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Efficiency of Anti-Epileptic Drug Topiramate in Treatment of Glioblastoma: An *In Vitro* Perception

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Abstract: Brain tumors account for 85% to 90% of CNS tumors. In 20-40% of the patients suffering from brain tumor (BT), seizures are common during the commencement of the disease. The relationship between the onset of seizures and brain tumor is poorly understood. These patients show a complex therapeutic profile making selection of the drugs very tough. The quality of life of the patient is highly compromised due to brain tumor-related epilepsy (BTRE) as it is drug-resistant and poses the challenging risk factor for everlasting disability. Hence, there arise the need of unique and multidisciplinary approach of proper selection of medications with minimum side effects. Glioblastoma Multiforme is a grade IV highly malignant tumor attacking the glia, which provides nourishment and assist in signal transmission. Glioma cells secrete glutamate and choose neurotransmitter receptors for their invasive growth. Glutamate binds to ionotropic receptors, activate calcium release mediating excitatory neurotransmission. The alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors are the major mediators of glutamate-mediated excitatory neurotransmission and are critical for spread of epileptic activity. Glutamate is the main culprit for both the occurrence of seizures and glioma metastases. Hence there lies an opportunity to use the anti-epileptic drugs which targets calcium permeable AMPA receptors for the treatment of glioblastoma as well as BTRE. Topiramate (TPM), a derivative of D-fructose is a novel broad spectrum anti-epileptic drug which shows antagonistic effect on AMPA receptor. When treated with TPM a dose-dependent decrease in live cell number, increase in the number of apoptotic cells, decrease in the calcium influx, reduction in phosphorylation of Akt, Erk1/2 expression were observed. Also phosphorylation of AMPA receptor in presence of EGF was observed. The data suggests that topiramate lead to decrease in proliferation and survival by decreasing calcium influx and inhibiting mitogenic and survival signalling in U87MG cells.
Keywords: Brain tumors, AMPA receptors, neurotransmission.

INTRODUCTION

Cancer is a group of diseases medically referred to as malignant neoplasm and is described by abnormal and uncontrolled cell growth. Among all cancers, central nervous system (CNS) tumors are rare, comprising 2% of all cancers, often linked with high mortality. Among them brain tumors account for 85% to 90% of CNS tumors [1]. According to Canadian Cancer Society, primary brain tumor is listed among top ten causes of death accounting to nearly for 2.3% of the total cancer-related death [2]. Glioblastoma Multiforme (GBM) or gliomas is a grade IV highly malignant and fast growing tumor attacking the glia or supportive tissues, which provide nourishment and assist in signal transmission. Gliomas generally affect people in the age group of 40-65. It covers 30%-40% of all intracranial tumors. Epileptic seizure is a major symptom shown by patients having brain tumors [3]. The term epilepsy denotes malfunctioning of the brain, which is conveyed in the form of periodic but unpredictable seizures. An epileptic seizure is an event caused due to abnormal neuronal action of the brain. Seizures are the outcome of the misbalance of the electrical discharges. These electrical discharges are produced by one brain cell to excite or inhibit other brain cells. Seizures are caused when there is misbalance between the excitation and inhibition of the neurotransmitters. Neurotransmitters which are present in the neurons are the major players in the commencement of epilepsy [4].

During this disease, 6% -10% of brain tumor patients faces the problem of epilepsy [5, 6]. There are several hypotheses suggesting how the brain tumors

relate to epilepsy however the genetics underlying the mechanism are unknown. Elevated levels of calcium and sodium have been found in the extracellular peritumoral space which may lead to neuronal excitability and ultimately seizures [5]. One of the probable hypotheses suggests the common pathways involve in the occurrence of seizures and brain tumors, like the role of certain excitatory and inhibitory neurotransmitters such as glutamate and GABA respectively [7].

Glioma cells secrete glutamate in the surrounding synapse, which is taken back into the astrocyte cells by excitatory amino-acid transporters (EAAT) but in glioma cells EAAT transporters malfunction due to which glutamate accumulates in the medium and is responsible for the neurocytotoxicity of neurons. Glutamate interacts with ionotropic and metabotropic receptors. The ionotropic receptors are *N*-methyl-D-aspartate (NMDA), 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid (AMPA) and kainite receptors. BTRE is drug resistant and affects the quality of life of people. Pharmacological resistance is a characteristic feature of BTRE [8]. Many antiepileptic drugs (AEDs) are released in the market on regular basis. The routinely used AEDs are zonisamide, phenobarbital, levetiracetam, topiramate, valproate which are used to control the seizures. Regardless of the progress made in treatment modalities, glioblastoma is still not curable, and the survival rate is very poor. The present criteria's for treating patient identified with glioblastoma are surgical resection, radiotherapy and chemotherapy [9]. Some of the anti-epileptic drugs are proven to be pro-cancer as well as anti-cancer [10].

Topiramate (TPM) (figure-1A) is a novel broad spectrum anti-epileptic drug which is a derivative of D-fructose [11-13]. It is effective as both monotherapy and adjunctive therapy [14]. TPM has good pharmacokinetic properties, high bioavailability, can be quickly absorbed, excreted mainly by kidney without any modification, and is highly tolerated by all age groups of the patients [15]. There are several mechanisms by which TPM acts as an anticonvulsant agent which includes blockage of calcium channels and AMPA receptors [16-19, 15]. However, the role of topiramate on glioma survival and proliferation was not studied yet. So, here we hypothesized that topiramate is effective against brain tumors by inhibiting the AMPA receptors induced Ca^{2+} influx hence inhibiting the proliferation of glioma cells and induce apoptosis.

MATERIALS AND METHODS

Chemicals and Reagents

Sodium chloride, acetic acid, ethylenediaminetetraacetic acid (EDTA), propidium iodide (PI), saponin, hydrochloric acid, sodium hydroxide, methanol, bromophenol blue, and molecular biology grade DMSO, disodium hydrogen phosphate (Na_2HPO_4), mono-potassium phosphate (KH_2PO_4), acridine orange, ethidium bromide , Bradford's reagent, Fura 2-AM bio-reagent, trypan blue, CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), nefidipine, pluronic acid F-127 and Topiramate (TPM) were procured from Sigma Aldrich, USA. Ethanol was from Merck Biosciences, USA. RNaseA was procured from QIAGEN Hilden, Germany. Trypsin-EDTA (0.25% Trypsin and 0.02% EDTA in Dulbecco phosphate buffered saline), Minimum essential medium (MEM) without glutamine was from Himedia. Fetal bovine serum (FBS), MEM powder from Gibco Life technologies, USA. S-AMPA (2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid was purchased from Abcam biotechnology, Cambridge, United Kingdom. All other chemicals were purchased from S. D. Fine-Chem. Ltd, India and SRL Pvt. Ltd. India.,

For the western blotting p-ERK1/2, total-Erk, phospo-Akt, total-Akt, β-actin was purchased from Cell signalling, PVDF membrane (0.45µm) was from Millipore, western chemiluminescent HRP substrate was purchased from Millipore Merck.

Cell line

U87MG human Glioblastoma multiforme (GBM) cell line was acquired from NCCS, Pune. The cells were grown in defined culture medium at 37°C, 95% relative humidity, 5% CO_2 atmosphere.

Stock and doses

Topiramate (TPM) was dissolved in DMSO at 100mM concentration and stored in 4°C until use. DMSO at final concentration of 0.1% was used for each treatment. The desired aliquot was freshly prepared on the day of experiment by diluting the stock solution to get the final working concentration for treatments. Doses of 25, 50 and 100 µM TPM were used in trypan blue assay, flowcytometry, acridine – orange and ethidium bromide assay. 100 µM TPM was used in the western blotting and live cell imaging by confocal analysis. All the treatments were performed in minimum essential medium without glutamine.

Treatments

For cell viability, flowcytometry the cells were seeded on 60 mm tissue culture treated plates. The cells were counted on haemocytometer and $1x10^5$ cells were seeded on the plates. Drug treatment was performed after 24 hours once the cells attached on the plate. The cells were treated with either DMSO alone or desired drug concentration for 48 hours in MEM without glutamine under regular serum conditions. The details of other cell seedings and treatments are given in the protocols of respective procedures.

Trypan Blue dye exclusion assay

Cells were seeded at a concentration of $1x10^5$ per plate in regular medium and serum conditions. After 24 hours the spent media was removed, and cells were treated with the desired concentration of drug or DMSO in MEM without glutamine with regular serum conditions and incubated for 48 hours. After the required incubation period the plates were removed spent media was taken and the cells were harvested by trypsinization. The cell suspension was centrifuged at 1500 – 1800 rpm for 5 minutes and the pellet was gently washed with ice-cold PBS, centrifuged and again resuspended in 1 ml 1X ice-cold PBS. Hundred µL of respective drug treated cell suspension was taken in 1.5 µL eppendorf tubes and kept on ice. Ten µL of trypan blue (0.4% solution in sodium chloride and potassium phosphate dibasic) was mixed to each tube one at a time and allowed to incubate at room temperature for 3-5 minutes. Tubes were placed on ice and cell counting was done to find out the number of live and dead cells in each dose by haemocytometer.

Acridine orange and Ethidium Bromide (AO/EB) staining for quantification of apoptotic cells

Cells were seeded at a density of $2x\ 10^5$ per 60mm plate. Drug treatment was performed as mentioned and after desired time the cells were completely collected by trypsinization and washed with 1X PBS. The cell suspension was centrifuged at 1200 rpm for 5 minutes and the cell pellet was resuspended in the residual fluid. Twenty-five µL cells were taken on a slide and mix with 2 µL of working stock solution of AO/EB mixture (1:1) (and mixed properly). Slides were prepared and covered with 10x10 mm cover slip and viewed under the 10X, 20X and 60X magnification under fluorescence microscope with Cy3 filter set up (excitation; 450-590 and emission: 540-690). The dye should be added only before visualizing and counting should be done immediately. The principle behind this staining is that AO is a vital stain which stains both live and dead cells. Ethidium bromide (EtBr) stains only dead cells. Hence AO is a DNA binding dye which intercalates into the live cell nucleus and stain uniformly green whereas EtBr stain apoptotic cells which have lost their membrane integrity and show yellow-orange stain. Approximately five-seven random fields were counted, and the cells were scored based on the color of the nucleus and chromatin condensation. Live nuclei appear bright green, live cells with apoptotic nuclei appear bright green with fragmented chromatin. Dead cells appear bright orange with organized chromatin. Based on dual staining and different morphological patterns it was possible to visualize four populations: live cells with normal nuclei (LN), live cells with apoptotic nuclei (LA), dead cells with normal nuclei (DN), dead cells with apoptotic nuclei (DA). The percentage of apoptotic and dead cells were calculated as follows:

$$\% \text{ Apoptotic cells} = \frac{LA+DA}{LN+LA+DN+DA}$$

$$\% \text{ Dead cells} = \frac{DN+DA}{LN+LA+DN+DA}$$

Calcium imaging by Fura 2-AM by confocal analysis

Cells were seeded at a final density of $1.5x10^5$ cells per 35 mm coverslip glass bottom dishes in regular media and serum conditions. The cells were culture for two days. At 46 hours single plate was treated with TPM in MEM without glutamine and serum. At 48 hours the media was removed and rinsed with serum free media without glutamine. The cells were mixed with serum free media with Fura2 AM (5µmol/L) (Cell signaling) reconstituted in 20w/v pluronic acid and incubated at 37°C for 30 minutes. The plates were removed and washed with 7% serum containing media and allowed to relax in the same media for 30 minutes. The cells were 15 minutes pre-treated with agonist and antagonist. The agonist and antagonist used for the experiment were S-AMPA (100µM) (Sigma), Nefidipine (calcium channel blocker; 30 µM) (Sigma), EGF (10ng/ml), TPM (100µM) alone and combinations of EGF+TPM, AMPA (100µM) +TPM (100µM), AMPA (100µM) +CNQX (10µM). Separate culture dishes were used for each treatment. The plates were kept on the Zeiss inverted microscope. The images were taken every one-minute interval for a period of 15-minute, emission at 539 nm.

Whole cell lysate preparation and Western blotting

Membranes were blocked in blocking buffer for 1 hours at R.T. or overnight at 4°C. Primary antibodies p-Erk (1:2000), p-Akt (1:1000), Erk (1:2000), Akt (1:1000), p-AMPA-R (1:1000), AMPA-R (1:1000) β-actin (1:200000) incubation with high speed shaking at R.T for 1 hours was followed by overnight incubation at 4°C. Next day, high speed shaking at R.T. was repeated for 1 hours and was followed by three times washing in 1X wash buffer. Blots were subsequently incubated with corresponding HRP-linked secondary antibody IgG mouse (1:3000) and IgG rabbit (1:5000) for 1-1.5 hours at high speed shaking and followed by three times washing in 1X wash buffer and processed for ECL detection. X-ray films were dried at R.T. and marked for molecular weight protein markers.

Statistical analysis

Data presented are mean ± SE of at least two experiments performed in triplicates. Unpaired t-test was done to indicate the statistical analysis. The differences were considered significant when $p < 0.05$ and indicated in the figures.

RESULTS

Topiramate inhibits cell growth and induces cell death in U87MG

With the purpose to assess the anti-cancer activity of topiramate (TPM), U87MG cells were treated with 25, 50 and 100µM topiramate for 48 hours. Topiramate treatment resulted in dose dependent decrease in the cell growth and proliferation. After 48 hours these doses lead to decrease in live cell count by16-28.7% ($P< 0.05$), total cell count by 13.2-21.3 % ($P< 0.05$) decrease in (Figure 1B). We also assessed the cell death and found a significant increase in cell death with the highest dose of 100µM. There was around 3-fold, 4-fold and 7- fold increase in the cell death from 2% in control to 6% in 25 µM, 8% in 50 µM and 15% in 100 µM (highest dose) ($P <0.05$) respectively (Figure-1B). The results indicate that topiramate strongly inhibits cell growth and proliferation and induces death in U87MG cells leading to decrease in total cell number.

The effect of topiramate on U87MG at 24 and 72 hours at 100µM was checked as well. Both the results showed that there was decrease in cell number and increase in percent cell death but not to a significant level (figure-1C).

Topiramate induces apoptosis in U87MG

An increase in dead cells by topiramate was observed in cell growth assay. Therefore, we examined whether topiramate induced death was through induction of apoptosis. Consistent with the cell death data, 25, 50 or 100 µM topiramate treatment for 48 hours showed a dose-dependent increase in apoptotic cells. Live cells show uniform bright green fluorescence (figure-2A). Apoptotic nuclei have extremely condensed chromatin that is consistently fluorescent. This may take the form of crescents around the boundary of the nucleus or the complete chromatin can be existing as one or group of featureless bright spherical beads. In advanced apoptosis the cell will have lost DNA or become fragmented into apoptotic bodies. Thus, in these way phenomena of early apoptosis as well as late apoptosis is identified. Live apoptotic cells have green fluorescence but condensed chromatin (figure-2B). Dead apoptotic cells have orange color chromatin (figure-2D).

We found that the apoptotic cells significantly increased at highest dose from 6.3% to 18.6 % ($P <0.05$) (Figure-2E). Also, at 48 hours the population of dead cells significantly increased by approximately 9-fold from 2% to 19% ($P <0.001$) at the highest dose (Figure-2F). Hence topiramate significantly increases the apoptotic and dead cells.

Effect of Topiramate on the calcium influx in U87MG

From the previous studies it was reported that glioma cells release excessive amount of glutamate and glioblastoma cells exploit glutamate for cell proliferation through AMPA receptors [20-22]. It was hypothesized earlier that glutamate release may activate glutamate receptors. Activation of these receptors may lead to influx of calcium $[Ca^{2+}]_i$ ions. Here we check the effect of topiramate on $[Ca^{2+}]_i$. Fura 2-AM dye was used which binds to calcium. Confocal image analysis was done at excitation at 409 nm and emission at 539 nm at intervals of 1 minute for a period of 15 minutes. Nefidipine (30 µM), a calcium channel blocker was used, which decreased the fluorescence to a minimal level suggesting the increase in florescence observed, is due to calcium influxing alone (figure 3H, I). U87MG cells were treated with S-(AMPA) (100 µM) which resulted in significant increase in fluorescence than compared to control ($P <0.05$) (figure-3E, I). When the cells were treated with topiramate (100 µM), a known antagonist of AMPA receptors there was significant decrease in the fluorescence as compared to control ($p <0.001$) suggesting decrease in calcium influxing (figure-3C, I). Also with TPM treatment we observed rounded clumped cells with their extensions lost suggesting death of the cells (figure-3C). When the cells were pre-treated with TPM (2 hours) and stimulated with AMPA for 15 minutes, we observe decrease in the fluorescence as compared to control ($P <0.05$) (figure-3F, I). With the combination of AMPA+TPM the cells are rounded, present in clumping with no extension (figure-3F). Also, when the cells were treated with CNQX (10µM), a competitive antagonist of AMPA receptor the fluorescence was almost inhibited (figure-3G, I).

As the glioblastoma cells are known to overexpress EGFR receptors (40%-50%), we wanted to check if there is a crosstalk between the AMPA and EGF receptors. Therefore, stimulation of EGF (100ng/ml), a ligand of EGFR was given for 15 minutes, non-significant change in calcium influxing as compared to control (figure-3B, I) was observed. Also, in the pre-treated TPM plates stimulation of EGF (100ng/ml) was given for 15 minutes and we observed that fluorescence increased as compared to control and EGF alone but there was increase in florescence intensity when compared to that TPM ($P <0.001$) (figure-3D, I). Also, we identified that when cells were treated with TPM few cells have completely lost their floroscence (white arrows) but some of the cells are having less floroscence as compared to control. Similarly, when treated with the combination of EGF+TPM (figure-3D), it can be observed that in some of the cells the floroscence intensity is much higher as compared to control (yellow arrows).

So, from here it may be concluded that TPM, an AMPA receptor antagonist decrease the proliferation of U87MG cells, due to decrease in $[Ca^{2+}]_{i.}$

Effect of agonist and antagonist of EGFR and AMPAR on cell growth and proliferation in U87MG

To check the final outcome of calcium influx on the growth and proliferation on U87MG cells, the cells were treated with EGF, AMPA, TPM, and combinations of EGF+TPM, AMPA+TPM, AMPA+CNQX and nefidipine (calcium channel blocker).

When the cells were induced with EGF and AMPA, number of total cell (1.4-fold) (1.1-fold) (figure-4A) and live cell (1.5-fold) (1.2-fold) (figure-4B) significantly increased respectively, as compared to control alone and the percentage of dead cells (figure 4C) decreased (0.6 %) as compared to control but with AMPA there was no much change in percentage of dead cells (figure 4C) when compared to control.

When the cells were treated with TPM there was significant decrease in both total cell (22%) (figure-4A) as well as live cell number (26.71%) (figure 4B) whereas percentage of dead cells (3- fold) (figure-4C) increased as compared to control, EGF, and AMPA alone. Also it was observed that there was a significant increase in the percentage of dead cells as compared to EGF and AMPA alone (figure-4C).

Even, when the cells were treated with combination of EGF+TPM and AMPA+TPM, the total cell (9.3%) (14.6%) (figure 4A) and live cell number (18.9%) (22.32%) (figure 4B) decreased significantly whereas cell death (4-fold) and 13% increased as compared to control. When compared with EGF, it was found that there was marked increase in the percent cell death in the combination of EGF+TPM (figure-4C). In both combination of EGF+TPM and AMPA+TPM around cell death was observed (figure-4C).

CNQX is a competitive inhibitor of AMPA receptor, when the cells were treated with combination of AMPA+CNQX, there was marked decrease in both total cell (25.8%) and live cell number (28.48%) as compared to control, AMPA and AMPA+TPM alone (figure-4A, B). The percentage of cell death increased in AMPA+CNQX as compared to control alone (figure-4C). When the cells were induced with nefidipine, a calcium channel blocker, number of live cells (30.5%) and total cell number (30.7%) considerably decreased (figure-4A, B) and number of dead cells increased as compared to control alone (figure-4C).

Effect of topiramate on cell survival and proliferation pathway

To confirm the result of action of topiramate on U87MG cell survival and proliferation, the expression levels of major molecules of these pathways namely Akt and Erk1/2 were checked by western blotting. The immunoblot and densitometry study showed that when treated with EGF (100 ng/ml) there was slight decrease in the level of expression of total Akt (30% decrease) as compared to control. When treated with topiramate (100μM) the level of total Akt was almost equal to that of control (0.9). Similarly, in the combination treatment of EGF+TPM the total Akt level was again same as that of EGF (30% decrease). However, when induced with AMPA (100μM) total Akt level decreased 70% as compared to control. Similarly, in the combination treatment of AMPA+TPM the total Akt level was found to be the same as that when treated with AMPA alone (figure-5A).

When induced with EGF there was no much difference in the phosphorylation of Akt at Ser-473 as compared to control (0.9). When treated with TPM, it was found that there were 40% decreases in the level of phospho-Akt as compared to control. When treated with the combination of EGF+TPM the level of phospho-Akt was found to be 0.8-fold increased as compared to control. And this increase in phosphorylation of Akt may be aiding in the apoptosis or cell death. When stimulated with AMPA the level of phosphor-Akt was found to be 0.3-fold increase as compared to control. When treated with the combination of AMPA+TPM the phosphorylation pattern of Akt was remain unchanged as when stimulated with AMPA alone (figure-9A). In the other treatments the level of total Erk1/2 was almost the same (figure-4B).

When induced with EGF, ligand of EGFR there was a significant increase in the phosphorylation of Erk1/2 at tyrosine202/threonine204 (1.4-fold increase) as compared to control. There was significant decrease in the phosphorylation pattern of Erk1/2 (80%) when treated with topiramate alone and in combination with EGF and topiramate. Similarly, when induced with AMPA, agonist of AMPA receptor there was a marked increase in phosphorylation pattern of Erk1/2 (1.3-fold) (figure-4B).

Similarly, when treatment with combination of AMPA + TPM resulted in the marked decrease in the p-Erk1/2 (70%) (figure-4B) while no change was observed in case of p-Akt (figure-4A) as compared to AMPA alone suggesting that one of the targets of topiramate may be the Erk1/2 pathway which may be playing a role in decreasing cell proliferation.

Effect of topiramate on the membrane receptors: AMPA and EGF receptors

When the cells were induced with EGF, there was increase in the phosphorylation of AMPA receptor at Ser-845, when the cells were pre-treated with TPM and then induce with EGF there was 70% decrease in the phosphorylation of AMPA receptor at Ser-845. Also, when the cells were stimulated with AMPA there was a marked increase in the phosphorylation of AMPA receptor at Ser-845 (3.3-fold increase). We observed that there was no phosphorylation of AMPA receptor (at Ser-845) in the control, and cells treated with TPM or AMPA+TPM.

We observed that the level of total AMPA receptors was almost equal in control, EGF induced cells and TPM treated cells. However, there was a marked decrease in the level of total AMPA receptors when the cells were pre-treated with TPM and induced with EGF (60%) or AMPA (40% fold), respectively.

DISCUSSION

Glioblastoma (GBMs) is the most malignant and dangerous tumors among all the brain cancers. The distinguishing features which make these tumors aggressive are uncontrolled cell proliferation, invasion into the nearby healthy brain tissues, angiogenesis, resistance to apoptosis, cells undergoing necrosis and extensive genetic alterations [23].

Most of the patients with brain tumor show a very distinct symptom of seizures [24]. The presence of epilepsy is considered as one of the important risk factor which can lead to prolonged disability in these patients and severely affect their quality of life [25-27]. In the patients suffering from brain tumor related epilepsy, it is very difficult to choose the proper medication, as they present a very intricate therapeutic outline and involves a multidisciplinary approach to treat the disease [28]. In this group of patients, it is necessary to design a treatment plan which targets both, the seizure as well as tumors. So, it is very important to choose an appropriate anti-epileptic drug which shows minimal side effects as brain tumor related epilepsy are drug-resistant [8].

But, the prophylactic usage of anti-epileptic drugs is not suggested in brain tumor patients, as there is risk of interactions of chemotherapeutic and enzyme inducing anti-epileptic drugs. The pharmacological approach to treat epilepsy must be still resolved, because a lot of controversy regarding drug-drug interactions has been proposed [29, 30]. It is necessary to make the perfect combination of these drugs to treat both cancer and cancer related epilepsy so that there is minimal side effect.

Many of the drugs which shows promising results which are in use involves gabapentin, lacosamide, oxcarbazepine, zonisamide and topiramate which have few drug interactions and side-effects [24]. But some of the recent studies in animal models have shown very shocking results. Some of the animal studies have reported that antiepileptic drugs like phenobarbital, phenytoin are categorized under tumor promoting agent, whereas valproic acid has tumor protective properties [29, 30].

So, the present study was done to explore the effects of an anti-epileptic drug topiramate on brain tumors. Also, there was no study done to see the effect of topiramate on U87MG cells. Further in the present study we tried to find out the role of topiramate on EGF receptors and AMPA receptors and their role in cell survival and proliferation. So, we evaluated the effect of topiramate in human glioblastoma U87MG cells.

Many reports have shown that glutamate and its receptors are important for GBM survival, proliferation and invasive behavior. Also, these tumors are resistant to the excitotoxicity caused by glutamate which helps in sustaining tumor growth by destroying the peritumoral neuronal cells by creating space for the tumor expansion [21]. In one of the study conducted by Lyon *et al*., it was showed that GBM cell lines including U87MG cells secrete nearly 100-300nmol/mg glutamate during a period of 4 hour [31]. It has already been proposed by Ischui et al that AMPA receptor antagonist can prevent cell proliferation and migration of glioblastoma cells [32]. So AMPA receptor antagonists have great therapeutic implications to prevent glioma growth and metastases.

Topiramate is a new anti-epileptic drug which effectively controls seizures and has very few side effects which makes them useful in epileptic patients. Chemically topiramate is a sulfamate substituted monosaccharide with several complex mechanisms which shows its anti-convulsant activity (figure-1A). The drug enhances γ-aminobutyric acid (GABA) activated chloride channels, it inhibits excitatory neurotransmission through kainite and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropanate (AMPA) receptors [33].

From the pharmacokinetic studies it was reported that, the mean plasma and renal clearance was found to be 22-36 ml/min and 17-18 ml/min respectively [34]. The mean plasma elimination half-life recorded was 19-23 hours in the normal human volunteers [35]. In one of the study it was shown that when the cells are grown in glutamate free medium with 10% fetal bovine serum for 4-5 days number of dead cells increased [22].

The anticancer efficacy of any agent can be assessed by checking its effect on cell growth and viability. The effect of topiramate on the cell line U87MG at 48 hours suggest that there is dose

dependent inhibition in growth and proliferation, however there is no much increase in the percent cell death (around 15%) (figure-1B). To check the effect of drug on the U87MG cell line at 24 and 72 hours the cells were treated with the most effective dose at 48 hours, i.e 100 μM. There was decrease in cell proliferation and increase in cell death but not to a significant level as it was seen in the 48 hours with the same concentration (figure-1C). This was in accordance to the previous experiments done to show that the 48 hours is the optimum time point for the action of the drug in the cell line.

To check the effect of drug on cell cycle, flow cytometric analysis was done. The flow cytometric analysis shows that there was very mild G2/M arrest at 48 hours (data not given). There was very less percent of cell death as shown in the trypan blue counting and hence very less percentage of cells were analyzed in cell cycle arrest. This suggests that topiramate does not have a significant role in inducing cell cycle arrest. Through this it can be proposed that there may be other mechanisms independent of cell cycle arrest which plays a key role in preventing cell proliferation and survival.

Other than the cell cycle arrest there are many ways a cell can undergo death and may be one of the way a cancer cell can be killed, one being apoptosis. Apoptosis is the programmed cell death in which there is a biochemical event which leads to the morphological changes in the cell and ultimately leads to the cell death. The morphological changes which occur in the cells are dwindling of cells, membrane blebbing, nuclear and chromatin condensation, fragmentation of chromatin. Apoptosis is controlled process and very different from necrosis in which the apoptotic bodies which are produced is phagocytosed by the phagocytic bodies and does not cause any injury to the nearby tissues. There may be many factors which lead to the apoptosis of the cells.

To check the effect of drug on apoptosis and percent cell death, acridine orange and ethidium bromide staining was used and it was observed that the percent cell death was almost equal to that was detected with trypan blue dye exclusion method. In the fluorescence microscope membrane blebbing, chromatin condensation (figure-2B), and chromatin fragmentation (figure-2D) was observed. Also observed that consistent with cell death data, there was a dose dependent increase in the number of apoptotic cells (figure-2E). Topiramate induces apoptosis in U87MG cells, this may be in part be mediated by decrease in Ca^{2+} influx.

Calcium has been identified as one of the mediators of antiproliferative activity. The blockage of the calcium influx may play a significant role in suppressing the glioma growth. In glioma cells, Ca^{2+} influx through alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropanate (AMPA) receptors stimulates cell proliferation, growth and motility through phosphorylation of Akt [32]. We studied the effect of topiramate on Ca^{2+} influx in U87MG cells by confocal analysis. The results demonstrated increase and decrease in calcium influx when stimulated with AMPA receptor agonist and antagonist, respectively. Also, when calcium channel blocker nefidipine was used there was minimum calcium influx (figure 3H). We observed decrease in Ca^{2+}influx when treated with topiramate (figure-3C), increase in the percent cell death (figure-4C). Decrease in Akt phosphorylation was confirmed by western blot analysis (figure-5A).

In glioblastoma phosphatidylinositol-3 kinase (PI3K) pathways is deregulated [36]. The pathway is activated through receptor tyrosine kinase signaling when ras is bound to GTP [37]. PI3K is recruited to the cell membrane and phosphorylate, phosphatidylinositol-4, 5-bisphosphate (PIP2) to PIP3. PIP3 recruit's serine/threonine kinase Akt to the plasma membrane where it is fully activated at Thr-308 and Ser-473 by mammalian target of rapamycin (mTOR) [38]. Elevated Akt phosphorylation is observed in 85% of the glioblastoma [39]. Already it has been shown that Ca^{2+} influx through calcium permeable AMPA receptors increase the phosphorylation of Akt at Ser-473. We could also observe that there was change in the phosphorylation of Akt at Ser-473 rather than at Thr-308 from the immunoblotting.

There is up regulation of epidermal growth factor receptor in 40%-60% of the glioblastoma patients [40, 41]. When the cells were treated with EGFR ligand EGF, we could observe there was no increase in calcium influx (figure-3I), but there was increase in cell number (figure-4A) which is due to the marked increase in Erk1/2 at Threonine202/Tyrosine204 activation as confirmed by western blotting (figure-4B). When the cells were treated with EGF and AMPA receptor antagonist, TPM we found that there was increase in the phosphorylation pattern of Akt (figure-4A). This suggests that, EGF induced Akt phosphorylation increases in combination with topiramate. And this increase in phosphorylation of Akt may be aiding in the apoptosis or cell death. This result is supported by a study in which it was reported that when there is overloading of calcium i.e. when the intracellular level of calcium increased to elevated levels it leads to the activation of PI3K/Akt, p53, and MAPKs pathways. The activation of PI3K/Akt pathway may give a pro-apoptotic signal to the cells leading to the initiation apoptosis. Similarly, there was an increase in the calcium influx when treated with combination of EGF and topiramate supporting the western blotting data. And this is further consistent with the results of cell

counting with trypan blue on treatment with EGF and topiramate (cell death, 12%) (figure-4C).

When treated with topiramate alone, there was decrease in the total and live cell number (figure-4A, B), also there was increase in the percent cell death as compared to control (Figure 4C), confirmed with the western blot, there was decrease in phosphorylation level of Akt (figure 5A) and Erk1/2 (figure-5B). This suggests that U87MG cells are very much dependent on AMPA signalling for its growth and survival. The cells were treated with AMPA, an agonist of AMPA receptor, we could observe that there was increase in total cell number (figure 4A), phosphorylation of Erk1/2 (figure-5B) and Akt (figure 5A). This again proves that AMPA dependent signalling is important in U87MG cells for growth and survival. There was significant increase and decrease in the mean florescence intensity for calcium influx when treated with AMPA and topiramate alone respectively (figure-3I), suggesting that Ca^{2+} influx is important for cell survival.

The mechanism of action of topiramate is through AMPA receptors in epilepsy [33]. When the cells were pre-treated with TPM and activated with AMPA, we noticed that there was a decrease in the calcium influx (figure-3I), a decrease in cell number and an increase in percent cell death (figure-4C). Also, there was a decrease in Erk1/2 and Akt activation as confirmed by western blotting. Thus, it can be concluded that Ca^{2+} influx is important for cell survival in U87MG cells. With nefidipine, a calcium channel blocker, we could observe that there was a strong decrease in calcium influx and cell survival which again supports that Ca^{2+} influx is important for cell survival.

To study the effect of topiramate on the AMPA receptors, we observed the phosphorylation pattern of AMPA receptors at Ser-845. When induced with EGF and EGF+TPM we could observe phosphorylation of AMPA receptor. So, there may be possibility of cross-talking between the membrane bound EGF and AMPA receptors. Also, we observed that topiramate was unable to inhibit the EGF-induced activation. Further studies are needed to find out the reasons why EGF-induced AMPA receptor activation is not inhibited by topiramate in the combination of EGF+TPM.

Majority of gliomas show amplification of the EGFR gene but there are possibilities of rearrangement of genes which results in the alterations of the transcript expressing both wild type and mutant form [42, 43]. Hence, a whole lot of separate study is needed to study the downstream activation through these receptors.

The present study shows that topiramate inhibits cell growth and proliferation, induces apoptosis, decrease Ca^{2+} influx in human glioblastoma U87MG cells. The effect of topiramate on Erk1/2 and Akt pathways were assessed. A decrease in phosphorylation level of Erk1/2 suggests that one of the antiproliferative mechanisms of action of drug may be the inhibition of Erk1/2 pathway (figure-5B).

Hence, from the data obtained, we can conclude that topiramate lead to decrease in proliferation and survival by decreasing calcium influx and inhibiting mitogenic and survival signaling in U87MG cells. However, further studies are needed to confirm the actual mechanisms of the drug and its possible use as an effective anti-cancer agent.

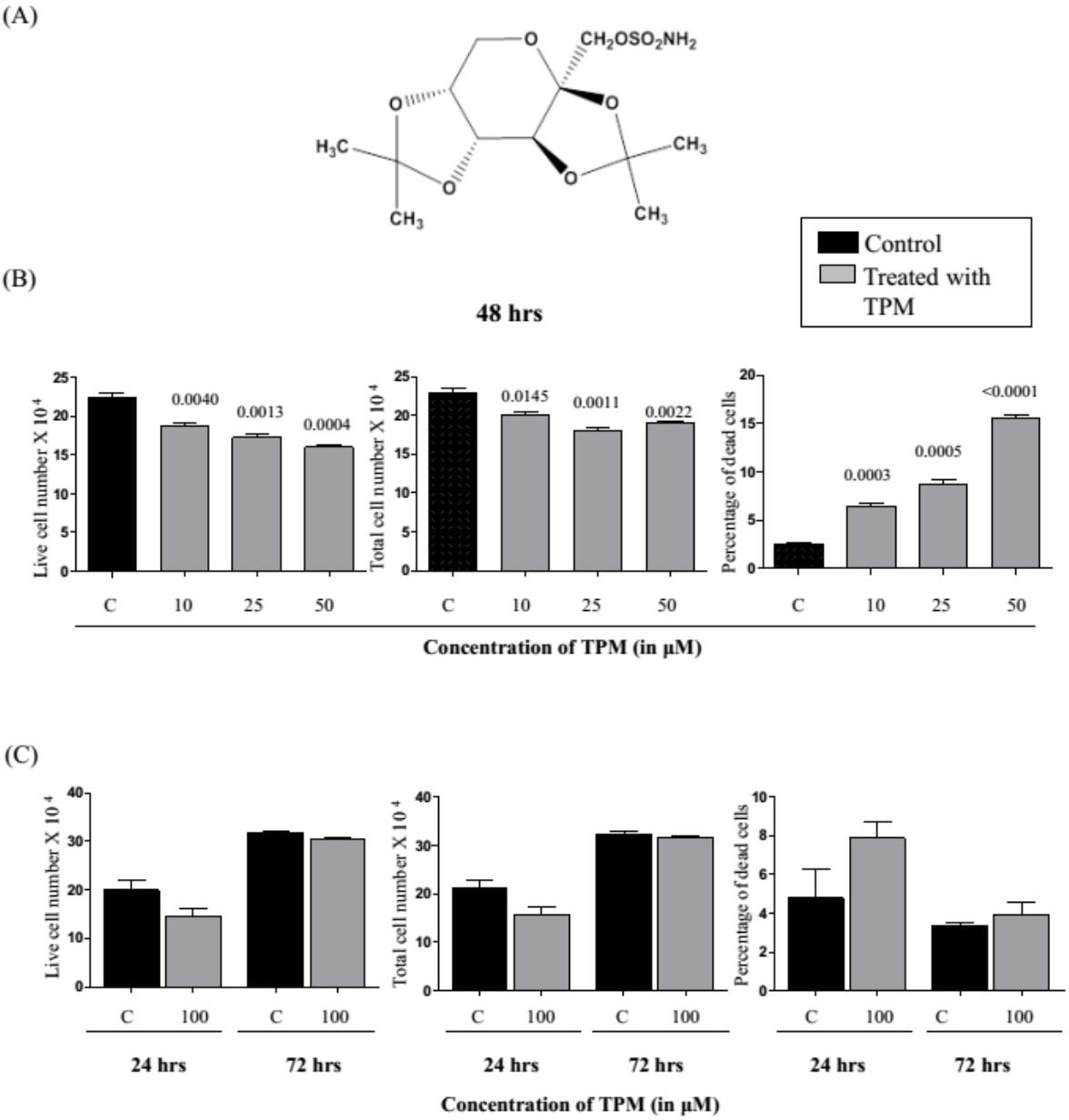

Figure 1: Trypan blue dye exclusion test. Effect of topiramate (TPM) on cell growth and death in human glioblastoma U87MG cells. Briefly, $1X10^5$ cells were seeded on 60mm culture dish. The cells were treated next day with DMSO (control) or 25, 50, 100 µM doses of TPM in minimum essential medium without glutamine. After 48 hours cells were collected and counted on haemocytometer. The cells were scored on the basis of trypan blue dye exclusion test (A) chemical structure of topiramate (B) 48 hours (C) 24 and 72 hours . Results are representative of three independent experiments. The data are presented as means of triplicate samples of each treatment. Statistical analysis, unpaired t-test; Columns, mean; Bars, SE. P values are shown at the top of the bar.

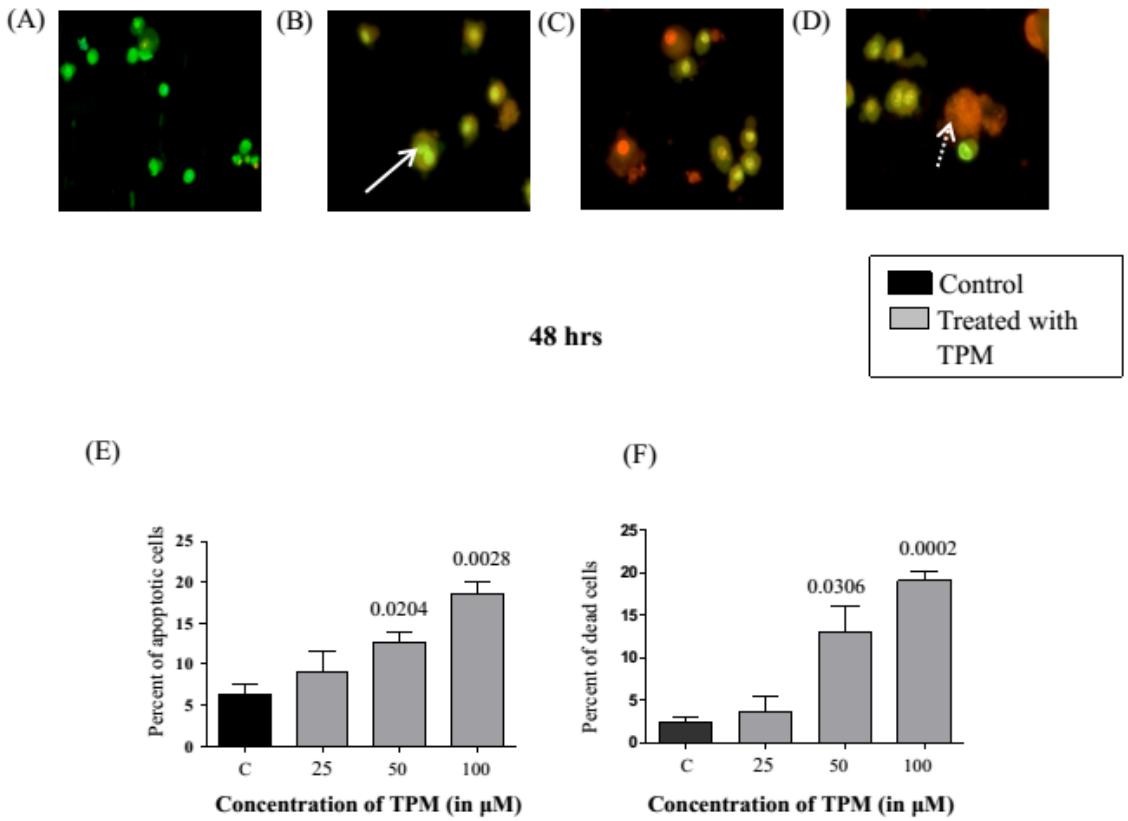

Figure 2: Acridine orange/ethidium bromide staining. To detect apoptosis U87MG cells were induced by different doses of topiramate: (A) control (B) 25 µM (C) 50 µM and (D) 100 µM TPM. Live cells (continuous arrow) are uniformly green, live apoptotic cells (dashed arrow) are characterized by green staining but fragmented chromatin, dead apoptotic cells (dashed and dotted arrows) yellow-orange staining due to chromatin condensation and loss of membrane integrity and necrotic cells (dotted arrows). Magnification 100X. Effect of topiramate (TPM) after 48 hours treatment on cell viability and apoptosis in human glioblastoma cells U87MG. Live cells and apoptotic cells were scored on the basis of acridine orange and ethidium bromide assay. (E) represents percent apoptotic cells (F) represents percent dead cells. The data are presented as means of triplicate samples of each treatment. Statistical analysis, unpaired t-test; Columns, mean; Bars, SE. P-values are shown at the top of the bar.

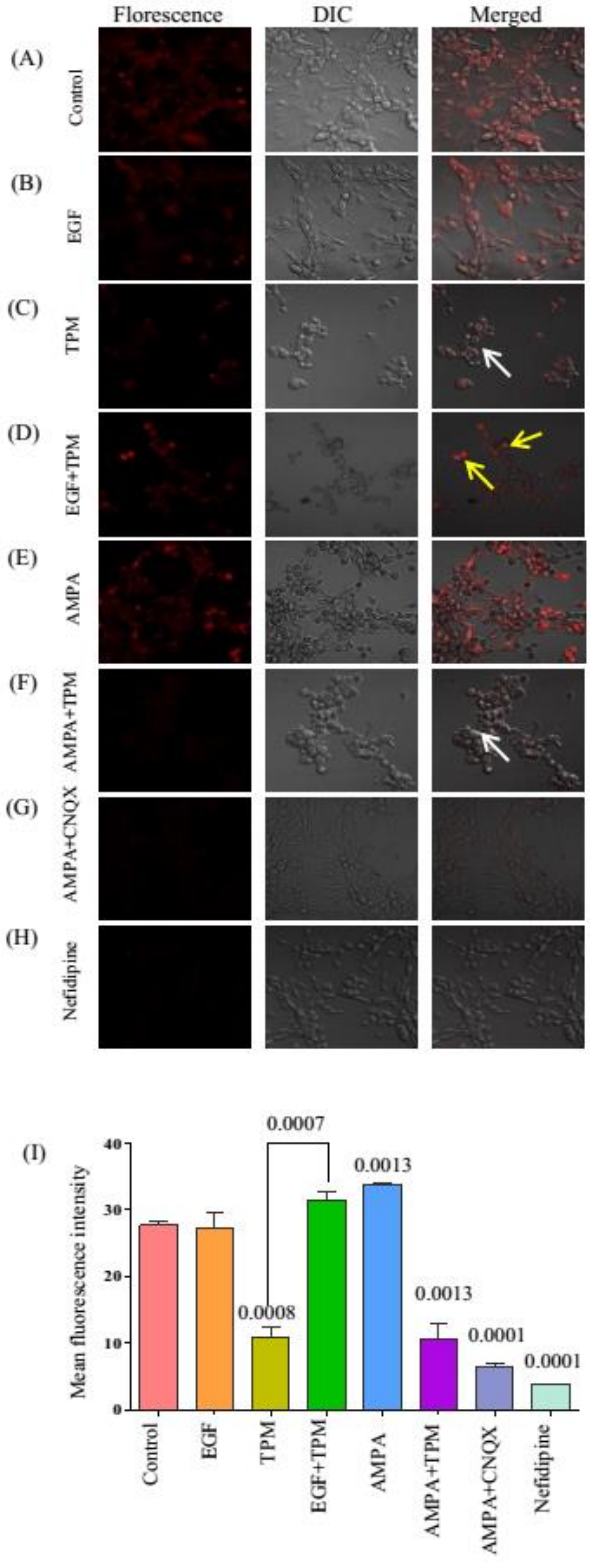

Figure 3: Fura-2AM staining. Intracellular imaging by confocal demonstrating calcium influx by fura2-AM in U87MG cells. About $1X10^5$ cells were seeded on 35 mm coverslip bottom glass dishes and cultured for two days. The cells were loaded with fura 2-AM as described in Materials and Methods. Images were taken at 23X magnification. (A) Control (B) EGF (100ng/ml) (C) Topiramate (TPM) (100 µM), cells treated with topiramate decrease calcium influx (D) EGF+TPM (E) AMPA (100 µM) agonist of AMPA receptor increase Ca^{2+} influx (F) AMPA (100 µM) + TPM (100 µM) decreases calcium influx (G) AMPA (100 µM)+CNQX (10 µM), antagonist of AMPA receptor inhibit Ca^{2+} influx (H) Nefidipine (30 µM), a calcium channel blocker inhibit Ca^{2+} influx. White arrows shows clumping of cells with decrease calcium influx, yellow arrows show cells with increase calcium influx. (I) The effect of topiramate on the mean florescence intensity of calcium influx in U87MG is measured. The data are representative of three independent experiments. Statistical analysis, unpaired t-test; Columns, mean; Bars, SE. P values are shown on the top of the bar.

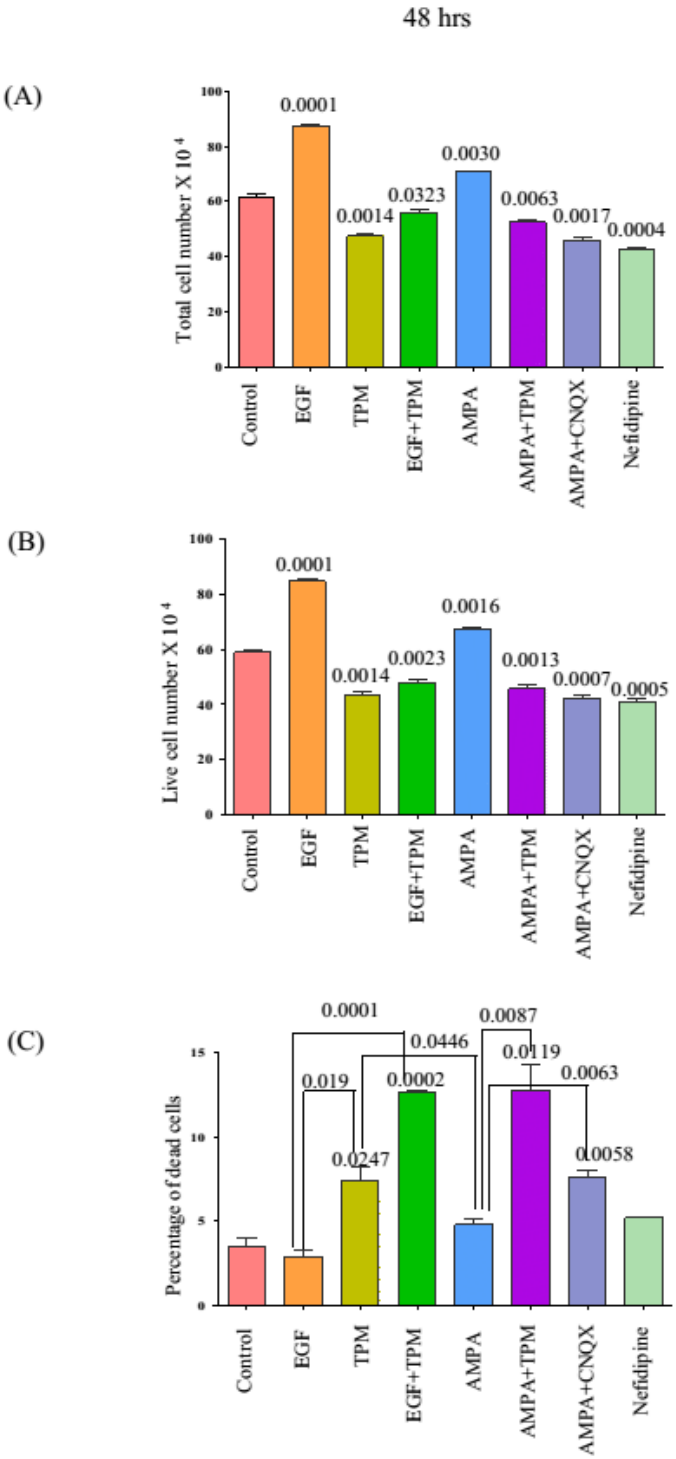

Figure 4: Trypan Blue dye exclusion test. The effect of agonist and antagonist of EGF, AMPA receptors and calcium channel blocker nefidipine on cell growth and proliferation at 48 hours in U87MG cells. (A) total cell number (B) live cell number (C) percent cell death. The data are representative of three independent experiments. Statistical analysis, Unpaired t-test; Columns, mean; Bars, SE. P values are shown on the top of the bar.

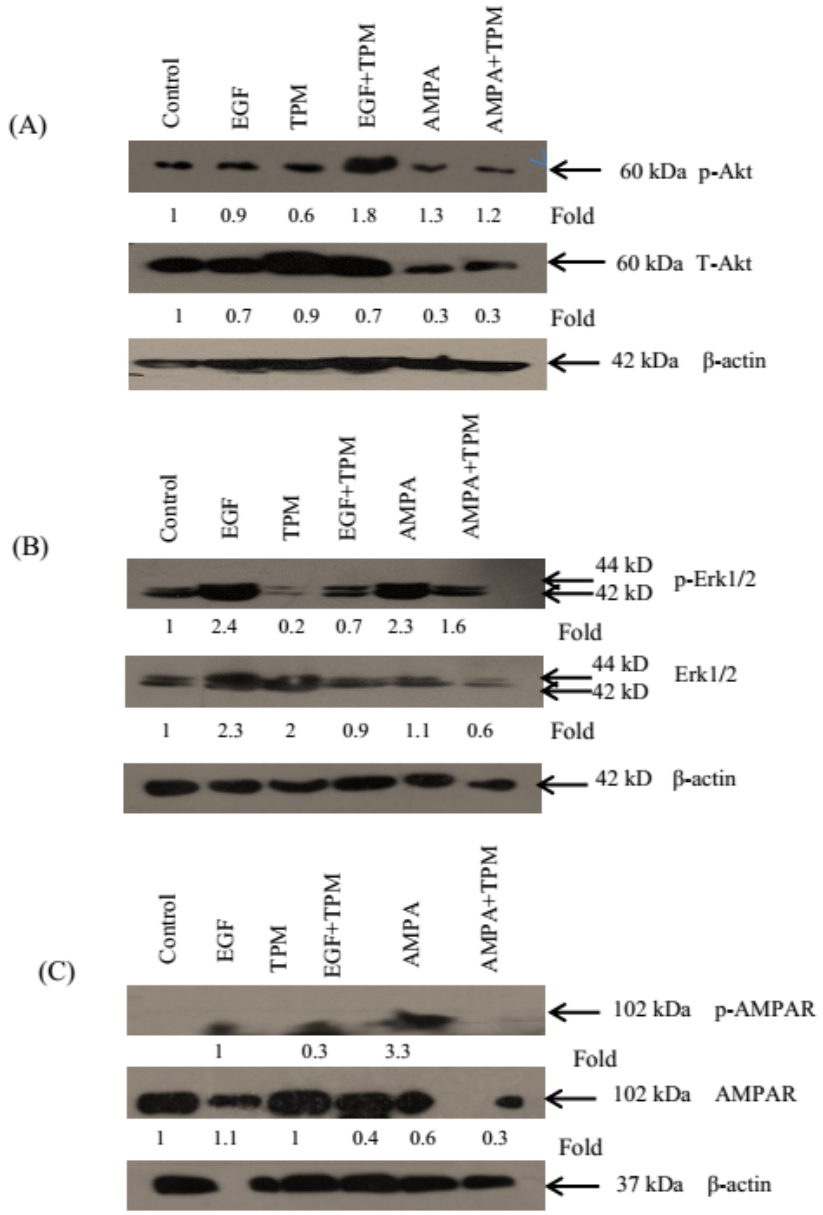

Figure 5:Western blotting to check protein expression on survival pathways and AMPA-R. Effect of topiramate on survival pathways (PI3/Akt and MAPK) molecules p-Akt and p-Erk1/2; and for p-AMPAR and AMPAR. Topiramate (100µM) treatment, stimulation with EGF (100ng/ml) and AMPA (100 µM) was given and its effect on p-Erk1/2 and p-Akt was observed. Whole cell lysates were prepared, 70-80µg/lane protein was loaded, electrophoresed on 12% SDS gel and analyzed for p-Erk1/2, Erk1/2, p-Akt, Akt. A) p-Erk1/2 and Erk1/2 B) p-Akt and T-Akt. The membranes were stripped, reprobed for T-Erk and β actin. β actin was used as the loading control. After normalization with β actin a fold induction is shown below the blots.

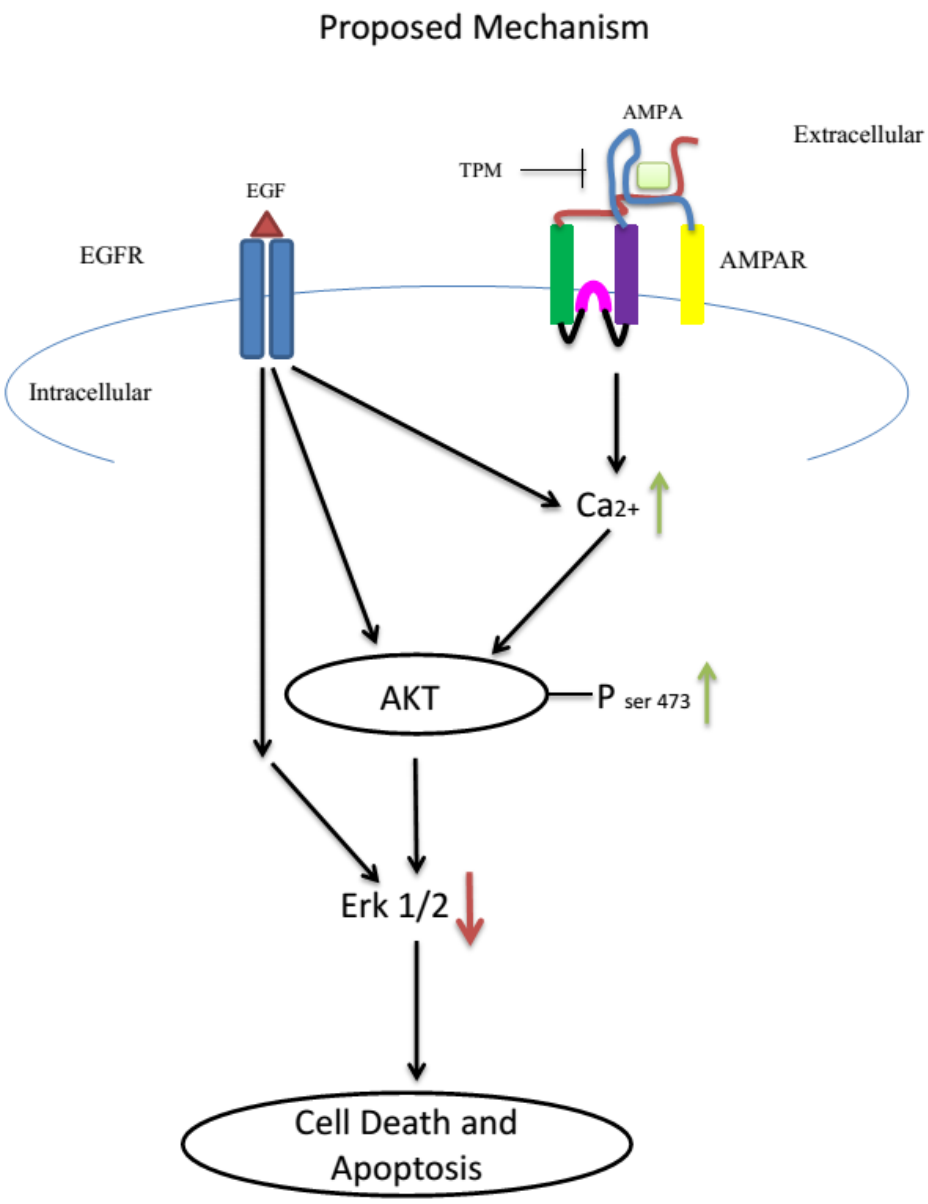

Figure 6: The proposed mechanism of Topiramate (100 µM) treatment on U87MG cell death (for discussion part)

CONCLUSION

The current study focuses on the role of topiramate (TPM) on the glioblastoma (GBMs) cells. We checked the effect of TPM on U87MG cells, a grade IV glioblastoma cell line.

We found a dose-dependent decrease in the live cell number and increase in the percent dead cells as shown by trypan blue dye exclusion assay. We observed that TPM has a very mild effect on the cell cycle as observed by flow cytometer analysis. We could also observe that there was a dose-dependent increase in the number of apoptotic cells and dead cells as measured by acridine orange and ethidium bromide assay. When seen in the fluorescence microscope, we could observe that there was chromatin fragmentation and membrane blebbing which are the indicators of cells undergoing apoptosis.

To check the effect of topiramate on calcium influx, Fura 2-AM dye which binds specifically to calcium, was used to measure the calcium influx. In the presence of TPM there was significant decrease in the calcium influx in the cells as seen from the confocal analysis.

Here, we investigated the role of topiramate on the cell survival and cell proliferation pathways. From the western blot analysis, we observed that there was a decrease in the phosphorylation of Akt and Erk1/2 when treated with TPM. So, it may be inferred

that there may be a possible role of TPM on these pathways.

We could also observe a decrease in live cell number and increase in percent dead cells in EGF induced, TPM pretreated cells. An increase in calcium influx was seen in these cells suggesting a possible interaction of TPM with EGFR pathway. In this treatment, we also observed a marked increase in the phosphorylation of Akt.

Further, in EGF and EGF+TPM treatments, we observed phosphorylation of AMPA receptor. This suggests the cross-talking between the membrane bound EGFR and AMPA receptor in presence of EGF. Also, we observed that topiramate was unable to inhibit the EGF induced activation of AMPA receptor (proposed mechanism of TPM action is shown figure 6). Overall, this is a preliminary study for the possible action of the antiepileptic drug, topiramate, on the growth and survival of glioblastoma cells. However, further studies are needed to prove its potential as anti-cancer drug.

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