Effective and selective targeting of Brain Tumor cells using a novel mTORC1/2 kinase inhibitor.

Michael Prendergast

Briarcliff High School

Abstract

Glioblastoma Multiforme (GBM), grade IV astrocytoma, is highly invasive and neurologically devastating. Despite the technological advances and novel therapeutics including neurosurgery, radiotherapy, and chemotherapy, GBM remains an incurable disease. The mammalian target of Rapamycin (mTOR), that exists in two multipotent complexes, namely (mTORC1, mTORC2), regulates cell growth and survival is found to be deregulated in GBM this offers a suitable therapeutic target. However, therapies, such as Rapamycin and its analog to target mTOR, have been unsuccessful in treatment of GBM. The failures of these drugs are attributed to either activation of mTORC2 or the MAPK pathway via the feedback loop. We sought to use a novel inhibitor, which unlike Rapamycin (RAPA), which is an allosteric site inhibitor for mTOR, uses ATP binding site mTOR pathway (PP242) inhibits mTORC2. In addition PP242 inhibits both mTORC1 (P70S6k) and mTORC2 (pAKT) substrate. GBM cell proliferation more completely than RAPA. Notably, at the molecular level, PP242 inhibits cap-dependent translation under conditions in which RAPA has no effect. Importantly, unlike RAPA, PP242 did not active MAPK pathway. The results analysis incorporation of EDU into the DNA showed that PP242 and RAPA inhibited the nucleus expression of this compound. Thus, the result of this finding demonstrates that the PP242 is a potent pharmacological agent which displays better results in inhibiting GBM cell in molecular as well as cellular and may serve as better target for the GBM cells.

1. Introduction

1.1 Review of Literature

Median survival time continues to be approximately one year after diagnosis. Overall, five-year survival is approximately only 2 %. GBM, the most frequent and malignant human

brain tumor, may develop de novo (primary GBM) or by progression from low-grade or anaplastic astrocytoma (secondary GBM). The genetic nature of GBM echoes the clinical dichotomy that exists between primary and secondary GBM. The PTEN and p53 tumor suppressors are among the most commonly inactivated or mutated genes in human cancers including GBM. Parallels between growth factor signaling elements implicated in GBM progression and those that control crucial stages in neural development are consistent with recent evidence signifying neural stem and/or progenitor cells as the cell type of origin for GBM (Galli et al., 2004; Holland et al., 1998; Holland, 2001; Sanai et al., 2005). Of the genomic alterations described in GBM, a tumor suppressor gene product PTEN (phophatase and tensin homolog on chromosone10) mutation and/or deletion is the most common, with an estimated frequency of 70–90% (Nutt and Louis, 2005; Hu and Pandolfi, 2005). PTEN promotes apoptosis primarily via inactivation of the PI3K/Akt cell survival pathway. Recent evidence suggests that PTEN may function independently of this pathway. The downstream activation of AKT and mTOR is observed frequently in GBM. AKT phosphorylates mTOR, which activates p70S6 kinase and 4E-BP1, which are both involved in protein synthesis. AKT activates downstream targets, other than mTOR, to independently enhance cell survival and proliferation.

The mammalian target of rapamycin (mTOR) is a serine-threonine kinase related to the lipid kinases of the phosphoinositide 3-kinase (PI3K) family. Deregulation of this kinase is often seen in many cancers. mTOR exists in two multiprotein complexes, mTORC1 (Guertin, and Sabatini 2007) and mTORC2 (Sarbassov 2004; Jacinto 2004) which are differentially regulated, have distinct substrate specificities, and are differentially sensitive to Rapamycin (RAPA). mTORC1 integrates signals from growth factor receptors with cellular nutritional status and controls the

level of cap-dependent mRNA translation by modulating the activity of key translational

components such as the cap-binding protein and oncogene eIF4E (Ruggero 2004) mTORC2 is insensitive to RAPA, and selective inhibitors of this complex have not been described.

Studies have shown that RAPA selectively inhibits the mTORC1 complex without inhibiting mTORC2 (Gulati et al 2006). In recent years, various therapeutic potentials for this drug in the treatment of GBM and other cancers have encouraged the development of derivatives for use in clinical trials. However, thus far, RAPA and its analogs have provided very little clinical response in patients. For example, the prolonged treatment with RAPA activated the mTORC2 complex and activated mitogenic pathway of MAPK (Gulati et,al 2006,: Albertet,al 2009). Molecular mechanism(s) of drug resistance remains not fully defined.

1.2 Hypothesis

The hypothesis here is to test that unlike allosteric inhibitor for mTOR (RAPA), small ATP binding inhibitor (PP242) that binds mTOR into the ATP site would inhibit mTOR pathway more potently. This hypothesis to be tested on GBM cells with respect to:

A. Downstream substrate, mTORC1/2 substrate (P70S6K and AKT) and the alternate activated pathway substrate (ERK).
B. GBM cell growth and proliferation.
C. GBM cell migration

2. Methods

2.1 Tumor samples and cell line

Samples of confirmed GBM were obtained as pathological discard from the Department of Pathology at Westchester Medical Center, Valhalla, NY. Sample usage was approved by the Institutional Review Board under the HIPAA waiver of authorization. The GBM cell lines.U373

and LN-18 (ATCC, Manassas, VA) were used to investigate the possible targets of mTOR signaling pathway in GBM progression.

2.2 Cell Culture

Cell lines were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and 1% penicillin/streptomycin/amphotericin in a humidified 5% CO_2 incubator at 37^{o}C. Cells were made quiescent by serum deprivation 24h prior to treatment with various combinations of RAPA (mTORC1 inhibitor), PP242 (mTORC1/2 inhibitor), LY294002 (LY, PI3K inhibitor), platelet-derived growth factor (PDGF), epidermal growth factor (EGF) (EMD Chemicals, Gibbstown, NJ) or fibronectin (FN, extra-cellular matrix) (Sigma-Aldrich, St. Louis, MO).

2.3 Chemotactic Migration: Directional migration was performed using a 48-well modified Boyden chamber kit (NeuroProbe, Gaithersburg, MD). Quiescent cells were subjected to RAPA-timed treatments (1, 3, 6, 12, 24h). Vehicle treated cells served as controls. Cells were aliquoted (3000 cells/μl) in either serum free media or their respective RAPA treated media. FN (20ng/ml, Sigma-Aldrich) is used as a chemoattractant and cells were allowed to migrate for 4h through a PVC membrane (8μm pore). The membrane was fixed in 70% ethanol, scraped along the non-migrated cell surface, and stained with DiffQuick (IMEB, San Marcos, CA). Migrated cells were imaged at 2.5X (Axiovert 100M) and analyzed as a percentage of total microscopic field occupied by migrated cells (ImageJ, NIH, Bethesda, MD).

2.4 Isolation of Protein: The cells were lysed with whole cell lysis buffer, containing phosphatase and protease inhibitors, in order to extract protein components. The protein concentration of the cells was determined using the modified Lowry Method.

2.5 Western Blot Analysis: Equal amounts (50-100 μg) of the protein content of the cell lysates were resolved on a 10% SDS-polyacrylamide gel and then electrotransferred onto nitrocellulose membrane. The membranes were incubated according to the manufacturer's instructions (Santa Cruz Biotechnology, CA; Cell Signaling Technology, Beverly, MA 01915). The membrane was then incubated with peroxidase-conjugated anti-rabbit IgG (1:3000 dilution), and bands were detected by chemiluminescence (ECL, Amersham). After immunoanalysis, the blots were stripped and re-probed with Ab-actin to ensure equal loading.

2.6 Immunohistochemistry: Routine immunohistochemistry was used to detect pAKT levels in tumor samples. Immunohistochemistry was carried out on 5 μm thick tumor sections on polylysine-coated slides. After routine deparaffinization and rehydration, antigen retrieval was performed. An overnight incubation with primary antibody against phospho-AKT s473 at a dilution of 1:500 was performed and biotinylated goat anti-rabbit IgG (1:300 dilutions) was used as secondary antibody (Vector Laboratories, Inc., Burlingame, CA, USA) was used.

2.7 Cell proliferation Assays: Cell proliferation was measured by MTT cell proliferation assay according to the manufacturer's protocol. This assay is based on the conversion of the yellow tretrazolium dye MTT to purple formazan crystals by metabolically active cells. Briefly, cells were seeded into 96-well plates at 3000 cells per well. On the day of harvest, 100 μl of spent medium was replaced with an equal volume of fresh medium containing 10 μl of MTT reagent. Plates were incubated at 37°C for 4 h, 100 μl of detergent reagent was added to each well, and plates were further incubated at room temperature in the dark for 2 h. Absorbance was measured at 570 nm.

2.8 Cell Viability Assay: Cell growth was measured by counting cells using hemocytometer

following the treatments over the period of four days. Treatments used were RAPA, LY, ATRA and ATO. Vehicle treated cells were used as control.

2.9 Statistical Analysis: Values are presented as the mean ± SEM. Student's t-test (unpaired, 2-tailed) was used to evaluate significant variations between control and treated groups. Values of $p<0.05$ were considered significant and $p<0.10$ was considered as a trend towards significance.

3. Results

3.1. Immunohistochemical Analysis indicated a significant number of GBMs expressed pAKT, thus signifying the presence of PTEN alterations

In our study, PTEN was found to be more expressed in both the cytoplasm and the nucleus. In the tumor area, there were regions of high pAKT and low pAKT expression zones. We found that maximum AKT expression was more commonly seen in primary GBM (75%) as opposed to secondary (Figure 1). This data suggest that in GBM AKT/mTOR pathway is activated.

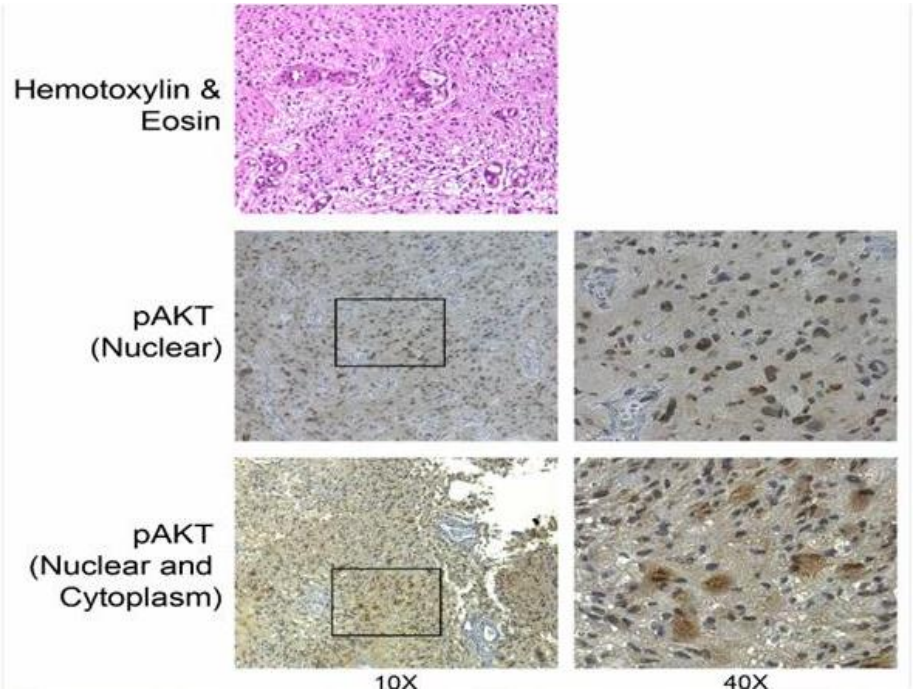


Fig 1**: Immunohistochemical analysis shows that significant number of GBM express pAKT levels suggesting mTOR pathway is hyperactive. H&E staining shows the typical characteristic of GBM. The figure shows both 10X and 40X expression of pAKT.**

3.2 ATP-Binding inhibitor is does not activate mTORC2 complex activation.

In this study, we have used a different inhibitor that binds to the ATP sites of the mTOR complex. We observed that the PP242 unlike RAPA was able to suppress the AKT activation as shown by decreased in levels of pAKT at ser473. Furthermore, the results demonstrated that in contrast with treatment with RAPA PP242 did not activate the mitogenic pathway of MAPK as shown by no increase in pERK levels rather, it suppressed the pERK levels in a dose dependant manner.

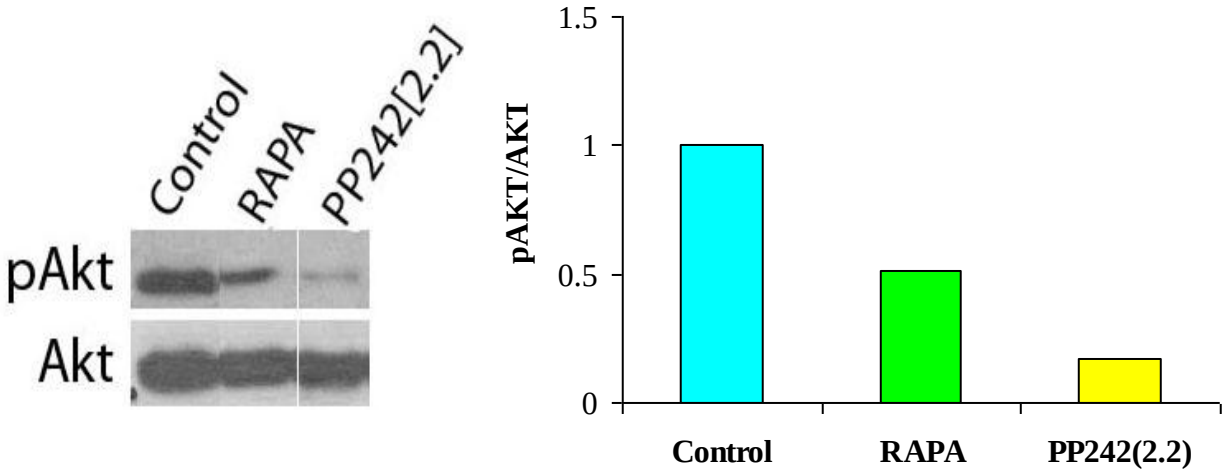


Fig 2**: Western Blot analysis shows substantial decreases in the expression of pAkt levels after treatment with Rapa or PP242. However PP242 has a much more potent effect in decreasing the levels of pAkt**

3.3 PP242 suppresses mTORC1 complexes more potently than RAPA

We treated GBM cells with RAPA at 10nM and PP242 at 2.2uM showed that both compounds inhibited downstream pathway from p-P70S6K but PP242 was more effective in suppressing the levels of P70S6K these levels regulate the growth of cells. Control cells showed activated levels of P70S6K in these cells. The total levels of P70S6K were similar in all three groups. GAPDH levels showed loading of protein was equal in all three lanes.

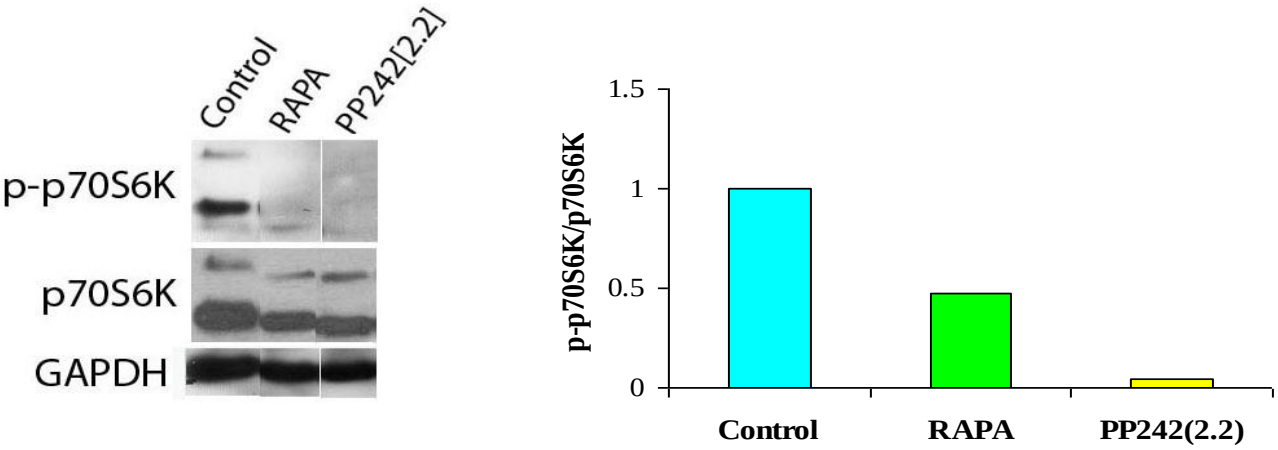


Fig 3**: The substrate of mTORC1, P70S6K was suppressed by PP242 more potently than RAPA following 24 h treatment**

3.4 ATP-Binding inhibitor is does not activate MAPK (ERK) of GBM cells.

Our previous study has demonstrated that RAPA, while suppressing mTORC1 activates mTOR2 as evidence of activation of AKT. Furthermore, we demonstrated that prolonged treatment with RAPA activates the mitogenic pathway of MAPK, thus causing tumor cell growth even more rather than suppressing.

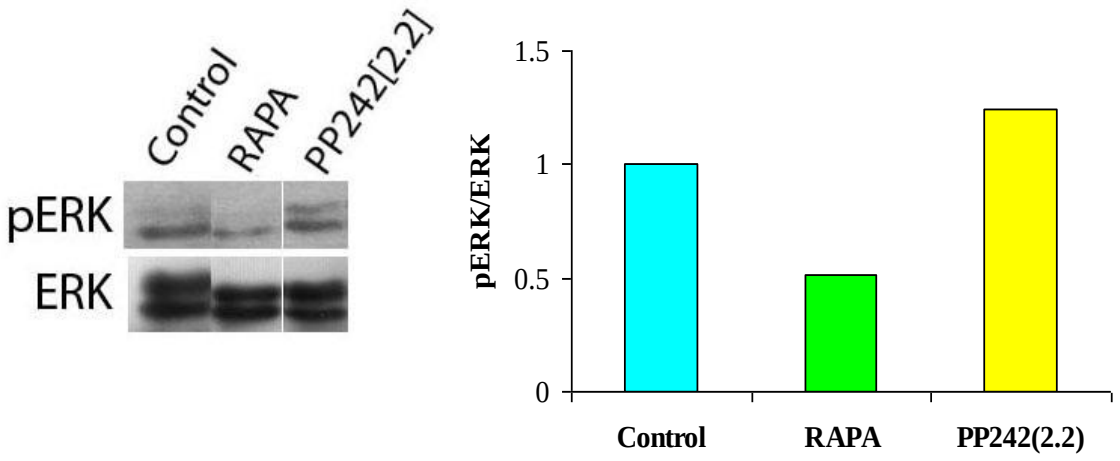


Fig 4: **Western Blot analysis shows that prolonged treatment with RAPA activates the mitogenic pathway of MAPK, thus causing tumor cell growth even more rather than suppressing**.

3.5 ATP-Binding inhibitor is more potent in suppressing growth of GBM cells.

We used two techniques to determine the cell growth of GBM. The cells were grown and were made quiescent by giving them serum free media, also called starve media for 24 hours. Cells were then subjected to treatment with RAPA or two doses PP242 (2.2uM or 1.1uM). MTT assay was performed after 24 hours later and our study showed that PP242 was more effective in reducing the cell growth of GBM cells than RAPA.

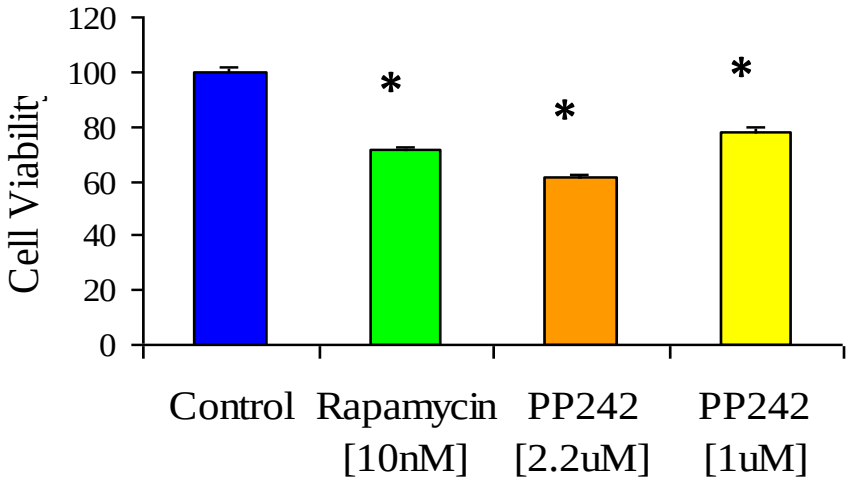


Fig 5: **The MTT analysis of GBM cells treated with RAPA, PP242 (2.2uM and 1uM) showed that PP242 at 2.2 was more effective than RAPA in suppressing GBM cell growth.**

3.6 PP242 suppresses GBM migration

In order to investigate whether PP242 can suppress the GBM cells, we needed to test the GBM cell migration. Since these cells are highly invasive and this invasion occurs to reoccurrence, we found that PP242 was more effective in suppressing GBM cell motility as compared to RAPA as done by chemotactic migration. Chemotactic migration allows the cells to move toward the attractant.

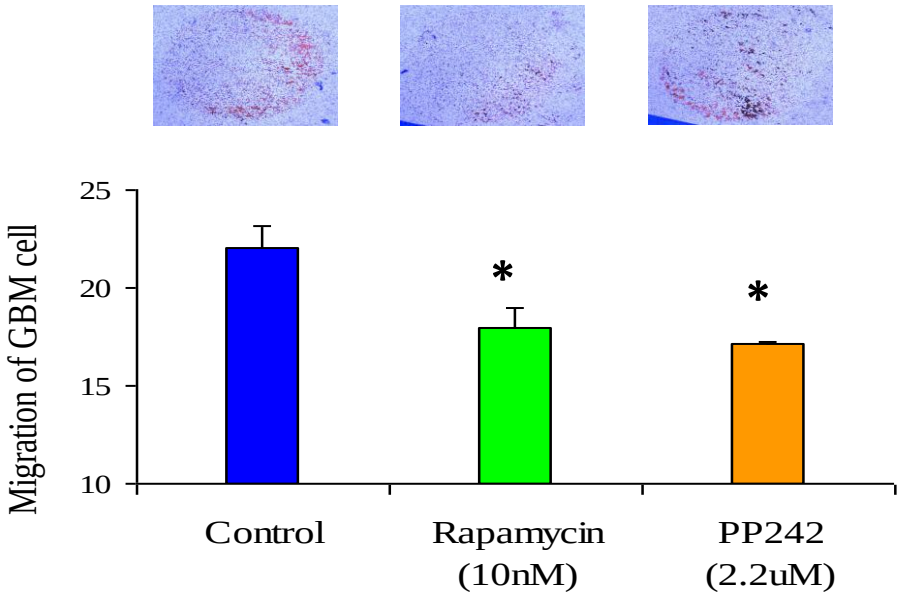


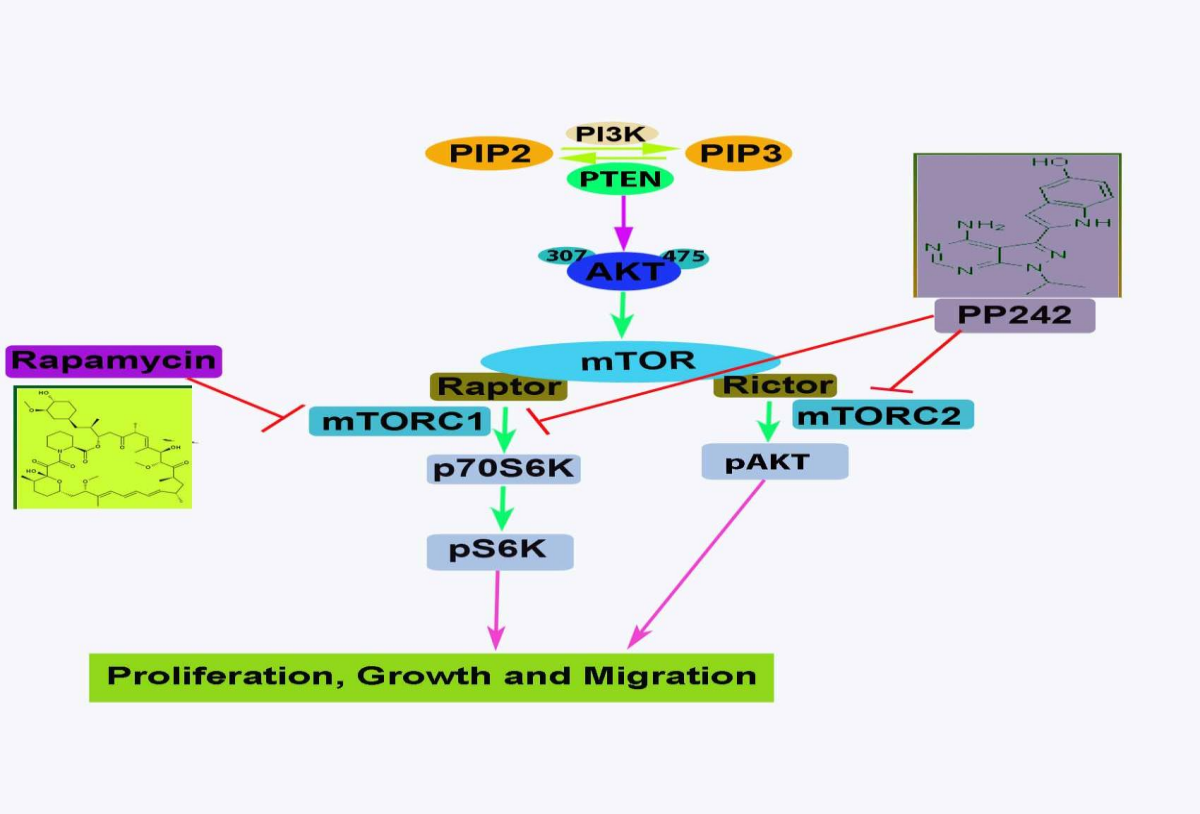
Fig 6: **GBM cell migration was suppressed by RAPA and PP242.**

We also investigated the heptotactic migration of the GBM cells. Cells were the cells were allowed to migrate on a plate with laminin coating. Laminin is extracellular coating. The

migration rate of cells was calculated by the measurement of the radius of cells in comparison to day 0.

4. Schematic diagram represents the action of PP242 as compared by RAPA.

As shown in this cartoon the RAPA inhibits only one complex of mTOR, which is mTORC1 while PP242 was more effective when suppressing GBM cell growth and motility, by inhibiting complexes, mTORC1 and mTORC2



5. Conclusion and Discussion

The result of this study demonstrated that PP242 ATP binding for mTOR was affected molecular as well as cellular levels. PP242 inhibited both complexes of mTOR. Furthermore, it did not activate and alternate pathway, which were the most important drawback in use of RAPA.

Partly because acute pharmacological inhibition of mTORC2 has not been possible, the functions of mTORC2 are less well understood than of mTORC1. mTORC2 is thought to modulate growth factor signaling by phosphorylating the C-terminal hydrophobic motif of some AGC kinases such as AKT [Sarbassov (2005) and SGK [Garcia-Martinez 2008). Growth factor stimulation of PI3K causes activation of AKT by phosphorylation at two key sites: the activation loop (T308) and the C-terminal hydrophobic motif (S473). Active AKT promotes cell survival in many ways, mainly by suppressing apoptosis.

The disruption of mTORC2 by different genetic and pharmacological approaches has variable effects on AKT phosphorylation. Targeting mTORC2 by RNA interference (RNAi) (Sarbassov et al; Gulati), homologous recombination Guertin 2006 et al., Jacinto 2006 et al.,) or long-term RAPA treatment results in loss of AKT hydrophobic motif phosphorylation (S473), strongly implicating mTORC2 as the kinase responsible for phosphorylation of this site Several small molecules have been identified that directly inhibit mTOR by targeting the ATP binding site.

The mTOR pathway is known to be activated in various cancers including GBM; it influences protein synthesis via its regulation of ribosome through S6K, which is downstream from mTOR (Guertin and Sabatini 2007). Inhibition of mTOR has been considered for GBM treatment, however clinical trials have only produced modest results (Galanis et al 2005). The

Ras/MAP Kinase pathway has also been shown to be activated in GBM and has also been demonstrated to play a role in GBM‘s malignant phenotype. These two signaling pathways involved in cellular growth appear to interact through Ras (Pelloski et al 2006). However, the exact nature of the interaction of these signaling pathways has not been well defined. Understanding these would provide a molecular basis of mTOR inhibitors’ ineffectiveness in GBM treatment.These findings underscore the possible strategy for GBM, the deadliest form of brain tumor and provide a molecular basis for treatment for inclusion of such compound (PP242) in treatment of GBM.