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# Exploring the Effect of Novel Small Molecules on Oligodendrocyte Precursor Proliferation

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UNIVERSITY SCHOLAR PROJECT

HONORS THESIS

# EXPLORING THE EFFECT OF NOVEL SMALL MOLECULES ON OLIGODENDROCYTE PRECURSOR PROLIFERATION

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**Introduction**

Gliomas are the most common primary brain tumor in adults, representing about 81% of malignant brain tumors (Ostrom et al., 2014). Gliomas are malignant and invasive tumors that arise from glial tissue. Although it is a relatively rare disease, it can be devastating. Severe forms of glioma spread throughout the brain, destroy normal brain tissue, and are resistant to treatment.

Gliomas can be classified in different ways. Gliomas can be categorized based on the predominant cell type in the tumors as astrocytomas, oligodendrogliomas, or oligoastrocytomas if the tumor has characteristics of both astrocytes and oligodendrocytes. The World Health Organization (WHO) has also developed a grading system to classify gliomas on the basis of malignancy (Louis et al., 2007). Grade I gliomas are benign and can be cured by surgical resection. Grade II gliomas have a low malignancy but are often incurable with surgery. Grade III gliomas are more proliferative and anaplastic than grade I. Grade IV gliomas are highly malignant and characterized by rapid cell proliferation, high vascularization, and necrosis. Grade IV gliomas are also known as glioblastomas.

The treatments currently available for malignant glioma are not very effective. The current standard treatment involves surgical resection if possible, radiotherapy, chemotherapy, and management of symptoms. Radiotherapy used with or without temozolomide (an oral alkylating agent) is the mainstay of glioma treatment (Wen and Kesari, 2008). Radiotherapy may increase survival from 3-4 months to 7-12 months, but after discontinuation of radiotherapy, about 90% of tumors reoccur (Stupp et al., 2005; Hochberg and Pruitt, 1980).

There have been numerous studies on the cause of gliomas. So far it has been shown that multiple molecular or genetic abnormalities can lead to the development of tumors. The Cancer

Genome Atlas (TCGA) Research Network analyzed 206 glioblastoma samples and found three core pathways that were altered in gliomas: 1) retinoblastoma (RB) pathway, 2) p53 pathway, and 3) receptor tyrosine kinase (RTK) signaling (Cancer Genome Atlas Research Network, 2008). The mutations found within the RTK signaling pathways led to increased cell proliferation and survival. One of the RTK pathways that they found to be altered was platelet derived growth factor (PDGF) signaling through PDGF receptors (PDGFR). PDGFR alpha (PDGFRa) alteration was also found to be a major feature of a molecular subtype of glioma classified as the proneural subtype (Verhaak et al., 2010).

PDGF and PDGFRs have an important role in normal physiology. There are four known members of the PDGF family: PDGF-A, B, C, and D (Hammacher et al., 1988). All PDGFs have a highly conserved domain, called the PDGF/VEGF homology domain, which is involved in forming the disulfide bridges to allow dimerization (Nazarenko et al., 2012). PDGF can form 30 kDa homodimers (PDGF-AA, BB, CC, DD) or heterodimers (PDGF-AB) that bind to PDGFRa or PDGFR beta (PDGFRb), or both. PDGF-AA, AB, BB, and CC can all bind to PDGFRa with high affinity, and PDGF-BB and DD bind to PDGFRb (Nister et al., 1991).

PDGFRs are membrane tyrosine kinase receptors (Heldin, 2013). PDGFRa is 1089 amino acids and PDGFRb is 1106 amino acids, but they are structurally similar (Claesson-Welsh, 1996). Both have a single transmembrane domain in the middle of the polypeptide. They have an extracellular domain consisting of five immunoglobulin-like domains (Claesson-Welsh, 1996). The intracellular portion of the receptors consists of the juxtamembrane domain, the tyrosine kinase domain, and the C-terminal domain (Claesson-Welsh, 1996). Ligands bind primarily to the extracellular Ig-like domains 2 and 3 (Heldin, 2013). Binding of the ligands to PDGFR induces dimerization of the receptor which allows transphosphorylation of the intracellular

tyrosine kinase domain. Autophosphorylation changes the conformation of the intracellular portion of the receptor which activates the kinase. The kinase activity of the receptor can initiate downstream intracellular signaling pathways.

Signal transduction molecules that get phosphorylated by activated PDGFR all have a homologous 100 amino acid stretch call the Src homology 2 (SH2) domain. This domain recognizes the phosphorylated tyrosine residues of the receptor. About 10 families of SH2 domain containing molecules have been found to bind to PDGFR and become activated. Some of the signal transduction molecules include Src family tyrosine kinases, Grb2, phosphatidylinositol 3-kinase (PI3K), phospholipase C-y (PLC-y), and GTPase activating protein (GAP) for Ras (Claesson-Welsh, 1996; Heldin, 2013). Receptors also bind signal transducers and activators of transcription (STATs) which translocate to the nucleus to act as transcription factors (Heldin, 2013). The activation of these signaling pathways leads to cell proliferation and survival, as well as cell migration.

In the CNS, PDGFRa is expressed on oligodendrocyte precursor cells (OPCs), which are a distinct glial cell type capable of differentiating into oligodendrocytes or astrocytes (Jackson et al., 2006). Not all OPCs differentiate, so a population of OPCs remains proliferative throughout life (Dawson et al., 2003). OPCs are the major source for oligodendrocytes, which are the cells that form the myelin sheath around neuronal axons for rapid neuron firing (Zhu et al., 2008). Survival and proliferation of OPCs is dependent on PDGF signaling (Calver et al., 1998). PDGF-AA is produced by neurons and astrocytes (Raff et al., 1988). PDGF-AA signaling maintains OPCs in a proliferative and undifferentiated state, and is important for the timing of oligodendrocyte development (Raff et al., 1988; Erlandsson et al., 2001; Vela et al., 2002). It has been shown that withdrawal of PDGF-AA from culture medium is sufficient to cause OPC

differentiation (Raff et al., 1988). PDGFRa is rapidly downregulated when OPCs begin to differentiate (Hart, 1989). PDGFRa is also expressed in some neural stem cells, which are cells found primarily in the subventricular zone that can self-renew and differentiate into multiple cell lines even in adulthood (Jackson et al., 2006; Reynolds and Weiss, 1992). PDGF signaling leads to the proliferation of these neural stem cells as well as induces them to enter oligodendrocyte lineage rather than neuronal lineage (Erlandsson et al., 2001; Menn et al., 2006).

There is significant evidence that overexpression or hyperactivity of PDGF ligands and receptors is a cause of gliomas. Activation of PDGF signaling pathways has been observed in more than 80% of oligodendrogliomas and in 50-100% of astrocytomas (Guha et al., 1995; Varela et al., 2004). Guha et al. compared glioma specimens of varying grades to non-neoplastic glial specimens (1995). They found that PDGF-A, PDGF-B, and PDGFRa were all more highly expressed in glioma than the non-neoplastic glial specimens (1995). The levels of PDGF-A, and PDGF-B, and PDGFRa were all slightly more elevated in the higher grades of glioma compared to the lower grades (Guha et al., 1995). Another analysis of human glioma tissue samples similarly revealed that PDGF ligands and PDGFRa were overexpressed in all grades of gliomas, but were found at the highest levels in glioblastoma (Hermanson et al., 1992; Hermanson et al., 1996). Another study found that the level of PDGFRa may be associated with malignancy of the tumor: amplification of PDGFRa was detected in 0% of Grade II tumors, 3.6% of Grade III tumors, and 9.15% of Grade IV tumors (Alentorn et al., 2012). Due to both PDGF ligand and PDGFRa overexpression together, it is likely that autocrine and paracrine loops are involved in activating this growth pathway and may be the reason for the uncontrolled growth of these tumors (Guha et al., 1995; Hermanson et al., 1996). Activation of these loops may be an early event in glioma development because PDGF and PDGFRa overexpression is observed even in

the low grades of glioma. These PDGF signaling loops may also be involved in tumor progression, because the highest expression is seen in the most severe grades.

Efforts to induce gliomas also revealed the role of PDGF signaling in glioma development. A retrovirus encoding PDGF-B was injected into newborn mice and resulted in highly malignant gliomas (Uhrbom et al., 1998). All of the tumors were found to express PDGF-B and PDGFRa mRNA, but not PDGFRb (Uhrbom et al., 1998). Since PDGF-B is a high affinity ligand for PDGFRa, this is more evidence of an autocrine pathway that leads to glioma development. A similar experiment was conducted where a retrovirus encoding PDGF-B was injected into the subcortical white matter of adult rats. 100% of the rats developed tumors that had similar characteristics to glioblastoma (Assanah et al., 2006). They found that the retrovirus predominantly infected OPCs, but most of the proliferating cells were uninfected OPCs that were recruited by paracrine signaling by PDGF-A and B that was secreted by the glioma cells (Assanah et al., 2006). Experiments that infused PDGF-A into the subventricular zone led to the proliferation of PDGFRα expressing neural stem cells that formed glioma like lesions (Jackson et al.,2006). It has also been shown that overexpressing PDGF leads to the proliferation of OPCs that can result in oligodendroglioma-like lesions in mice (Appolloni et al., 2009; Dai et al., 2001).

These findings suggest that blocking the activity of the tyrosine kinase portion of the receptor would reduce PDGF signaling and therefore reduce the proliferation of glioma cells. However, tyrosine kinase inhibitors (TKIs) that are currently in clinical trials have been ineffective when given to glioma patients (Nazarenko et al., 2012). These agents have had response rates of only 0-15% and no prolongation of 6-month progression free survival (Wen and Kesari, 2008). Small molecule TKIs, like imatinib, sunitinib, and sorafenib, are thought to be

ineffective because they are non-specific inhibitors of multiple kinases with lower potency for PDGFR, and also because resistant mutations of PDGFR often arise that prevent binding of the drugs (Heldin, 2013).

Although inhibition of PDGFR kinase activity has been unsuccessful, decreasing this signaling pathway though other mechanisms could be an alternate path for inhibiting glioma cell proliferation. An initial screen previously conducted by the Nishiyama lab led to identification of small molecule compounds that inhibit PDGFRa transcription (Medved, 2014). Some of the compounds that were found to downregulate PDGFRa had similar chemical structures based on N-methyl-N-benzylguanidine structure (guanidine compound). 12 guanidine derivatives were synthesized by the Wright lab for further investigation. These derivatives were found to have differing effects on PDGFRa transcription, as seen through luciferase assay and qPCR (Medved, 2014). DW002, DW003, DW004, DW005, DW007, DW008 significantly decreased PDGFRa transcription, while DW001, DW006, DW009, DW0010, DW0011, and DW0012 did not have a significant effect (Figure 1).

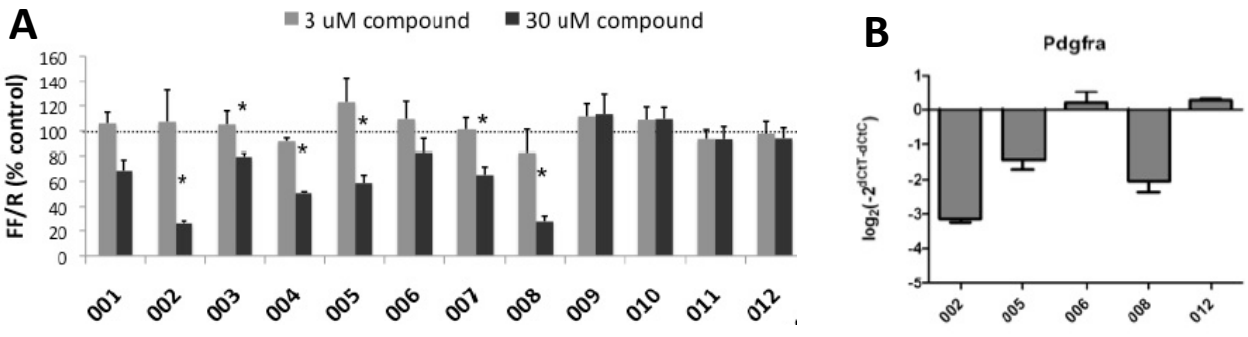


**Figure 1. Guanidine derivative compounds have different effects on PDGFRa transcription**. A) Oli-neu cells were treated with 3 uM or 30 uM of 12 different guanidine derivative compounds for 2 days. A luciferase assay was conducted to measure PDGFRa transcription. 30 uM of DW002 and DW008 caused the greatest downregulation of PDGFRa. Data is presented as relative to DMSO control (100%). B) mRNA was collected from Oli-neu cells treated with five different guanidine derivative compounds and PDGFRa transcription was analyzed by qPCR. The results reflect the luciferase assay results. DW002 and DW008 cause a robust decrease in PDGFRa transcription, while DW006 and DW012 do not. dCtC = delta Ct of DMSO control; dCtT = delta Ct. Bars represent average result obtained from 3 independent experiments. Error bars are standard errors of the mean. * $p < 0.05$, one sample t-test. (Modified from Medved, 2014).

As previously discussed, increased signaling through PDGFRa and its overexpression is a common event involved in the pathology of glioma formation. Therefore, these compounds that were found to decrease transcription of PDGFRa are interesting because they could limit PDGFRa signaling and possibly have a therapeutic effect in glioma. I have chosen to focus in depth on two of the guanidine derivatives tested: DW002 and DW006. DW002 caused the most robust decrease in PDGFRa transcription while DW006 did not have a significant effect. However, these compounds only differ in the placement of a methoxy group on the benzyl ring (Figure 2). Discovering the mechanism that leads to their different outcomes could help us to find a pathway that leads to downregulation of PDGFRa.

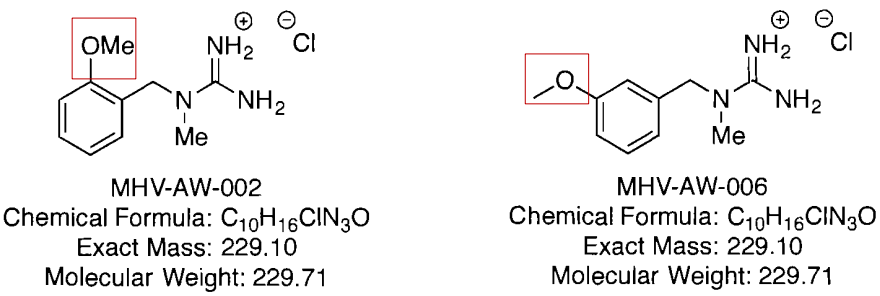


**Figure 2. Chemical structures of compounds of interest**. The chemical structure of DW002 (left) and DW006 (right) differ by the placement of the methoxy group on the aromatic ring (indicated by box).

In this project, I have conducted cell viability and cytotoxicity assays to determine if DW002 leads to decreased cell proliferation without causing cell death. I also used qPCR to determine if the compounds are specifically targeting PDGFRa transcription and not PDGFRb transcription. To investigate the mechanism by which DW002 may work, I have treated the cells with kinase inhibitors to block different signaling pathways. This was also explored by iTRAQ (isobaric tags for relative and absolute quantification) analysis, a proteomics technology that will allow us to compare protein expression of samples treated with each compound.

## Materials and Methods

### *Cell culture*

Oli-neu cells were used as a model for OPCs. Oli-neu cells are derived from primary murine OPCs that have been transformed with an ErbB2 oncogene, t-neu (Jung et al., 1995). Cell culture dishes were coated with 30 ug/ml polylysine solution prior to plating cells. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM)/F12 medium containing 1% N2 supplement, 1% horse serum, 1X penicillin/streptomycin, and 1X glutamine. Cells were kept at 37°C with 5% CO2. When the cells became ~80% confluent, they were split by trypsinization.

### *DW002 and DW006 compounds*

DW002 and DW006 were synthesized by the Wright lab and provided to us as a 50 mM stock dissolved in dimethyl sulfoxide (DMSO).

### *Cell viability assay*

Cells were plated in triplicate at 10,000 cells/well in a 96-well white plate and incubated overnight. Cells were also plated at the same time in triplicate in a clear plate. Cells in both plates were then treated with DW002 or DW006 for two days. Cell viability was determined using the Cell Titer Glo 2.0 Assay (Promega) according to the manufacturer's protocol. In addition to the assay, cell morphology changes were visualized under light microscope using the cells in the clear plate. The concentration of compound that inhibited 50% of cell viability (IC50) was calculated with GraphPad Prism 6 using the equation: Y=Bottom + (Top-Bottom)/(1+10^((LogIC50-X)*HillSlope)). Statistical significance of a difference between DW002 and DW006 was determined using an unpaired Student's t-test for each concentration.

Since the DMSO control of the previous experiment appeared to significantly decrease cell viability, a DMSO assay was conducted to determine the effects of the DMSO that the compounds were dissolved in. Cells were plated in triplicate at 10,000 cells/well in a 96-well white plate and incubated overnight. Cells were then treated with concentrations of DMSO that corresponded to the amount of DMSO added with each of the treatments with DW002 and DW006. The Cell Titer Glo 2.0 Assay was conducted according to the manufacturer's protocol after two days of treatment.

*Cytotoxicity assay*

Cells were plated in triplicate in horse serum free medium at 10,000 cells/well in a 96-well black plate as well as a 96-well clear plate and incubated overnight. Cells in both plates were then treated with digitonin, lysis buffer, DW002, and DW006 for one or two days. Cell death was measured using the CellTox Green Cytotoxicity Assay (Promega) according to the manufacturer's protocol for cells in the black plate. Cells in the clear plate were also visualized under a light microscope to observe morphological changes.

*Quantitative PCR (qPCR)*

mRNA was previously collected from Oli-neu cells treated with DMSO control, 50 uM DW002, and 50 uM DW006 for two days and converted to cDNA. Three independent sample sets were analyzed, each in triplicate. Reactions were prepared with 100 ng cDNA, 3 uM of primers, and SsoAdvanced Universal SYBR Green Supermix for a total reaction volume of 20 ul. PDGFRb and GAPDH primers were used (in table below). The data is expressed as raw Cq values, as well as normalized to GAPDH.

| Gene | Forward primer | Reverse primer |
|---|---|---|
| PDGFRb | CACCTTCTCCAGTGTGCTGAC | CGGAGTCCATAGGGAGGAAG |
| GAPDH | TGACAACTTTGGCATTGTGG | ATGCAGGGATGATGTTCTGG |

*iTRAQ optimization*

In preparation for iTRAQ analysis, a series of experiments were conducted to determine the best practice for plating the cells in serum free medium (SFM). SFM is DMEM/F12 medium containing 1% N2 supplement, 1X penicillin/streptomycin, and 1X glutamine. Cells were plated in triplicate at 100,000 cells/well in a 24-well clear plate in serum-containing medium. The medium was changed to SFM at three different times: upon plating, 2 hours post plating, and 24 hours post plating. Phase contrast pictures were taken one, two, and three days after plating.

Next, cells were plated in triplicate at 10,000 cells/well in a 96-well white plate, switched to SFM two hours after plating, and incubated overnight. Cells were treated with DW002 +/- PDGF growth factor for two days. Cell viability was determined using the Cell Titer Glo 2.0 Assay according to the manufacturer's protocol.

After determining the conditions for sample preparation, the cell samples were prepared. Four different samples were prepared in four different 10 cm plates. $4x10^6$-$5x10^6$ cells were plated in each plate, medium was changed to SFM after two hours, and then incubated overnight. Each plate of cells received a different treatment: DMSO control (0.2%) incubated for 20 hours, 30 uM 002 incubated for 20 hours, 30 uM DW006 incubated for 20 hours, or 30 uM DW002 incubated for 30 hours. Cells were harvested from the plates and snap-frozen in liquid nitrogen. This was repeated to create an identical set of plates. Eight samples were generated (four treatments in duplicate). The frozen samples were sent to the Beth Israel Deaconess Medical

Center Proteomics Center and Dana Farber/Harvard Cancer Center Cancer Proteomics Core for iTRAQ analysis.

Data from iTRAQ analysis was reported as ratios between the different treatments for each protein. 245 proteins were analyzed. The ratios of interest were identified (114:113, 118:117, 116:113, 121:117, 115:113, 119:117, 114:115, and 118:119). For each list of ratios between two treatments, GraphPad Prism 6 was used to calculate the $25^{th}$ and $75^{th}$ percentile. These cut offs were used to flag proteins as "abnormal". Protein "hits," meaning proteins that were more or less expressed after DW002 treatment, were identified in two ways. In Method 1, proteins were classified as hits if they were abnormal after both short and long incubations with DW002, but not with DW006. In Method 2, proteins were classified as hits if they were abnormal for both replicates of DW002:DW006. They were classified as strong hits if the ratios for both replicates were <0.5 or >2 (arbitrarily chosen).

*Inhibitor treatment*

Inhibitor compounds were purchased from SelleckChem and dissolved in DMSO to obtain 50 mM stock solutions. 10 fold dilutions of the 50 mM were created to make additional stocks. Cells were plated in triplicate at 10,000 cells/well in a 96-well white plate and incubated overnight. Cells were treated with inhibitors of p38 MAP kinase (SB202190), JNK (SP600125), PI3K (LY294002), and PDGFRa (ponatinib, axitinib) for two days +/- DW002. Inhibitors +/- DW002 were diluted in medium then added to the cells to obtain the correct final concentrations. Cell viability was then measured using Cell Titer Glo 2.0 Assay.

## Results

*DW002 has a more potent effect on cell viability than DW006*

We first wanted to determine if the compounds had different effects on cell proliferation, so dose response was evaluated using a cell viability assay. Oli-neu cells were plated in a 96-well plate and allowed to adhere overnight. The next day, cells were treated with 10-fold dilutions of DW002 and DW006 ranging from 0.1 uM to 1000 uM and incubated for two days before conducting the Cell Titer Glo 2.0 Assay (Figure 3A). DW002 and DW006 had differing effects on cell viability that can be seen visually on the graph. At lower concentrations, neither DW002 nor DW006 appeared to affect cell viability, but DW002 appeared to have an effect at a lower concentration than DW006. At higher concentrations, DW002 appeared to be associated with a greater decrease in cell viability than DW006. The difference in effect between DW002 and DW006 was analyzed using an unpaired Student's t-test for each concentration. It was found that the compounds had a statistically different effect on cell viability at 100 uM.

From the initial dose response assay, we noticed the strong decrease in cell viability that occurred with the DMSO control. The DMSO control was 2% DMSO, which was the highest concentration of DMSO added to the cells due to the compounds being dissolved in DMSO. When the cells were viewed under the light microscope, the DMSO seemed to have a cytotoxic effect. Cells were rounded and less adherent to the plate, indicating cell death. We wanted to ensure that the effect seen due to the compounds was not due to the DMSO that they were dissolved in. Therefore, the cell viability assay was conducted again, but this time cells were treated with DMSO concentrations that corresponded to the amount added with each compound treatment (Figure 3B). From the results, it can be seen that DMSO did not have a toxic effect on the cells expect at the highest concentration of 2%, which was the amount added when cells were

treated with 1000 uM of compound. Therefore, it is reasonable to think that the effects seen at lower concentrations are due to the compound itself rather than DMSO toxicity.

To further explore the dose dependent effects of the compounds, we next focused on a more narrow concentration range. Cells were again plated in triplicate in a 96-well plate and incubated overnight. The next day, the cells were treated with 3-fold dilutions of DW002 and DW006 ranging from 1 uM to 300 uM. After two days, cell viability was analyzed (Figure 3C). It can be seen that DW002 has a more potent effect than DW006. DW002 is associated with a dose dependent decrease in cell viability, whereas DW006 does not show a strong dose-dependent effect at these concentrations. The data was analyzed using an unpaired Student's t-test for each concentration. There is a statistically significant difference in effect for 10, 30, 100, and 300 uM. The most significant difference between the two compounds was at 100 uM ($p<0.0001$, unpaired Student's t-test). Dose response curves were created and the IC50 was determined using GraphPad Prism 6 (Figure 3D). The IC50 for DW002 is much lower than DW006, indicating that it has a more potent effect on cell viability.

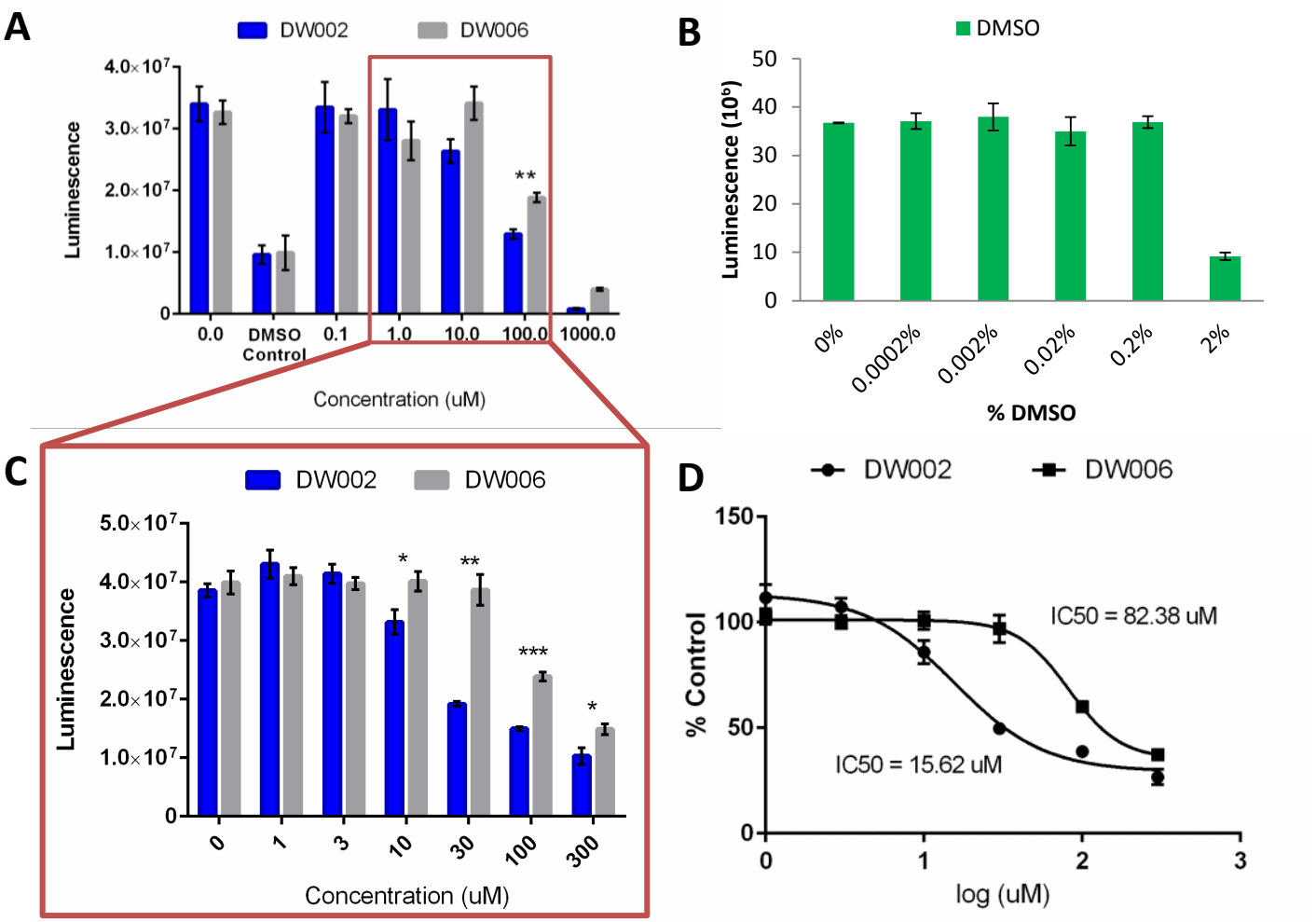


**Figure 3. DW002 decreases cell viability in a dose dependent manner not exhibited by DW006**. A) Cells were treated with increasing concentrations (0.1-1000 uM) of DW002 and DW006 for 2 days. DMSO control = 2% DMSO, corresponding to the largest amount of DMSO added with the compounds. Cell viability was measured with Cell Titer Glo 2.0 Assay. Luminescence corresponds to the number of viable cells. It can be visually seen that DW002 and DW006 differ in their effect on cell viability. There is a statistically significant difference in effect for 100 uM and 1000 uM (**, P<0.001; unpaired Student's t-test). B) Cells were treated with DMSO concentrations corresponding to the compound treatments in A. The data in B confirms that the effect on cell viability was due to the compounds except for the highest concentration (1000 uM). C) Cells were treated with increasing concentrations (1-300 uM) of DW002 and DW006 for 2 days. This range expanded a narrow section of concentrations from A (red box). Cell viability was measured with Cell Titer Glo 2.0 Assay. Luminescence corresponds to the number of viable cells. It can be visually seen that DW002 has a more potent effect on cell viability than DW006. There is a statistically significant difference in effect for the higher concentrations (*, P<0.05; **, P<0.001; ***, P<0.0001; unpaired Student's t-test). D) Effect of DW002 and DW006 on cell viability expressed as percent of control (0 uM). The IC50 of DW002 is 15.62 uM and the IC50 of DW006 is 82.38 uM. Fitted curves were generated using 4-parameter nonlinear regression. Data represent means of the triplicates and error bars represent standard deviation.

*DW002 and DW006 did not cause increased cell death*

After determining that the compounds did decrease cell viability, the next goal was to determine if this effect was due to a cytotoxic property of the compounds or whether they were inhibiting proliferation. To investigate this, we conducted a cell toxicity assay. Cells were plated in a 96-well black plate and incubated overnight. The next day, cells were treated with lysis buffer, digitonin, and 100 uM of DW002 and DW006. Cell death was measured after one and two days (Figure 4). Cell death upon treatment with either compound is comparable to untreated cells (control). This indicates that the compounds have minimal cytotoxic effect.

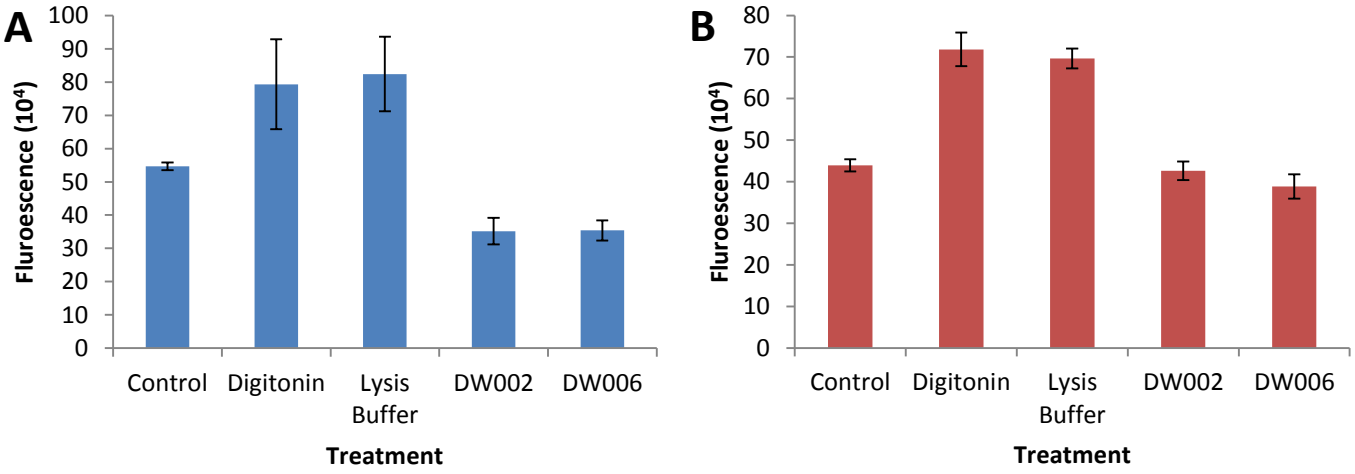


**Figure 4. DW002 and DW006 do not cause increased cell death after 24 hours or 48 hours of treatment.** A) Cells were treated with 100 uM of DW002 and DW006 for one day. B) Cells were treated with 100 uM of DW002 and DW006 for two days. In both A and B, cell death was measured using the CellTox Green Cytotoxicity Assay. Fluorescence corresponds to the number of dead cells. Digitonin (30 ug/ml) and lysis buffer (1:25) were used as positive controls for cell death. Control = untreated cells. Bar graph values represent means of the triplicates and error bars represent standard deviation.

*DW002 is selective for PDGFRa over PDGFRb*

Previous research conducted by Jelena showed that DW002 and related compounds decreased transcription of PDGFRa. PDGFRa and PDGFRb are structurally very similar but have very different physiologic functions. Therefore, our next goal was to determine if DW002 decreased transcription of both PDGFRs or just PDGFRa. To examine this, qPCR was conducted using cDNA samples taken from the same cells that Jelena studied. There were three different treatments analyzed: untreated, 50 uM DW002, or 50 uM DW006. Of each treatment, there were three independent samples. Astrocytes are known to express PDGFRb, so cDNA from cultured astrocytes were used as a positive control. GAPDH was used as an internal control. 100 ng of cDNA was used with 3 uM of each primer. qPCR was run in triplicate. From the graph of the Cq results, it does not appear that the compounds change the transcription of PDGFRb in comparison to the control (Figure 5). It is important to note however that the results show that the average Cq value for the astrocyte samples was 22.92, while the average Cq value for the control Oli-neu samples was 35.15. This indicates that the expression of PDGFRb in Oli-neu cells is very low. Therefore, it is difficult to detect any effect that the compounds may have on PDGFRb transcription. However, since PDGFRb is not expressed in Oli-neu cells, we can be fairly confident that the effect of the compounds is specific to PDGFRa and that the results so far were not confounded by acting on PDGFRb.

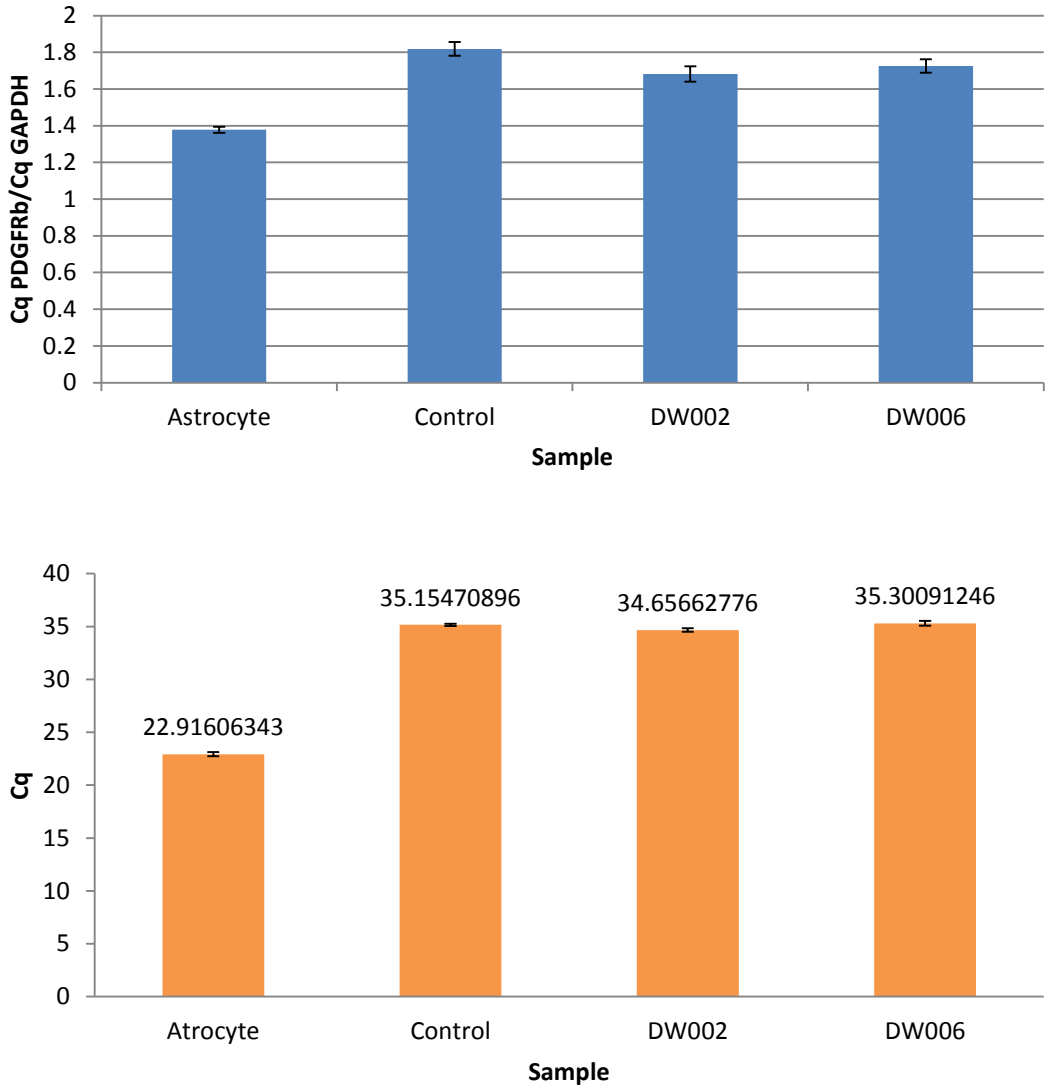


**Figure 5. PDGFRb is not expressed in Oli-neu cells.** mRNA was collected from astrocytes, Oli-neu cells treated with DMSO control, and Oli-neu cells treated with 50 uM DW002 or DW006 for two days, and converted to cDNA. The cDNA samples were analyzed by qPCR to detect if the compounds affected PDGFRb expression. The Cq values are expressed as normalized to GAPDH (A) as well as the raw values (B). By comparing the Cq values to the positive control (astrocytes), we concluded that PDGFRb is not expressed in Oli-neu cells. Therefore, the effect of DW002 and DW006 on Oli-neu cells is likely due to its effect on PDGFRa transcription. Bar graph values represent means of the triplicates and error bars represent standard deviation.

*DW002 maintains its activity in serum free medium*

We next wanted to determine the mechanism by which DW002 was able to decrease PDGFRa transcription, because this appeared to be linked to its activity of inhibiting cell proliferation. To investigate this, we decided to utilize iTRAQ analysis, a proteomics technology. This method labels all of the protein in a sample with an isobaric tag, and then determines the amount and the identity of the proteins in the sample through mass spectrometry. Different tags allows for the comparison of different samples. The samples that we chose to compare were: DMSO control, 30 uM DW002, 30 uM DW006, and 30 uM DW002 after a longer incubation.

iTRAQ is more effective for serum free samples, so before preparing the previously mentioned samples, we had to confirm that the Oli-neu cells would survive and that the activity of DW002 remained unchanged in SFM. To test this, I plated cells in triplicate in a 24-well plate and switched to SFM at different times to determine the best conditions for the cells. I switched the medium upon plating, 2 hours post plating, and 24 hours post plating. I took phase contrast pictures one, two, and three days after plating the cells and compared them to the cells in regular medium (Figure 6). Cells plated immediately in serum free medium survived for one day but then seemed to decline, evident from the clumped appearance. Cells in SFM two hours after plating appeared to survive and increase in density each day, suggesting that they were able to proliferate normally, although they did not reach the same density as the cells in regular medium. They also remained adherent to the plate and extended processes. Cells in SFM 24 hours after plating also increased in density and extended processes, indicating they were healthy and proliferative. I chose to proceed with the iTRAQ sample preparations by changing the medium of the Oli-neu cells to SFM two hours after plating.

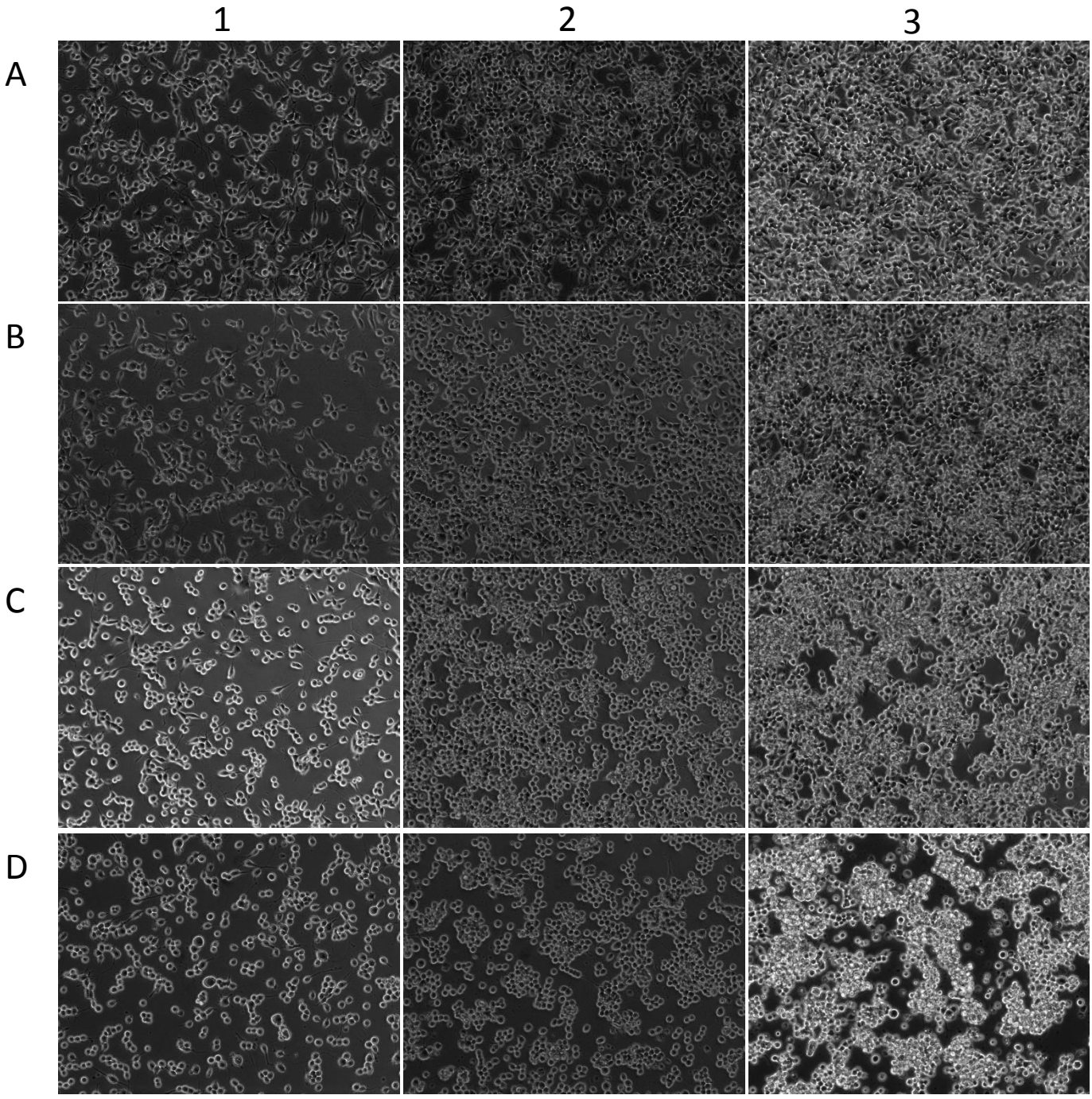


**Figure 6. Oli-neu cells survive and proliferate when switched to SFM 2 or 24 hours after plating.** Oli –neu cells were plated under different conditions (A-D) and phase contrast pictures were taken 1, 2, and 3 days after plating. A) Cells were plated in regular medium and used as a control. The cells proliferated rapidly. B) Cells were plated in regular medium and switched to SFM after 24 hours. These cells appeared healthy and proliferated to about the same extent as the control cells. C) Cells were plated in regular medium and switch to SFM after 2 hours. These cells also appeared healthy and proliferated quickly. D) Cells were plated immediately in SFM. These cells did not appear as healthy as the other treatments. They clumped together and did not adhere well to the plate, indicating cell death.

Next I confirmed that DW002 maintained its activity by conducting the cell viability assay again. Cells were plated in triplicate in a 96-well plate and medium was changed to SFM two hours later. The cells were incubated overnight. The next day they were treated with DMSO control (0.2%), 100 uM DW002, 20 ng/ml PDGF, and 100 uM DW002 + 20 ng/ml PDGF. Cell viability was measured after two days. It can be seen that DW002 still decreases cell proliferation in SFM (Figure 7). Of additional interest, it can be seen that DW002 maintains this effect even in the presence of the growth factor PDGF. However, PDGF did not lead to a strong increase in cell proliferation.

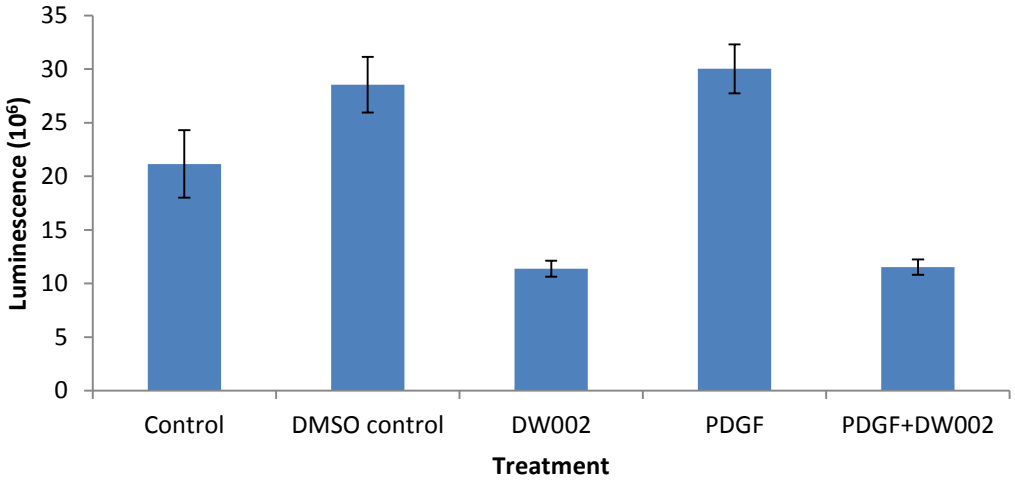


**Figure 7. DW002 maintains its effect on cell viability under serum-free conditions.** Cells in SFM were treated with 100 uM DW002 +/- 20 ng/ml PDGF. DW002 still decreased cell viability in SFM, even in the presence of growth factor. Control = untreated cells, DMSO control = 0.2% DMSO. Bar graph values represent means of the triplicates and error bars represent standard deviation.

After confirming that the cells would survive and that DW002 effect could still be observed in SFM, the four different samples (in duplicate) were sent for iTRAQ analysis. 245 proteins were analyzed. The iTRAQ data was reported back to us as ratios between samples, which allowed us to identify proteins that were more or less expressed in one treatment compared to another. Proteins were expressed at the same level if the ratio was 1. The samples were labeled as follows: DMSO control was 113 and 117, 30 uM DW002 short incubation was 114 and 118, 30 uM DW006 was 115 and 119, and 30 uM DW002 long incubation was 116 and 121. Before comparing any proteins, I first determined if the data was normally distributed. From analysis with GraphPad Prism 6, I found that the data was not normally distributed. Therefore, I calculated the $25^{th}$ and $75^{th}$ percentile for each ratio. I used these values as cut off points to classify a protein as being more ($>75^{th}$ percentile) or less ($<25^{th}$ percentile) expressed, which I will call "abnormal".

The data was analyzed in two ways to find proteins whose expression may be altered by DW002 treatment. Because samples were taken at two different times after treatment with DW002, I wanted to determine if there were any proteins that were consistently abnormal at both times compared to control, that were unchanged when treated with DW006. Therefore, I focused on the ratios: 114:113, 118:117, 116:113, 121:117, 115:113, and 119:117. I flagged proteins as "hits" if protein ratios were abnormal for 114:113, 118:117, 116:113, 121:117, but not 115:113 and 119:117. From this method (Method 1), I identified 6 hits (Table 1). Three of these proteins were more expressed, and three were less expressed. The second method (Method 2) that I used to analyze the data was to focus directly on the ratios between DW002 and DW006, which were 114:115 and 118:119. I flagged proteins as hits if they had abnormal expression in both replicates. I identified 34 proteins using this method (Table 1). Of these, 17 were more expressed

and 17 were less expressed. Because of the relatively large number of proteins I identified as hits, I attempted to find the most important proteins by identifying what I will call "strong hits." I defined this as protein ratios <0.5 or >2, which were values arbitrarily chosen. I identified 6 strong hits, 2 that were more expressed and 4 that were less expressed. I used UniProt to find the function of proteins found to be hits using Method 1 and strong hits using Method 2. The descriptions of the proteins from UniProt are listed in Table 2.

Of the 6 hits I identified from Method 1, 5 were also hits identified by Method 2. However, only 2 of the hits identified from Method 1 were also strong hits under Method 2. This is summarized in Figure 8. From analysis using Method 1, I did not identify any proteins that were abnormal at the longer incubation with DW002 but not at the short incubation, which may indicate that protein changes occur early after treatment with DW002.

| Method 1 | Method 2 |
|---|---|
| **Alpha-enolase OS=Mus musculus GN=Eno1 PE=1 SV=3** | Heat shock cognate 71 kDa protein OS=Mus musculus GN=Hspa8 PE=1 SV=1 |
| **Acyl-CoA-binding protein OS=Mus musculus GN=Dbi PE=1 SV=2** | **Alpha-enolase OS=Mus musculus GN=Eno1 PE=1 SV=3** |
| **40S ribosomal protein S19 OS=Mus musculus GN=Rps19 PE=1 SV=3** | Heterogeneous nuclear ribonucleoproteins A2/B1 OS=Mus musculus GN=Hnrnpa2b1 PE=1 SV=2 |
| **Ubiquitin-40S ribosomal protein S27a OS=Mus musculus GN=Rps27a PE=1 SV=2** | Tropomyosin alpha-3 chain OS=Mus musculus GN=Tpm3 PE=1 SV=1 |
| Calmodulin OS=Mus musculus GN=Calm1 PE=1 SV=1 | **Acyl-CoA-binding protein OS=Mus musculus GN=Dbi PE=1 SV=2** |
| **Histone H4 OS=Mus musculus GN=Hist1h4a PE=1 SV=2** | Glyceraldehyde-3-phosphate dehydrogenase OS=Mus musculus GN=Gapdh PE=1 SV=1 |
| | Pyruvate kinase PKM OS=Mus musculus GN=Pkm PE=1 SV=4 |
| | Nuclease-sensitive element-binding protein 1 OS=Mus musculus GN=Ybx1 PE=1 SV=3 |
| | **40S ribosomal protein S19 OS=Mus musculus GN=Rps19 PE=1 SV=3** |
| | Elongation factor 1-alpha 1 OS=Mus musculus GN=Eef1a1 PE=1 SV=3 |
| | **Ubiquitin-40S ribosomal protein S27a OS=Mus musculus GN=Rps27a PE=1 SV=2** |
| | 40S ribosomal protein S3 OS=Mus musculus GN=Rps3 PE=1 SV=1 |
| | Adenosylhomocysteinase OS=Mus musculus GN=Ahcy PE=1 SV=3 |
| | Isoform 2 of Heterogeneous nuclear ribonucleoprotein K OS=Mus musculus GN=Hnrnpk |
| | ATP synthase subunit delta, mitochondrial OS=Mus musculus GN=Atp5d PE=1 SV=1 |
| | Isoform 4 of Hepatoma-derived growth factor-related protein 2 OS=Mus musculus GN=Hdgfrp2 |
| | Isoaspartyl peptidase/L-asparaginase OS=Mus musculus GN=Asrgl1 PE=1 SV=1 |
| | **Histone H4 OS=Mus musculus GN=Hist1h4a PE=1 SV=2** |
| | G7b alternative form OS=Mus musculus GN=Lsm2 PE=1 SV=1 |
| | Short-chain specific acyl-CoA dehydrogenase, mitochondrial OS=Mus musculus GN=Acads PE=1 SV=2 |
| | Putative mitochondrial import inner membrane translocase subunit Tim8 A-B OS=Mus musculus GN=Timm8a2 PE=3 SV=1 |
| | Cell adhesion molecule 4 OS=Mus musculus GN=Cadm4 PE=1 SV=1 |
| | Phytanoyl-CoA hydroxylase-interacting protein OS=Mus musculus GN=Phyhip PE=1 SV=1 |
| | 40S ribosomal protein S13 OS=Mus musculus GN=Rps13 PE=1 SV=2 |
| | Putative RNA-binding protein Luc7-like 2 OS=Mus musculus GN=Luc7l2 PE=1 SV=1 |
| | Isoform Short of Cyclin-dependent kinase 16 OS=Mus musculus GN=Cdk16 |
| | Protocadherin Fat 3 OS=Mus musculus GN=Fat3 PE=1 SV=1 |
| | N-acetylglucosamine-6-sulfatase OS=Mus musculus GN=Gns PE=2 SV=1 |
| | Protein Vmn1r204 OS=Mus musculus GN=Vmn1r204 PE=4 SV=1 |
| | FH2 domain-containing protein 1 OS=Mus musculus GN=Fhdc1 PE=2 SV=3 |
| | Guanylate cyclase OS=Mus musculus GN=Gucy2d PE=3 SV=1 |
| | Molybdopterin synthase catalytic subunit OS=Mus musculus GN=Mocs2 PE=2 SV=3 |
| | Notchless protein homolog 1 OS=Mus musculus GN=Nle1 PE=1 SV=4 |
| | Opioid growth factor receptor OS=Mus musculus GN=Ogfr PE=2 SV=1 |
| 6 | 34 |

**Table 1. Expression of certain proteins was altered after treatment with DW002 but not DW006**. iTRAQ analyzed expression of 245 proteins. Ratios were calculated between each treatment (DMSO control, 30 uM DW002 short incubation, 30 uM DW006 short incubation, and 30 uM DW002 long incubation). In Method 1, ratios between DW002 and control and DW006 and control were analyzed. Proteins were identified as hits if they were in the bottom or top 25$^{th}$ percentile after treatment with DW002 (at both incubation times) but not treatment with DW006. Six proteins were hits using this method. In Method 2, ratios between DW002 and DW006 were analyzed. Proteins were identified as hits if they were in the bottom or top 25$^{th}$ percentile, and strong hits if ratios were <0.5 or >2. Using the method, 34 proteins were hits and 6 were strong hits. Bolded protein names are those found using both methods. Blue = more expressed, orange = less expressed. For Method 2, the dark blue and orange represent strong hits.

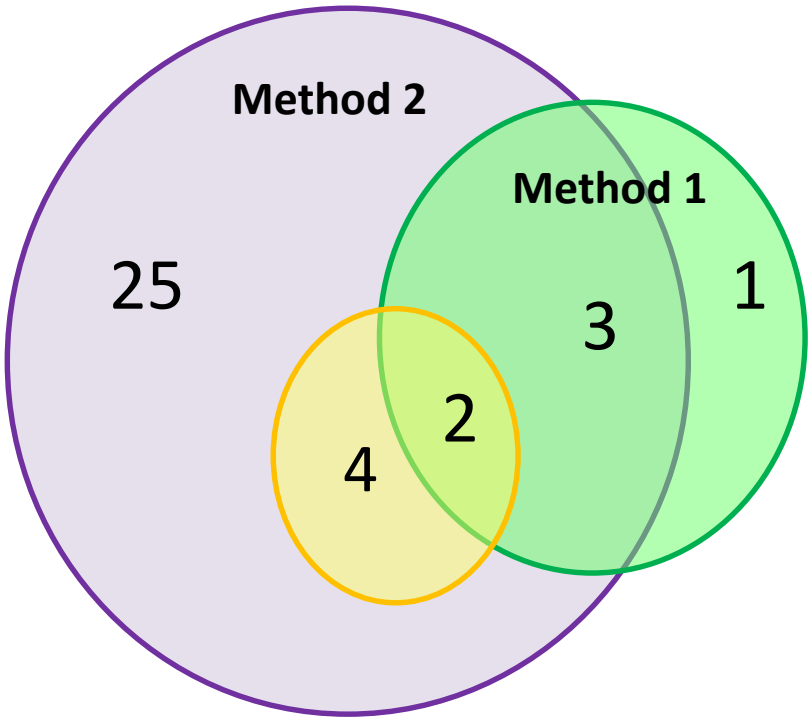


**Figure 8. Method 1 and Method 2 of iTRAQ data analysis identified some of the same and some different protein hits.** The purple circle represents protein hits found using Method 1, the green circle represents hits found by Method 2, and the yellow circle represent hits found by Method 1. 5 of the 6 hits found by Method 1 were also identified using Method 2. 2 of the 6 hits from Method 1 were strong hits identified by Method 2.

| | |
|---|---|
| **Alpha-enolase** | Multifunctional enzyme that, as well as its role in glycolysis, plays a part in various processes such as growth control, hypoxia tolerance and allergic responses. May also function in the intravascular and pericellular fibrinolytic system due to its ability to serve as a receptor and activator of plasminogen on the cell surface of several cell-types such as leukocytes and neurons. Stimulates immunoglobulin production. |
| **Acyl-CoA-binding protein** | Binds medium- and long-chain acyl-CoA esters with very high affinity and may function as an intracellular carrier of acyl-CoA esters. It is also able to displace diazepam from the benzodiazepine (BZD) recognition site located on the GABA type A receptor. It is therefore possible that this protein also acts as a neuropeptide to modulate the action of the GABA receptor. |
| **40S ribosomal protein S19** | Required for pre-rRNA processing and maturation of 40S ribosomal subunits. |
| **Ubiquitin-40S ribosomal protein S27a** | Ubiquitin: Exists either covalently attached to another protein, or free (unanchored). Polyubiquitin chains, when attached to a target protein, have different functions depending on the Lys residue of the ubiquitin that is linked: Lys-6-linked may be involved in DNA repair; Lys-11-linked is involved in ERAD (endoplasmic reticulum-associated degradation) and in cell-cycle regulation; Lys-29-linked is involved in lysosomal degradation; Lys-33-linked is involved in kinase modification; Lys-48-linked is involved in protein degradation via the proteasome; Lys-63-linked is involved in endocytosis, DNA-damage responses as well as in signaling processes leading to activation of the transcription factor NF-kappa-B. |
| Calmodulin | Calmodulin mediates the control of a large number of enzymes, ion channels, aquaporins and other proteins by Ca2+. Among the enzymes to be stimulated by the calmodulin-Ca2+ complex are a number of protein kinases and phosphatases. |
| **Histone H4** | Core component of nucleosome. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling. |
| Pyruvate kinase PKM | Glycolytic enzyme that catalyzes the transfer of a phosphoryl group from phosphoenolpyruvate (PEP) to ADP, generating ATP. Stimulates POU5F1-mediated transcriptional activation. Plays a general role in caspase independent cell death of tumor cells. The ratio betwween the highly active tetrameric form and nearly inactive dimeric form determines whether glucose carbons are channeled to biosynthetic processes or used for glycolytic ATP production. The transition between the 2 forms contributes to the control of glycolysis and is important for tumor cell proliferation and survival. |
| Adenosylhomocysteinase | Adenosylhomocysteine is a competitive inhibitor of S-adenosyl-L-methionine-dependent methyl transferase reactions; therefore adenosylhomocysteinase may play a key role in the control of methylations via regulation of the intracellular concentration of adenosylhomocysteine |
| Isoform 2 of Heterogeneous nuclear ribonucleoprotein K | One of the major pre-mRNA-binding proteins. Binds tenaciously to poly(C) sequences. Likely to play a role in the nuclear metabolism of hnRNAs, particularly for pre-mRNAs that contain cytidine-rich sequences. Can also bind poly(C) single-stranded DNA. Plays an important role in p53/TP53 response to DNA damage, acting at the level of both transcription activation and repression. When sumoylated, acts as a transcriptional coactivator of p53/TP53, playing a role in p21/CDKN1A and 14-3-3 sigma/SFN induction (By similarity). As far as transcription repression is concerned, acts by interacting with long intergenic RNA p21 (lincRNA-p21), a non-coding RNA induced by p53/TP53. This interaction is necessary for the induction of apoptosis, but not cell cycle arrest. |
| Molybdopterin synthase catalytic subunit | Catalytic subunit of the molybdopterin synthase complex, a complex that catalyzes the conversion of precursor Z into molybdopterin. Biosynthesis of molybdopterin, an essential cofactor for the catalytic activity of some enzymes, e.g. sulfite oxidase, xanthine dehydrogenase, and aldehyde oxidase. |

**Table 2. Functions of protein hits identified by Method 1 and strong hits identified by Method 2**. Expression of these proteins were found to be altered after treatment with DW002. The methods that were used to identify these proteins have been previously explained. The functions of these proteins was identified using UniProt (www.uniprot.org). Bolded protein names were found using both methods. Blue = more expressed, orange = less expressed. Dark blue and orange represent strong hits.

*Inhibition of specific intracellular signaling pathways did not change the effect of DW002*

While iTRAQ analysis was being conducted, we explored the mechanism of DW002 by inhibiting intracellular signaling pathways. We hypothesized that if inhibition of a signaling pathway eliminated the effect of DW002, DW002 may be altering that pathway in some way to decrease PDGFRa transcription. Cells were plated in triplicate in a 96-well plate and incubated overnight. They were then treated with a wide range of concentrations of inhibitors for various kinases or receptors, with or without DW002. The various inhibitors blocked: p38 MAP kinase (SB202190), JNK (SP600125), PI3K (LY294002), and PDGFRa (ponatinib, axitinib). The concentrations of the inhibitors were based off of their reported IC50 values. Cell viability was measured after two days. None of the inhibitors blocked the effect of DW002 (Figure 9). DW002 retained its ability to decrease cell proliferation even in the presence of inhibitors that blocked many major signal transduction pathways. Another interesting result was that inhibition of PDGFRa did not inhibit cell proliferation, even though blocking PDGFRa in OPCs would be expected to have that effect.

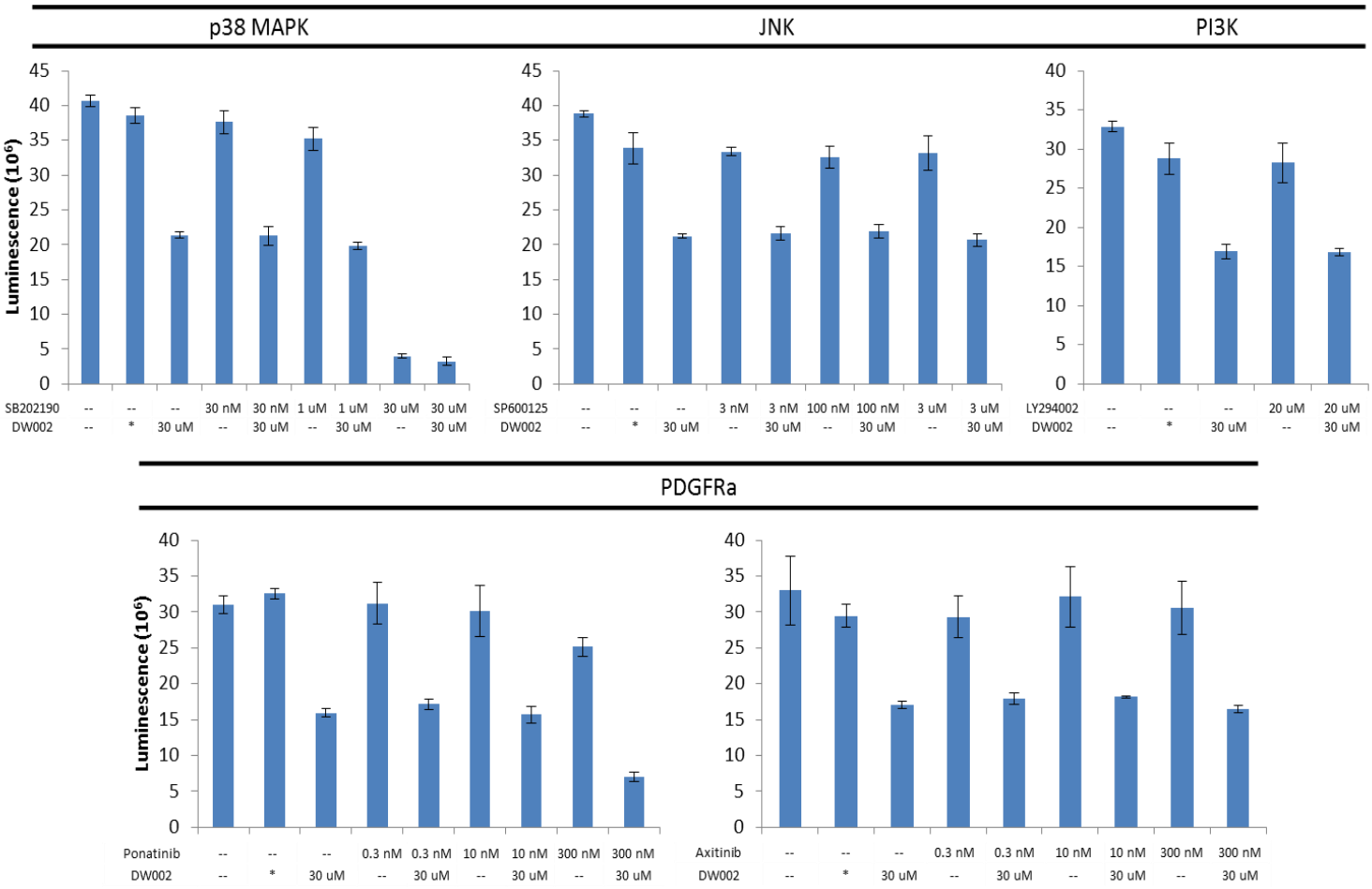


**Figure 9. Treatment with inhibitors does not block the effect of DW002.** Cells were treated with increasing concentrations of potent and selective inhibitors with or without 30 uM of DW002 for 48 hours. Cell viability was measured using the Cell Titer Glo 2.0 Assay. Luminescence corresponds to the number of viable cells. If DW002 acted through the pathway blocked by the inhibitor, we would expect to DW002 to no longer decrease cell viability. Treatment with p38 mitogen activated protein kinase (MAPK) inhibitor (A), c-Jun N-terminal kinase (JNK) inhibitor (B), phosphatidylinositol-4,5-bisphosphate 3-kinase(PI3K) inhibitor (C), and PDGFRa inhibitors (D, E) did not block the effect of DW002, suggesting that DW002 does not act through these signaling pathways. *, DMSO control=0.0012%. Bar graph values represent means of the triplicates and error bars represent standard deviation.

## Discussion

*DW002 decreases cell proliferation likely through downregulation of PDGFRa*

From the cell viability and cytotoxicity assays, we found that treatment with DW002 led to decreased cell viability but not increased cell death. From these results, we concluded that the compound must be inhibiting cell proliferation. The untreated cells were able to grow normally, leading to an increased number of cells and increased cell viability. In contrast, the cells treated with DW002 stopped proliferating, leading to fewer cells after two days of treatment and a decreased cell viability compared to control. It is unlikely that DW002 decreased cell viability through a cytotoxic effect because cell death was not increased with treatment with DW002. We expected DW002 to inhibit proliferation because it was already observed that DW002 decreases transcription of PDGFRa. As mentioned in the introduction, PDGFRa is important for OPC proliferation and differentiation. Proliferation of OPCs is dependent on signaling by PDGF-AA through PDGFRa (Calver et al., 1998). Therefore, it makes sense that decreased expression of PDGFRa due to DW002 would lead to decreased proliferation.

We also investigated the specificity of DW002. PDGFRa and PDGFRb are structurally very similar, so we wanted to determine that the activity of DW002 was specific to PDGFRa. Therefore, we used qPCR to determine if DW002 also led to decreased transcription of PDGFRb. However, we found that PDGFRb is not expressed on Oli-neu cells. This result is somewhat helpful, because now we know that DW002 could only be affecting PDGFRa transcription in our model. This supports our hypothesis that the PDGFRa downregulation caused by DW002 is responsible for the decreased cell proliferation. However, we have not determined if DW002 does affect PDGFRb transcription. This is important to know because the two receptor subtypes have different function in disease as well as in normal physiology. We

want any therapeutic compound to treat glioma to have targeted activity against PDGFRa to decrease tumor growth. Downregulating PDGFRb may have negative consequences. Therefore, in would be useful to conduct more studies in the future with perhaps a different model to determine the effect of DW002 on PDGFRb transcription.

*DW002 does not appear to affect p38MAPK, JNK, PI3K, or PDGFRa signaling*

We conducted the assays with the inhibitors to determine if DW002 had an effect on certain cell signaling pathways. The cell signaling pathways we decided to inhibit were chosen based on evidence that it may lead to PDGFRa transcription or because they were known to lead to OPC proliferation. OPC proliferation in response to PDGF-AA is primarily mediated by activation of PI3K which leads to Akt activation (Hill et al., 2013). DW002 may inhibit a component of this signaling pathway, which may be why it inhibits cell proliferation. However, we did not find that PI3K inhibition affected the proliferation of these cells. Therefore, it is unlikely that DW002 acts through that mechanism.

p38MAPK is involved in OPC differentiation into oligodendrocytes (Baron et al., 2000). It has been shown that p38MAPK phosphorylation increases as PDGFRa expressing progenitor cells differentiate (Chew et al., 2010). PDGFRa is downregulated when OPCs begin to differentiate (Hart, 1989). Therefore, it may be possible that p38MAPK may be involved in OPC differentiation by regulating the transcription of PDGFRa. In fact, there is evidence to support this. Activation of p38MAPK has been associated with a decrease in PDGFRa mRNA (Vela et al., 2002). In addition, p38MAPK activates a transcription factor C/EBPβ (CCAAT/enhancer-binding protein) which represses PDGFRa transcription (Afink et al, 2004). p38MAPK also inhibits the activity of JNK, which normally activates ATF2 (activator of transcription factor 2)

(Chew et al, 2010; Lau et al., 2012). ATF2 stimulates PDGFRa transcription (Maekawa et al., 1999). We thought it may be possible that DW002 decrease PDGFRa transcription by activating the p38MAPK-C/EBPβ pathway or activating the p38MAPK/JNK/ATF2 pathway to decrease PDGFRa transcription, and therefore inhibit cell proliferation. However inhibition of p38MAPK did not block the effect of DW002 on cell proliferation. Inhibition of JNK similarly did not affect DW002 activity. Therefore, it is unlikely that the mechanism of DW002 activity involves modulation of these pathways. A summary of the pathways inhibited is depicted in Figure 10.

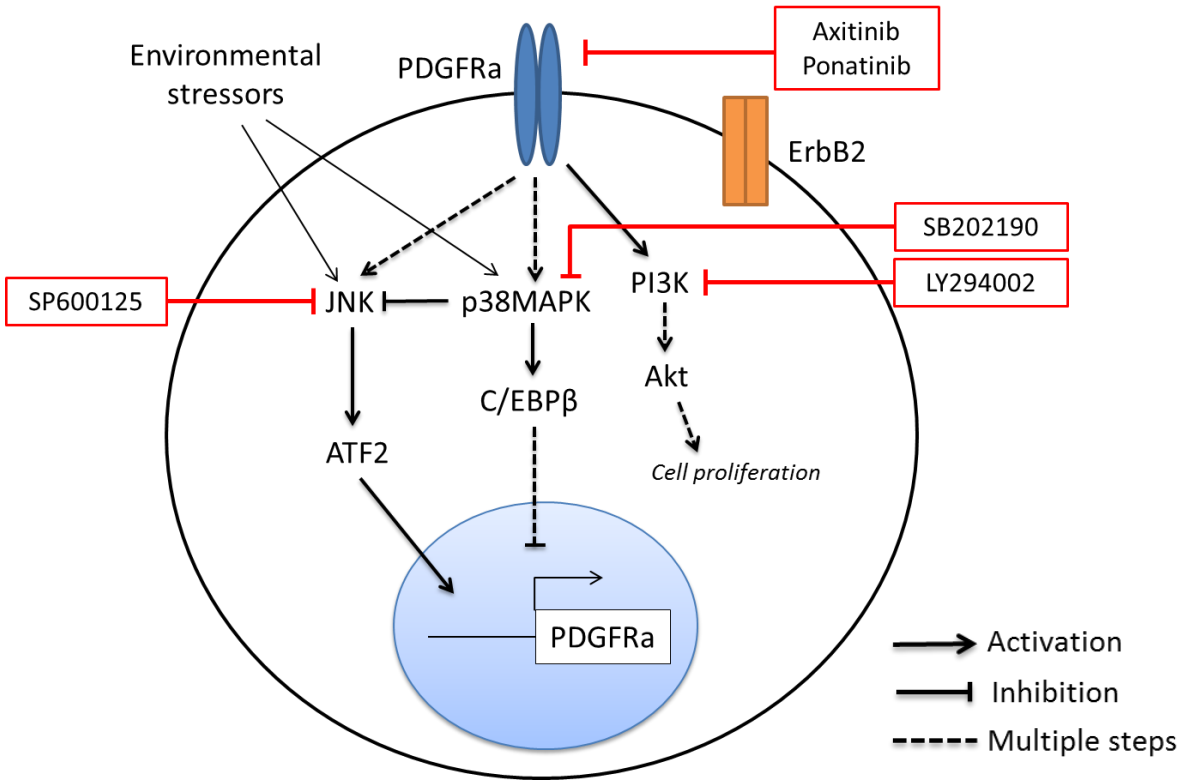


**Figure 10. Simplified diagram depicting the signaling pathways that DW002 may be affecting to cause downregulation of PDGFRa.** Stimulation of PDGFRa by PDGF ligands leads to activation of JNK, p38MAPK, and PI3K. JNK and p38MAPK can alternatively be activated by environmental stressors. It has been previously shown that activation of JNK leads to increased transcription of PDGFRa, while activation of p38MAPK leads to decreased transcription of PDGFRa through different transcription factors (ATF2 and EBPβ). p38MAPK decreases the activity of JNK. Oli-neu cells were treated with various inhibitors (red boxes) against PDGFRa, JNK, p38MAPK, and PI3K with or without DW002.

There are some possible limitations to these experiments with the inhibitors. One is that the inhibitors may inhibit more than the target that we intended. We tried to choose inhibitors that were selective for each kinase or receptor, but there are very few inhibitors that are entirely selective. For example, Ponatinib is an inhibitor of PDGFRa with an IC50 of 1.1 nM, but it also inhibits VEGFR2 (IC50-1.5 uM) and FGFR1 (IC50=2.2 uM). Another limitation is that the concentrations chosen may not adequately inhibit the intended target. We used a wide range around the IC50 of the inhibitors, but a higher concentration may be needed to adequately inhibit the target in the cells.

A major limitation to this experiment, as well as the other experiments, is that our model, an Oli-neu cell line, may not perfectly represent human OPCs. From the data, we saw that PDGFRa inhibitors did not inhibit the proliferation of the cells. However, previous experiments have found that OPCs are dependent on PDGF-AA signaling through PDGFRa to proliferate. Oli-neu cell growth independent of PDGFRa signaling may be because it is a cell line transformed with an ErbB2 oncogene (Jung et al., 1995). This oncogene may provide enough signals for proliferation so that other growth factor signaling pathways are not necessary. The fact that Oli-neu cells do not require PDGFRa signaling to proliferate indicates that PDGFRa downregulation may not be the mechanism by which DW002 inhibits cell proliferation. To investigate this further, we will test an inhibitor of ErbB2 to determine if blocking that pathway inhibits growth of the cells. If so, DW002 may be affecting a component of that signaling pathway, therefore causing the decreased cell proliferation.

*iTRAQ analysis did not yield any clear protein changes involved in DW002 mechanism of action*

Along with the inhibitor treatments, we also used iTRAQ analysis to elucidate the possible mechanism of action of DW002. We were able to identify proteins that were up or down regulated after treatment with DW002 but not treatment with DW006. I used UniProt to find the function of proteins found to be hits using Method 1 and strong hits using Method 2. The descriptions of the proteins from UniProt are listed in Table 2. The proteins that are less expressed after treatment with DW002 appear to be proteins involved in glycolysis, transcription, and cell division. This is expected, because we already know that DW002 leads to decreased cell proliferation. Cells treated with DW006 or untreated cells undergo more cell proliferation, so they require the activity of proteins involved in growth and division. Of note, one of the proteins identified through Method 2 that was downregulated was Notchless Protein Homolog 1. Evidence has shown that this protein downregulates p21, which is a cyclin dependent kinase inhibitor. This finding is interesting because Jelena previously found that DW002 decreases transcription of p21 (Medved, 2014). Taken together, this supports the idea that DW002 blocks cell cycle progression of the cells and leads to decreased proliferation.

Of the upregulated proteins after DW002 treatment, Histone H4 makes sense because with less cell division, there is less DNA replication and transcription, so the DNA can stay more tightly coiled around histones. It is unclear why 40S Ribosomal Protein S19 is upregulated. I would expect it to be downregulated because there is less transcription and translation occurring after DW002 treatment.  Molybdopterin synthase catalytic subunit is also upregulated. This cofactor is necessary for some metabolizing enzymes. It may be upregulated because the cells are metabolizing the DW002 compound. The last protein that is upregulated is calmodulin. This could be involved in a multitude of processes. It is difficult to determine why it is upregulated

with DW002 treatment. It may be useful to further investigate the pathways that calmodulin is involved in.

There were no clear signaling pathways that were up or down regulated due to DW002 treatment. Many of the proteins that had altered expression were just indicators of decreased cell proliferation, and did not provide any mechanistic information. However, before drawing any final conclusions, it is necessary to further analyze the data we received from iTRAQ. The methods that I used to identify protein hits may not be the best way to find altered protein expression. This was just preliminary work to get an idea of what may be occurring. While the criteria to classify proteins as "abnormal" was objective, choosing the criteria of less than the $25^{th}$ percentile and greater than the $75^{th}$ percentile was a subjective decision. It would be useful to use actual proteomics software to glean more information, and to identify a way to actually determine if protein expression after DW002 is significantly different from the control or treatment with DW006.

In the preparation for iTRAQ, the cells were treated with DW002 for a long and short incubation. The short incubation was about 20 hours and the long incubation was about 30 hours. Both of these incubations were shorter than how the cells were treated for all of the previous experiments. Therefore, we may not have captured proteins that were altered at later time points.

*Future directions and significance*

Through the methods and experiments described so far, we have been able to confirm that DW002 decreases Oli-neu cell proliferation. It has a much more robust and potent effect than DW006, despite having almost identical structures. This indicates that the small difference in the structure of DW002 is important for its activity. We have also investigated the mechanism

by which DW002 has this effect, but so far have been unsuccessful in determining a clear target. More research needs to be conducted to elucidate DW002's mechanism of action. As previously mentioned, Oli-neu cells are not a perfect representation of OPCs. Therefore, we will next try to determine the pathways that regulate Oli-neu cell proliferation to identify possible pathways that DW002 may be modulating. We will use lapatinib to inhibit ErbB2 and see if the effect of DW002 is maintained. We will also use a different model, mouse slice culture, to retest the previous inhibitors and see if the results match the Oli-neu cell results. It would also be helpful to test DW002 on glioma models or samples to see if it has a true beneficial effect.

Further investigation into the mechanism of DW002 may help to identify new targets for the development of glioma treatments. Once we can identify how DW002 is decreasing proliferation, we can try to improve its activity through structure activity relationship studies. We could also develop new compounds to target other components of the pathway that DW002 modulates. The goal is to develop therapeutic compounds that could decrease the proliferation of glioma cells, thereby inhibiting tumor formation. Future studies into DW002 may also be beneficial for other CNS diseases where PDGFRa plays a role. For example, downregulation of PDGFRa may inhibit the proliferation of OPCs, allowing them to differentiate into oligodendrocytes that remyelinate nerves, a possible therapy for multiple sclerosis. Therefore, the research conducted thus far is the beginning of work that could help to find therapeutic compounds for glioma and other diseases as well.

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