Effect of Fasudil on Glioma Cell Migration

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Abstract

Gliomas are tumors arising from glial cells that are highly infiltrative within the brain. Their extensive migratory patterns greatly limit the treatment options and prognoses for patients. It has been shown that glioma cells undergo migration in a unique manner that relies heavily on non-muscle myosin II. Since functionality of non-muscle myosin II requires the phosphorylation of its regulatory light chain by enzymes like Rho-Kinase, we have treated glioma cells with the Rho-Kinase inhibitor, fasudil, and tested its effects. The results indicated that fasudil is a potent inhibitor of migration and also accordingly decreases regulatory light chain phosphorylation in glioma cells. This suggests that fasudil could be a good potentia l candidate for future in vivo studies and contribute to current therapeutic options for the treatment of gliomas. However, residual cell motility and phosphorylation despite treatment with fasudil also suggest that more work must be done to assess the importance of other pathways in glioma cell migration.

Introduction

Gliomas are brain tumors that arise from glial cells and malignant gliomas are associated with poor prognoses due to their prolific migration within the brain [1]. Though the tumor cells often do not migrate outside of the central nervous system, they spread across white matter tracts and are highly infiltrative [1]. In patients, this extensive migration makes it difficult for a complete surgical tumor resection, which can eventually cause recurrent tumors and disease progression [6]. This makes gliomas particularly hard to treat and limits available therapeutic options [6].

Glioma cells exhibit a rather unique migratory pattern due to the tightly packed and constraining physical environment they are exposed to within the brain parenchyma which forces them to migrate through sub-micrometer spaces [1]. Consequently, cell migration occurs in two steps where the cytoplasm first protrudes outward and the cell body follows in saltatory fashion [4]. Interestingly, this type of migration closely resembles that of glial progenitors [4].

Non-muscle myosin II (NMII) has been shown to be particularly important in glioma cell migration and plays a key role in the second step, in which it forces the large nucleus and cell body through small spaces within the brain [1]. NMII consists of three distinct isoforms (A, B, C), defined by their heavy chain, but only NMII A and NMII B seem to be involved in cell migration [13]. Three distinct genes encode the respective heavy chains, and the three isoforms share 60-80% of the same amino acid sequence identity [8]. All NMII molecules have two globular heads and a helically coiled tail and assemble into bipolar filaments through interactions between their coiled-coil tails [11]. Each head consists of an amino-terminal portion of a heavy chain, one essential light chain and one regulatory light chain, and the tail consists of the carboxyl terminal portions of the two heavy chains [11].

When NMII regulatory light chain (RLC) is phosphorylated, actin-dependent Mg^{2+}-ATPase motor activity that is associated with the globular head domain increases. This induces the unidirectional movement of the motor domain on its associated actin filament resulting in actin filament contractility [8, 9]. Though originally it was thought that only the Ca^{2+}-dependent Myosin Light Chain Kinase (MLCK) phosphorylated the regulatory light chains (RLCs), it was later found that a significant amount of RLC phosphorylation occurs through a Ca^{2+}-independent pathway [5]. This pathway is now known to be mediated by a small GTPase, RhoA,

which had previously been associated with many cellular activities including cell motility, cytokinesis, regulation of stress fibers and formation of focal adhesions [5]. Activated GTP-RhoA can activate Rho-associated protein kinase (ROCK), which then phosphorylates NMII RLCs directly and also indirectly by inactivating myosin phosphatase [5]. Both MLCK and ROCK can mono-phosphorylate the Serine 19 site or di-phosphorylate the Serine 19 and Threonine 18 sites on the RLC [5]. Regardless, previous studies have demonstrated promising results in which inhibition of just ROCK can efficiently inhibit cell motility [1].

In the Beadle et al. study, the treatment of cells with Y-27632—a potent inhibitor of ROCK—proved to be an efficient inhibitor of cell migration and motility. However, no studies have tested what the effect of the drug, fasudil, also a ROCK inhibitor, will have on glioma cell migration. Since fasudil is already an FDA-approved drug, it is valuable to evaluate it further and determine whether or not it has any effect on migratory patterns.

Materials and Methods
Cell culture
MGPP3 (PDGF$^+$/PTEN$^{-/-}$/p53$^{-/-}$) and PTYB (PTEN$^{fl/fl}$/YFP$^+$) glioma cells were used in this study. These cells were isolated from gliomas generated in mice [10]. Cells were grown in BFP medium (DMEM containing 2.5% FBS, N-2 supplement, 10 ng/mL FGF, 10 ng/mL PDGF-B, and antimycotic-antibiotic supplement) at 37°C, 5% CO_2 in a humidified atmosphere.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay
MGPP3 cells (1x10^3) in 100 µL/well were plated in a 96-well plate for 12 hours with 3 wells dedicated to each of the 13 treatment conditions. Treatment conditions included no drug and the indicated concentrations of either fasudil or Y-27632. 20 µL of reagent (CellTiter 96 Aqueous One Solution Reagent) was added to each well and the plate was incubated for 6 hours with reagent. The reagent of the assay is a yellow tetrazole that is reduced to purple formazan in living cells [12]. Consequently, wells that appeared more purple in hue contained more viable cells. Absorbances were recorded at 490 nm using a 96-well plate reader.

Transwell Migration Assay
MGPP3 cells (1x10^5) in a total volume of 200 µL 10% fetal bovine serum (FBS) were seeded on top of 3 µm fluoroblok transfilters (BD Biosciences, San Jose, CA). 500 µL of 10% FBS was added to the bottom well. A plate was set up with three transfilters per condition—control, 100 µM fasudil and 500 µM Y-27632. For conditions that require drug treatment, drug was added both above and below the transfilter membrane. The entire plate containing all transfilters was incubated for 6 hours at 37°C. After incubation, both sides of the transfilter were washed three times with phosphate-buffered saline (PBS). Cells were then fixed with 4% paraformaldehyde for 15 minutes on ice. After another three washes in PBS, cells were stained with 4,6-diamidino-2-phenylindole (DAPI) for 20 minutes. The transfilter membranes were removed and mounted on slides. Images of the bottom part of the filters were acquired on a Nikon widefield-inverted microscope using a 4× objective and the nuclei within five fields per membrane were counted and averaged together.

Phosphomyosin staining
PTYB cells (3.4x10^4) were plated on poly-L-lysine coated coverslips overnight. Cells were then treated with either fresh BFP media, BFP supplemented with 60 µM fasudil, or BFP supplemented with 50 µM Y-27632 for three hours. Cells were fixed with 4% PFA and stained overnight at 4°C with phospho-serine 19 antibody (pS19, mouse antibody; Cell Signaling Technology) or phosphor-Threonine 18-serine 19 antibody (pT18pS19, rabbit antibody; Cell Signaling Technology). Later, cells were incubated at room temperature with Alexa-568 conjugated secondary antibodies (InVitrogen). Images were acquired on a Nikon widefield-inverted microscope using a 20× objective under identical excitation conditions. Images were analyzed for the integrated density of the signal using ImageJ (National Institute of Health), and results were imported into Microsoft Excel and values were normalized to the control condition (BFP only), n=3.

Statistical Tests
Significance was determined by student's t-test (paired, two-tailed unless otherwise

indicated) and a $p < 0.05$ was considered significant.

Results

In order to test the motility of untreated and treated cells and confirm fasudil would inhibit glioma cell migration to a significant degree, Transwell Migration Assays (TMA) were the assay of choice. Transfilters provide 3 µm pore sizes comparable to sizes of pores that exist in the brain through which glioma cells would need to migrate [1]. Figure 1 demonstrates the relative size of the glioma cells compared to the small pore that it must migrate through. The TMAs evaluate chemokinetic migration as either side of the filter membrane contains media supplemented with growth factors that act as chemoattractants. This approach mimics a native environment similar to that which exists in the brain.
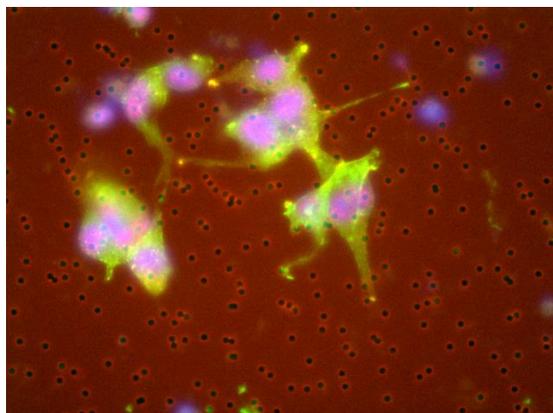

Figure 1: Glioma migration through 3 µm Transwell membranes. Representative image of the top of a transfilter membrane stained with pS19-RLC antibody showing the relative sizes of the control glioma cell and the 3 µm pore. Purple color represents cell nuclei; green color represents phosphorylation at Serine 19 site; black dots represent 3 µm pores through which cells migrated.

In order to identify non-toxic drug concentrations for the TMA experiments, we conducted a dose response assay, specifically a colorimetric MTT Assay. From this assay, we determined that 100 µM fasudil is the highest concentration that can be used without affecting cell survival (Figure 2). Cells treated with

equivalent amounts of Y-27632 demonstrated more cell viability although cells tolerate both inhibitors at high concentrations (Figure 2). 500 µM was chosen as the designated Y-27632 concentration because previous studies used 50 µM Y-27632 as an established, effective concentration for inhibition and a tenfold increase would create an extreme concentration point that could help further elucidate the effect of fasudil [1].
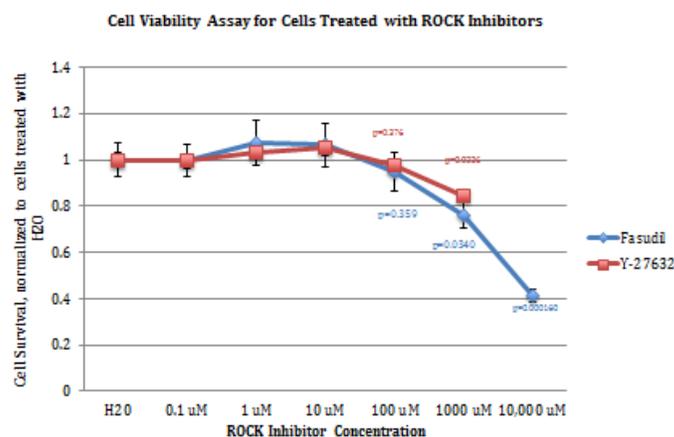

Figure 2: MTT Assay demonstrating cell viability for different concentrations of ROCK inhibitors. Dose response curves for MGPP3 cells treated with either fasudil or Y-27632 for 6 hours. Each point on the curve represents the average of five trials for each condition and the error bars represent standard error. Cell survival (y-axis values) was normalized to the absorbance levels measured in cells treated with only H_2O, p-values for concentrations greater than 100 µM determined by a student's t-test (unpaired, one-tailed) are labeled in the corresponding color above the points.

As Figure 3 shows, 100 µM fasudil reduces trans-membrane migration of cells to 25.36% compared to the control condition ($p = 0.009$). 500 µM Y-27632 also significantly reduces cell migration to 17.14% ($p = 0.0055$). On the other hand, the difference in motility between fasudil-treated cells compared to Y-27632-treated cells is just under $p = 0.05$ ($p = 0.04985$), despite the much higher dosage of Y-27632.

A. Effect of ROCK inhibitors on cell migration through 3 µm Transwell membranes.

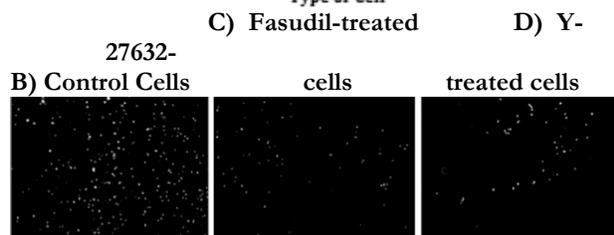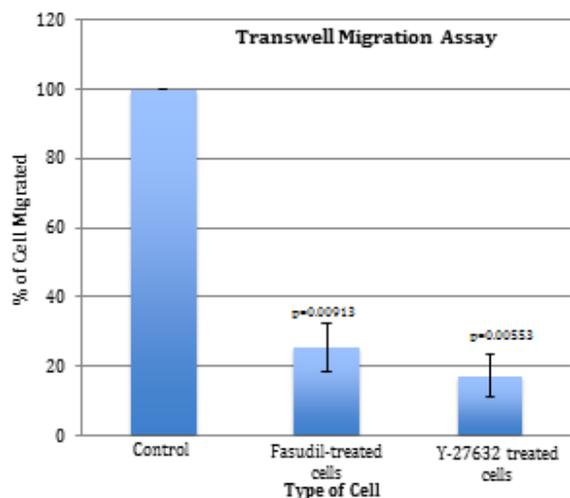C) Fasudil-treated cells   D) Y-27632-treated cells

B) Control Cells

Figure 3: Cell Migration in Transwell Migration Assays. (A.) Percent migration was normalized to the number of migrated control cells, error bars represent standard error of the averages of the nuclei count from five representative fields of each filter at 4x magnification and p-values are listed above the bars. Representative images of the bottom portion of transfilters used in TMA experiments after MGPP3 cells were incubated for 6 hours at 37°C with either 10% FBS and were (B.) uninhibited (control) cells, (C.) FBS+100 µM fasudil-treated cells, or (D.) FBS+500 µM Y-27632-treated cells. Cells were fixed and stained with DAPI. Each experimental condition was assessed in triplicate for a total n=3.

Aside from observing the difference ROCK inhibitors can make on migration, it is also important to ascertain whether the predicted pathway is operating as expected when inhibition occurs. Since phosphorylated RLCs (phospho-RLCs) are expected to be responsible for much of the NMII-dependent contractions within glioma

cells, fasudil and Y-2632 inhibition of ROCK should decrease motility after decreasing RLC phosphorylation.

In order to quantify this observation and corroborate this with previous findings, PTYB cells were stained with the same pS19 and pT18pS19 antibodies after treatment with 60 µM fasudil (Figure 4A,B) or 50 µM Y-27632 (data not shown) which were concentrations used in other studies [1]. Representative images were captured using a wide-field microscope and integrated density of the signal was calculated (Figure 4A,B). The results show a 47.99% decrease in phosphorylation of the Serine 19 site when cells were treated with 60 µM fasudil compared to control (Figure 4D; p=0.006). Similarly, 50 µM Y-27632 demonstrated a 41.93% decrease in mono-phosphorylation (Figure 4D; p=0.0046).
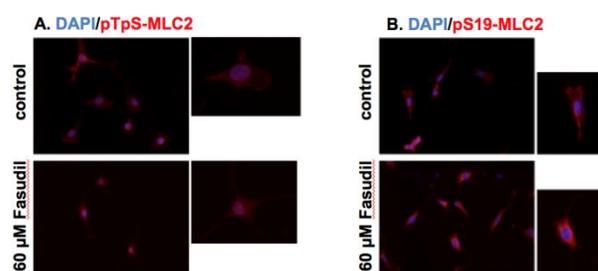
C. Phosphorylation of cells w/ pT18pS19-RLC
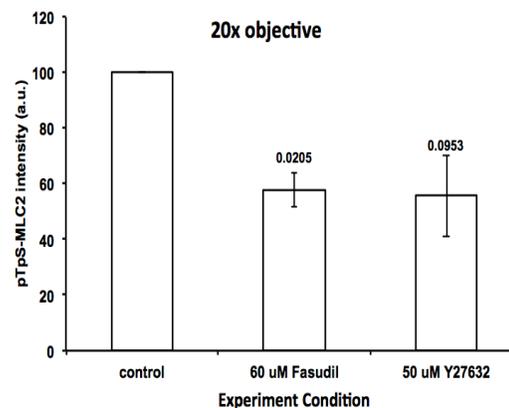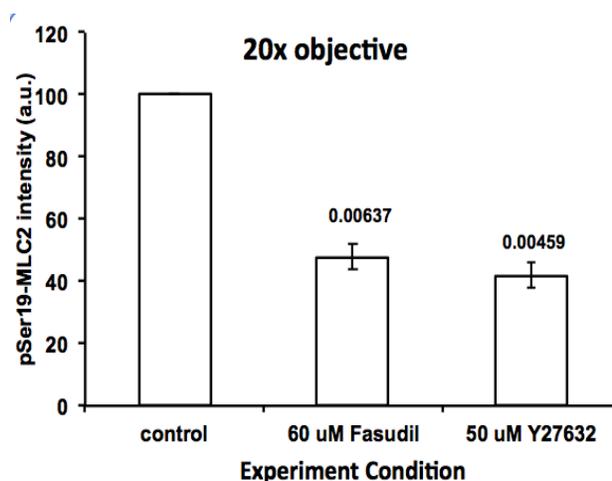
D. Phosphorylation of cells w/ pS19-RLC

Figure 4: Phospho-RLC Staining.
Representative images of PTYB cells treated with BFP media, or BFP+60µM fasudil for 3 hours before fixing and staining with DAPI and the primary antibodies (A.) pT18pS19-RLC or (B.) pS19-RLC. Underlying blue color represents DAPI staining of nuclei; red color represents phospho-RLC. Integrated density of the (C.) di-phosphorylated-RLC signal or (D.) mono-phosphorylated-RLC signal was measured from 20 cells per condition, averaged and normalized to control PTYB cell phosphorylation. Error bars represent standard error of measurements for each condition and p-values are listed above the corresponding bars.

After measuring the integrated density of cells incubated with the di-phosphorylated antibody, the results demonstrated 60 µM fasudil decreased phosphorylation to 57.9% of the control (Figure 4C; p=0.02). On the other hand, 50 µM Y-27632 decreased phosphorylation to 55.82%, which was not significant on a paired t-test (p=0.095; Figure 4C), but was significant on an unpaired t-test (p=0.039). Fasudil, therefore, effectively decreases the mono- and di-phosphorylation of the RLCs.

Discussion

Through the various Transwell Migration Assays, the results indicate that chemokinetic motility is indeed inhibited, though not completely abolished, when glioma cells are treated with 100 µM fasudil. These results do yield promising

implications for fasudil as its efficacy suggests it could make a good inhibitor of cell migration in *in vivo* studies as well. The concentrations used in the Transwell experiments as well as the MTT assay can help inform the drug dosages that could be administered to a living system for maximal inhibition and minimal side effects.

It is surprising, however, that the negative control of 500 µM Y-27632-treated cells produced results that represented a statistically significant difference from that of fasudil but did not represent a large difference even though the Y-27632 concentration had been purposefully and dramatically increased. These results suggest that even at a much higher concentration of ROCK inhibitors, glioma cell migration cannot be completely inhibited. Preliminary results of TMA experiments of cells treated with a heightened concentration of 680 µM fasudil corroborate with this finding (5.5% residual migration, n=1).

Similarly, the PTYB cells stained with phospho-RLC antibodies demonstrated cells treated with fasudil or Y-2763 exhibited a lower level of total phosphorylation (Figure 4). The phospho-RLC images also demonstrate a clear change in cell morphology after treatment with fasudil or Y-27632. Cells begin to form long, thin spindle-like extensions and lose their more rotund cell shape (Figure 4). This suggests that decrease in NMII RLC phosphorylation and the decrease in NMII activity is qualitatively related to the cytoskeleton structure of glioma cells. In addition, the NMII activity decrease potentially is related to the decrease in focal adhesions and stress fiber formation, as detailed in other studies [2].

In images of cells stained with pT18pS19, however, there was evidence of non-specific nuclear staining that most likely overestimated the phosphorylation signal during quantification. Future experiments can be done to corroborate the results shown in Figure 4 by measuring phosphorylation levels through other methods such as Western blots using antibodies that can detect mono- or di-phosphorylation. Decreased ROCK activity after treatment with fasudil could also be assessed through Western blots with a ROCK antibody like Ser1366, antibodies for an alternate substrate of ROCK such as LIM kinase, through a quantitative ROCK activity assay [3].

It is also possible, however, that the residual phosphorylation and migration across transfilters is due to RLC phosphorylation from

other pathways. One important pathway to consider is the MLCK mediated route, which is also known to phosphorylate RLCs at the same sites as ROCK. This could be tested by assessing phosphorylation and migration after treating cells with both fasudil as well as an MCLK inhibitor such as ML-7 [7].

Finally, it is important to note that though fasudil is an effective drug target in short-term or acute treatment, we still do not know if it will work after a more long-term treatment. It is possible that cells will eventually compensate for ROCK inhibition. This information will be particularly important for future *in vivo* studies or clinical trials because it can greatly influence how the drug is delivered, how often it is delivered, or whether it should be administered along with another drug. Subsequent experiments can be done to test motility of glioma cells after prolonged periods of treatment with fasudil. Further experiments in motility could also be conducted with fasudil that has been incubated for periods of time at biological temperatures to test the biostability of the drug and to see if its efficacy decreases with prolonged incubation. These experiments also have very important implications for future *in vivo* or clinical studies. Thus, though fasudil is a very promising drug, more work must be done in order to elucidate the prolonged effects of fasudil and determine the cell's precise response to the drug and its potential as a cancer drug in living systems.

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