post op RT. 35 Gy (with 2-deoxyglucose as radio-sensitizer); GLA given 12 months after surgery	<40%	None	Slight reduction of contrast enhancement	No follow up
6	33/F	Left parieto-occipital GBM	2 cytoreductive surgeries; 50 Gy RT.; Gamma Knife boost; PCV chemotherapy- 4 cycles; GLA given 4 yrs after 1st surgery	<40%	None	Slight reduction of contrast enhancement and mass effect	Died 4 weeks after completion of therapy
7	5/M	Right thalamic GBM	Stereotactic Biopsy; Cytoreductive surgery; No RT; GLA given 1 month after cytoreductive surgery	<40%	None	Improvement in neurological status. Reduction in contrast enhancement	Died 4 months after completion of therapy
8	42/M	Bifrontal GBM	Cytoreductive surgery; 60 Gy RT; Etoposide/Cisplatin/CCNU; GLA given 8 months after 1st surgery	<40%	None	Reduction of contrast enhancement	Had recurrence, re-operated 2 months after GLA. Well for 34 months, lost for follow-up
9	60/F	Right Parietal GBM	Cytoreductive surgery; 60 Gy RT; GLA given 7 months after surgery	<40%	Slight deterioration of neurological status after GLA	No change	Died 3 months after completion of therapy

ceived a total of 10 mg of GLA. Since none of these patients showed any side effects, the 6th patient of this study received 1 mg of GLA per day for 20 days and thus, he was given a total dose of 20 mg, who also did not show any side effects despite receiving higher dose compared to the others. Thus, in this Phase I open label clinical study a dose escalation study was performed and none of these patients had any significant side effects and all showed good response to GLA. Details of the six patients studied in this phase I study are given in Table 6.

In the second open label clinical study, which was performed in patients who satisfied radiological criteria of diagnosis of malignant glioma and who did not undergo a surgical procedure prior to inclusion in the study (primary tumor), were subjected to debulking neurosurgical procedure with subtotal or near total excision of the tumor depending on the feasibility and accessibility [41]. Before the closure of the dura, 1mg of GLA was instilled into the tumor bed and a cerebral catheter was placed in the excised tumor cavity with the reservoir located on the bone flap under the ga-

Table 11. Comparison of clinical studies 1, 2, and 3 with GLA.

Study 1	Study 2	Study 3
6 patients	15 patients	9 patients
Recurrent glioma	Primary glioma	Recurrent glioma but had far more advanced disease than in study 1
Had previous surgery and radiotherapy/ chemotherapy before GLA	No prior therapy before GLA	Had multiple surgeries, radiation and chemotherapies before GLA
Total Dose: varied from 4.5 mg to 20 mg per patient, rate varied	Total Dose: 10 mg per patient at the rate of 1 mg per day	Total Dose: 7 mg per patient at the rate of 1 mg per day
After GLA, no other therapy was given	After GLA, radiotherapy was given	After GLA, no other therapy was given
No side effects due to GLA	No side effects due to GLA	No side effects due to GLA

lea for subsequent injections. On the 7th postoperative day, following suture removal, a CT scan of the brain with and without contrast was obtained for pre-GLA therapy baseline morphology. One milligram (1mg) of GLA in 2–3 ml of sterile saline was instilled everyday through the cerebral reservoir under strict aseptic conditions. Following 10 days of GLA therapy, a repeat CT scan was performed with and without contrast for post-GLA therapy morphology. After the completion of the GLA therapy all the patients received standard radiotherapy and were instructed to return for regular follow up. All the 15 patients included in the study tolerated the treatment well and no side effects or complications due to the therapy were noted. In all the patients, a striking change in the morphology of the tumor bed was noted in the CT scan. The high-density regions of the gliomas had turned into lesser density areas and an increase in the cystic area was observed when pre-GLA and post-GLA treatment scans were compared. The mass effect and the midline shifts of the brain tissue were also reduced to a significant degree. A significant increase in the cyst size, an indication of necrosis of the tumor, and a remarkable decrease in the midline shift, a parameter that denotes a decrease in the mass effect of the tumor, was noted following GLA therapy in all the patients studied (Tables 7 and 8). Only 1 out of the 15 patients studied required reexploration after 11 months of radiotherapy with features of raised intracranial pressure. This patient was operated on again and a debulking surgery was performed after 8 months of GLA therapy that did not reveal any recurrence of the tumor. Histopathological examination of the mass removed at surgery showed areas of gliosis and necrosis with thickened blood vessels with no evidence of tumor recurrence suggesting that those changes are only secondary to radiation. Of the 15 patients studied, 3 patients died 5–8 months following GLA and radiotherapy. Details of the 15 patients are given in Table 9.

The results of this second study showed that intra-tumoral injections of GLA could significantly prolong the survival of the patients with glioma (approximately from 2 to 4 years). Although CT scans do not give precise quantitative changes in the tumor size, they are useful in routine neurosurgical practice to monitor the progress of the disease. Further, the appearance of increasing low attenuation areas following GLA therapy on CT scans does not seem to be the natural growth pattern of the tumor. The fact that 12 out of 15 patients with grade III to IV malignant brain tumors did not show any recurrence of the tumor during the follow up period suggests that GLA is useful in the treatment of human gliomas. During the period of this study, it was also established that intra-tumoral injection of GLA does not produce any significant side effects or toxicity [41]. Since this study was done in those with primary glioma who received injections of GLA after debulking surgery, it qualifies to be a Phase II/III study. The results of this study have also clearly demonstrated that the survival of patients could be improved significantly (~2 ½ years after therapy) compared to a historical survival of not more than 40 weeks (~1 year). Furthermore, none of the patients studied showed any significant side effects. In fact, some of the patients received GLA as an outpatient procedure wherein the patient came to hospital everyday for the intra-tumoral administration of GLA through the Ommaya reservoir.

In the study 3, 9 patients who had advanced grade IV glioma were studied. All patients had recurrent glioma after surgery, radiation, and chemotherapy, and these patients were not expected to live for more than 4–6 weeks at the time of inclusion in the study. These patients received GLA 1 mg/day for 7 days (a total of 7 mg) into the tumor bed. In this study, 4 patients lived for 3, 4, 10, and 11 months; 3 died within 4 weeks after GLA therapy; 1 was alive for 11 months when lost for follow-up; 1 patient was lost for follow-up immediately after receiving GLA therapy. In all the patients studied, post-GLA therapy CT/MRI scans showed considerable decrease in the tumor mass and decrease in contrast enhancement, suggesting necrosis of the tumor. No side effects were observed [47]. The results of this study are given in Table 10.

The differences in the survival of the patients after GLA therapy can be attributed to the differences in the size of the tumors, previous treatments taken by the patients, aggressiveness of the tumors and differences in the study protocol. For instance, in the 1st clinical study, the patients with recurrent glioma were recruited into the study i.e. these patients already underwent surgery for debulking of the tumor, radiation, and chemotherapy and then had a recurrence of the tumor when were included in the study. In the 2nd clinical study, patients recruited had primary glioma. In

other words, in this study once the diagnosis of glioma was made, they underwent surgery following which they were given GLA and then were given radiation therapy. Thus, these patients received GLA for the primary tumor and so they showed much better response. In the 3rd clinical study, the patients already had 2 or 3 surgeries, radiation and chemotherapy and were in terminal stages of life and were given GLA as the last alternative. Most of these patients were not expected to survive beyond 2 to 6 weeks, had large tumors, dose of GLA was probably insufficient (received only a total of 7 mg) compared to the those included in the 2nd clinical study (a total of 10 mg) and hence their response was much less dramatic compared to the first two clinical studies. In Table 11, the results of the three clinical studies and the differences in their results are given for easy reference.

Conclusions

It is evident from the preceding discussion that GLA induced apoptosis of tumor cells with little or no action on normal cells. This selective tumoricidal action of GLA is not always dependent on cyclo-oxygenase (COX) and lipoxygenase (LO) enzymes suggesting that GLA by itself could be active. In majority of the tumor cells studied, the cytotoxic action of GLA could be blocked by anti-oxidants; and GLA-treated tumor but not normal cells produced 2-3-fold increase in free radicals and lipid peroxides, suggesting that GLA-induced tumor cell apoptosis is a free radical-dependent process [11,22,24,28]. In addition, GLA decreased the anti-oxidant content, suppressed the expression of oncogenes *ras*, and *Bcl-2*, enhanced the activity of p53, and formation of lipid peroxides due to augmented free radical generation in tumor cells [25,48–50]. GLA and other PUFAs seem to produce mitochondrial depolarization, lipid accumulation, and overexpression of C-MYC and P53 [51–53] to bring about their tumoricidal action. Furthermore, incubation of deoxyguanosine 5'-monophosphate with various unsaturated fatty acids in the presence of ferrous sulphate led to the formation of different amounts of acrolein (Acr), crotonaldehyde (Cro), and trans-4-hydroxy-2-nonenal (HNE) adducts derived from these fatty acids depending on the number of double bonds present in the PUFAs. It was also noticed that HNE-dG adducts are preferentially formed in a sequence-specific manner at the third base of codon 249 in the p53 gene providing further evidence that GLA and other PUFAs modulate p53 expression [54] by forming various lipid peroxides.

The protective action shown against radiation and chemical-induced DNA damage, ability to potentiate the cytotoxic action of anti-cancer drugs and reverse tumor cell drug resistance suggests that GLA could be useful in the management of drug-resistant tumors. Animal studies revealed that GLA is effective against skin pailloma, prevented chemical-induced hepatocarcinogenesis, and ascitic tumors [55–58]; and intravenous administration of lithium GLA to patients with cancer was found to be relatively non-toxic [59,60]. Furthermore, GLA induced tumor regression and preserved the surrounding normal brain tissue in animal glioma model, and three open-label clinical studies [41,46,47] showed that intra-tumoral injection of GLA is effective in reducing the size of the glioma without any significant side effects, suggesting that further studies are needed to explore anti-cancer actions of GLA.

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