Andrews University
Digital Commons @ Andrews University

Honors Theses | Undergraduate Research

4-1-2022

Analysis of Invasion Proteins MMP2, MMP9, ADAM12, and ADAM17 in Glioblastoma U87MG Cells treated with anti-cancer compound 3,4-dimethoxybenzaldehyde

Andras Muranyi
Andrews University, andras@andrews.edu

Follow this and additional works at: https://digitalcommons.andrews.edu/honors

Part of the Biology Commons, and the Cancer Biology Commons

Recommended Citation
Muranyi, Andras, "Analysis of Invasion Proteins MMP2, MMP9, ADAM12, and ADAM17 in Glioblastoma U87MG Cells treated with anti-cancer compound 3,4-dimethoxybenzaldehyde" (2022). *Honors Theses*. 265.

https://digitalcommons.andrews.edu/honors/265

This Honors Thesis is brought to you for free and open access by the Undergraduate Research at Digital Commons @ Andrews University. It has been accepted for inclusion in Honors Theses by an authorized administrator of Digital Commons @ Andrews University. For more information, please contact repository@andrews.edu.

J. N. Andrews Honors Program
Andrews University

HONS 497

Honors Thesis

Analysis of invasion proteins MMP2, MMP9, ADAM12, and ADAM17 in glioblastoma U87MG cells treated with anti-cancer compound 3,4-dimethoxybenzaldehyde.

Andras Muranyi

April 1st, 2022

Advisor: Dr. Denise Smith

Primary Advisor Signature: Denise L Smith Ph.D.

Department: Biology

Abstract

This research tested the effectiveness of novel compound 3,4-dimethoxybenzaldehyde, demonstrated to have anti-cancer properties. U87MG Glioblastoma cells were exposed to the compound at its LC_{50} concentration, then processed to collect proteins from the cells. Proteins were analyzed via Western blotting for specific protein levels of matrix metalloproteinase 2 and 9 (MMP) and disintegrin and metalloprotease 12 and 17 (ADAM). Previous research indicates these proteins are involved in the invasive properties of glioblastoma cells. Westerns were quantified with ImageJ and compared using a one-way ANOVA. Results indicate the compound has minimal effect upon the expression of MMP2, MMP9, ADAM12, ADAM17 proteins.

Introduction

Glioblastoma is an aggressive form of brain cancer which affects about 100,000 people per year. At present, no effective treatment for glioblastoma exists; although surgical resection and radiotherapy are techniques which have helped extend lifespan, they have done so with only mild success. Woodward (1996) demonstrated through mathematical models that surgical resection and radiotherapy alone was unable to put glioblastoma into remission without the use of chemotherapy. Currently, temozolomide is used to treat patients with glioblastoma; although temozolomide is a targeted treatment, it does not increase lifespan beyond a few months (Dinnes, Cave, Huang, & Milne, 2002). Because of the need for a more powerful chemotherapeutic agent, this project aims to provide insight into the effectiveness of other anticancer compounds like 3,4-dimethoxybenzaldehyde that were used as targeted chemotherapeutic agents against glioblastoma.

Glioblastoma's highly invasive behavior is caused by the expression of disintegrin and matrix metalloprotease families ADAM and MMP; these proteins degrade extracellular tissue, draw nutrients toward the tumor, or encourage growth, creating a more robust cancer (Chen et al., 2013). More specifically, the ADAM (a disintegrin and metalloproteinase) protein family contains transmembrane proteins which are known to release or "shed" other proteins on the membrane. These released proteins may then signal other areas of the cell to encourage growth. For example, ADAM17 sheds epidermal growth factor receptor (EGFR) proteins from the surface of the cell; overexpression of ADAM17 leads to excessive shedding of EGFR proteins which then signal the cell to induce growth (Chen et al., 2013). ADAM12 acts similarly, but it instead releases proteins which bind to heparin-binding epidermal growth factor (HB-EGF). As expected, HB-EGF proteins will trigger their respective receptors to induce cell growth, much

like EGFR proteins (Nakada et al., 2007). The behavior of the matrix metalloproteinase (MMP) protein family demonstrates effectiveness in degrading the extracellular matrix (ECM) which surrounds the outside of glioblastoma cells. Both MMP2 and MMP9 have been shown to have specialized enzymatic sites which allow them to degrade the ECM rapidly; this degradation generates space for new cell growth, allowing glioblastoma to migrate from the original progenitor site (Nakada et al., 2007). With both MMP and ADAM proteins combined, glioblastoma cells become capable of inducing rapid growth while generating more area for growth in the process.

Past research in the Smith-Murray lab attempted to combat glioblastoma by developing novel anti-cancer treatments though experimentation of various chemical functional groups. Previous graduate students investigated dihydropyridine derivatives (Hunyenyiwa 2019), benzothiazoles (Kyi 2019), and fluorinated α-cyanostilbenes (Mayor 2019) in attempts to develop anti-cancer compounds. Their research both developed novel hybrids and determined the LC_{50}, a value which indicates the concentration of the compound which kills half of the total number of cells in a cell culture. The LC_{50} is useful in assessing the lethal effectiveness and toxicity of a compound; typically, lower concentrations indicate a greater toxicity, meaning the compounds have greater anticancer potential. Compounds from Meyer, Hunyenyiwa, and Kyi all had low LC_{50}, the highest being 0.08125 mg/ml and the lowest being 0.00197 mg/ml (Hunyenyiwa 2019; Kyi 2019; Mayor 2019). These values suggested that the synthesized compounds had potential to develop into anti-cancer agents and that further research into their affects on glioblastoma cells was required.

Though past research developed novel compounds, analysis concerning their molecular action was lacking. My project sought to analyze the effects of one novel anti-cancer drug to

determine its affects on protein expression. By doing so, protein analysis could determine if the drug induced changes in glioblastoma protein expression which could reduce its aggressive spread. To do so, I selected the most promising compound, 3,4-dimethoxybenzaldehyde (Mayor), which had the lowest LC_{50} for analysis in my project. Then, I proceeded to treat U87MG cells with this compound, harvest their proteins, then analyze their expression using Western blotting to determine the upregulation or downregulation of ADAM and MMP proteins after treatment.

Methodology

To compare the effects of the novel hybrid 3,4-dimethoxybenzaldehyde on cellular levels of protein expression, glioblastoma cells were grown and treated; first, total cellular protein was extracted from the cultured cells after exposure to the compound, and then target proteins were isolated and identified using Western blotting. The expected results provided qualitative blots which were compared between control and experimental groups.

Cell Culture: U87MG Glioblastoma cell lines were grown with a mixture of minimum essential media (MEM; Gibco) with 10% fetal bovine serum (Gibco) and 1% penicillin / streptomycin antibiotics (Gibco). The cells were grown in 100mm culture plates (Nunc) in an incubator (Thermo Fisher Scientific) at 37 ºC, 5% CO_2 (Hunyenyiwa, 2019). Each culture plate's MEM was changed regularly at least every two days and replaced with 8ml of fresh MEM. Once a plate reached maximum confluency (when cells completely covered the base of the plate), the cell population was halved by removing the MEM and adding 2ml 1X Trypsin solution (Gibco). This solution suspended the cells off the plate and allowed for half of the cells to be removed via

micropipette. The cells were then transferred to a new culture plate and provided 8ml MEM or otherwise were destroyed. Any solutions or cells which were no longer needed were killed in bleach and flushed down the drain. Used culture plates were disposed of in biohazard waste.

Cell Treatment and Protein Extraction: A series of three experimental and control plates were grown to about 95% cell confluency (cells filling about 95% of the culture plate). Once the cells grew to the expected confluency, three experimental plates were treated with 3,4-dimethoxybenzaldehyde which was solvated with dimethyl sulfoxide (DMSO) at the LC_{50} concentration. Another three control plates remained completely untreated without 3,4-dimethoxybenzaldehyde or dimethyl sulfoxide. (The DMSO was purposefully omitted because past research by Kyi (2019) has shown that it has no significant effect upon U87MG glioblastoma cells when under 1% concentration in the culture solution. The solvated 3,4-dimethoxybenzaldehyde has a DMSO concentration much lower than 1% in total solution.) The entire series of plates was then incubated for 24 hours; after that period, MEM was removed from each plate. These plates were treated with NP40 cell lysis buffer (Thermo Fisher Scientific), rupturing the cells and releasing their proteins (Chen et al., 2013). The subsequent protein-rich cell lysate was centrifuged and the supernatant (remaining liquid product) was stored in a -80 ^{o}C freezer.

Protein Assay: Following extraction of the cellular protein, the sample protein concentrations from both experimental and control cell groups needed to be quantified to know how much protein to analyze. This was done via a protein assay kit (Pierce). A bovine serum albumin protein standard from the kit was used in graduations from 2000 ug/ml to 5 ug/ml; both the

standard and sample proteins were pipetted in triplicate into a 96-well plate (a plate containing 96 individual containment wells) in 10ul volumes. After pipetting each protein sample and recording its position on the 96-well plate, 200 ul of dye reagent substrate also provided in the kit was added to each well containing protein. The plate was incubated for 30 minutes and afterward scanned using a spectrophotometer to create absorbance values based on the light absorbed by the proteins in each sample. Absorbance values from the protein standards created a linear regression by plotting the absorbance values against the known protein standards' concentrations. The absorbance value of each sample's triplicate set was averaged and then graphed on the linear regression model to determine the final concentration of protein in each individual sample. In essence, this process compared a known protein concentration's optical density to the experimental protein's optical density. Then the experimental protein concentration was mathematically calculated by that comparison. Through this process I ascertained that each protein sample had at least 20ug/ml of protein to produce adequate results during Western blotting.

Western Blotting: After determining the sample protein concentrations, the sample protein sets (experimental and control) were analyzed using a Western blot. First, the protein samples were separated by electrophoresis using a sodium dodecyl sulfide polyacrylamide gel (SDS-PAGE; Invitrogen). This system allowed for different proteins to be separated by size for ease of distinction. Following electrophoresis, the separated proteins were transferred from the gel to a nitrocellulose membrane via a membrane transfer apparatus (BioRad). During transfer, the nitrocellulose membrane captured all proteins from the gel, allowing the proteins to be blotted. Following the transfer, positive confirmation of total transfer of the protein bands onto the

membrane was visualized using Ponceau S (Thermo Fisher Scientific), then destained with lab-made tris-buffered saline. Polyclonal rabbit antibodies (GenScript) were used to bind and detect ADAM17, ADAM12, MMP2, or MMP9 proteins on the membrane in sequence. These bound antibodies were visualized using a chemifluorescent agent via the Pierce Fast Western Blotting kit (Pierce); the resulting complex formed blots on the membrane which were photographed using Kodak Scientific Imaging Film for analysis (Chen et al., 2013). The resulting blots from visualization allowed for comparison between control and experimental groups. For example, lighter bands indicated reduced expression after treatment while darker blots indicated an abundance of protein expression and ineffective treatment.

Reuse of Nitrocellulose Membrane: The nitrocellulose membrane was capable of being blotted twice while providing usable results for analysis. Following a Western blot, the membrane was stored in phosphate buffered saline at 4 ^{o}C in a refrigerator until ready to be used. On use, the membrane blot antibodies were removed with Restore Stripping Buffer (Thermo Fisher Scientific) by shaking the membrane in solution for 20 minutes. Afterward, the membrane was control stained with Ponceau S, then destained with tris-buffered saline. The Western blot could then take place.

Digital and Statistical Analysis: Films from Western blots were digitally imaged in a lightbox, then stored on a computer hard drive for further analysis. Digital film .tiff files were imported into paint.NET software, converted into black and white images, then exported as .jpeg files at the highest quality possible. The .jpeg film images were then imported into ImageJ software. The image contrast and brightness were adjusted accordingly as needed in each image to detect

differences between bands in each well. Then, each well (control and experimental) was selected and sampled using the rectangle tool; the selection area remained constant across each well. Data sampling provided mean gray value and min/max gray values for each well. Resulting values were tabulated and a ratio of control to experimental mean gray values was created for each respective well (e.g. mean gray value of control well 1 was divided against the mean gray value of experimental well 4 to create the ratio). A one-way ANOVA was used to compare the control wells against the experimental wells for each protein to determine statistical significance. Values where $p < 0.05$ are considered statistically significant. All statistical and mathematical calculations were done in Microsoft Excel software.

In summary, glioblastoma cells were exposed to 3,4-dimethoxybenzaldehyde in triplicate, and the impact on the cellular expression of target proteins was assessed. After cell culturing and treatment, the target proteins were extracted from the cells, separated from other cellular components, and visualized by Western blotting to determine if there was a difference in protein expression between treated and non-treated glioblastoma cells. Differences between control and experimental blots qualitatively and quantitatively indicated the protein expression of ADAM and MMP proteins; this allowed conclusions to be drawn regarding the effects of 3,4-dimethoxybenzaldehyde on U87MG glioblastoma cell protein expression.

Results

During protein content analysis, a Bradford assay indicated protein level concentrations of our samples at >1000ug/mL exceeding our threshold of minimum protein necessary (20µg/mL) for protein analysis. Furthermore, Ponceau S stains consistently indicated proper protein

transfer following each membrane preparation. Western blot film images were difficult to qualitatively compare because of high background (Fig. 6). Ratios of control to experimental mean gray area varied within a range of 0.85 to 1.15, indicating minimal differences between control and experimental bands (Fig. 5). Furthermore, one-way ANOVA tests (Fig. 1-4) show no statistically significant differences between control and experimental for MMP2 ($F(1, 4) = 0.865$, $p = 0.405$), MMP9 ($F(1, 4) = 3.184$, $p = 0.149$), ADAM12 ($F(1, 4) = 0.0373$, $p = 0.856$), or ADAM17 ($F(1, 4) = 3.348$, $p = 0.141$).

Discussion

This experiment was able to successfully collect and quantify protein samples for analysis. However, there was difficulty in successfully replicating Western blotting techniques consistently. Often signals from the blotted membrane were non-existent or extremely weak due to the use of faulty Western kits or a lack of protein on the membrane because of improper transfer. After a number of methodological revisions, including getting a better Western blot kit and adding Ponceau S as a positive indicator, more consistent results began to appear. However, even these results are difficult to interpret because of high background, either during film exposure or because of the kit / methodology itself. Furthermore, the antibodies used in this experiment failed to be as selective because of their polyclonal nature. In every film image, full wells are seen as opposed to the individual bands one would expect from a monoclonal antibody.

Because of the cryptic and unclear nature of the data collected, the results seem to be inconclusive. The lack of specificity of the antibodies used in combination with poor imaging techniques and high background calls into question the viability of the data. Regardless, the data itself suggests that there is minimal difference between treated and untreated cells. Only minor

variation between groups in the C/E ratios as well as the lack of significant change across all proteins suggest that the compound 3,4-dimethoxybenzaldehyde does not have strong anti-cancer properties. However, it is unclear whether the lack of difference is due to poor techniques or because there truly is no difference.

If this research were to be continued, future work should consider a longer exposure time to the chemotherapeutic agent to exaggerate potential differences between control and experimental groups. Furthermore, using an ELISA assay before Western blotting may provide a less technically complicated assessment of immediate differences before continuing to more difficult blotting procedures. Once Western blotting is pursued, careful selection of a quality Western blot kit including monoclonal antibodies may help reduce background and produce more specific results. Furthermore, better film-exposure techniques may help reduce dark bands from developing along the edges of the film during exposure, obscuring data.

Overall, this research provided no definitive results concerning the mechanism of 3,4-dimethoxybenzaldehyde on the molecular level; however, this research has produced viable protein samples from U87MG glioblastoma cells treated with other novel anti-cancer compounds which could also be analyzed in future work to assess their anti-cancer capability.

Acknowledgements

Great thanks to Priscilla Kyi, Tendai Hunyenyiwa, Joseph L. Mayor, and Dr. Desmond Murray for the basis of this research project. Thanks to Dr. Peter Lyons for lending equipment to complete the Western blots. Large thanks to Dr. Denise Smith for mentoring me and this project with all its challenges, even after retiring early due to COVID19. Thanks to the Department of Biology at Andrews University for their fiscal and material support. And finally, much thanks to

my friends, family, and loved ones for continuing to encourage and inspire me even in the most difficult times. Thank you.

Literature Cited

Chen, X., Chen, L., Chen, J., Hu, W., Gao, H., Xie, B., . . . Wang, X. (2013). ADAM17 promotes U87 glioblastoma stem cell migration and invasion. Brain Res, 1538, 151-158. doi:10.1016/j.brainres.2013.02.025

Dinnes, J., Cave, C., Huang, S., & Milne, R. (2002). A rapid and systematic review of the effectiveness of temozolomide for the treatment of recurrent malignant glioma. British Journal of Cancer, 86, 501-505. doi:10.1038/sj/bjc/6600135

Hunyenyiwa, T. (2019). Investigating the anti-cancer properties of novel hybrid 1,4-dihydropyridine derivatives in U-87MG glioblastoma cell lines.

Kyi, P. (2019). Synthesis and screening of novel hybrid benzothiazoles. (MS Graduate Thesis). Andrews University, Berrien Springs, MI.

Mayor, J. L. (2019). Anticancer activity of fluorinated a-cyanostilbenes. (Honors Thesis). Andrews University, Berrien Springs, MI.

Woodward, D. E. (1996). A mathematical model of glioma growth: the effect of extent of surgical resection. Cell Proliferation, 29, 269-288.

Figures and Graphs

SUMMARY – SINGLE FACTOR ANOVA, MMP2

Groups	Count	Sum	Average	Variance
Control	3	91.125	30.375	2.858644
Experimental	3	94.883	31.62767	2.584108

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2.353761	1	2.353761	0.864916	0.405011	7.708647
Within Groups	10.8855	4	2.721376			
Total	13.23927	5				

Fig. 1 – One-way ANOVA results of MMP2 mean gray area comparing control and experimental means.

SUMMARY – SINGLE FACTOR ANOVA, MMP9

Groups	Count	Sum	Average	Variance
Control	3	92.175	30.725	0.839604
Experimental	3	99.442	33.14733	4.689334

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	8.801548	1	8.801548	3.183811	0.148932	7.708647
Within Groups	11.05788	4	2.764469			
Total	19.85942	5				

Fig. 2 – One-way ANOVA results of MMP9 mean gray area comparing control and experimental means.

SUMMARY- SINGLE FACTOR ANOVA, ADAM12

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	100.247	33.41567	2.481157
Experimental	3	99.445	33.14833	3.266532

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.107201	1	0.107201	0.037302	0.856261	7.708647
Within Groups	11.49538	4	2.873845			
Total	11.60258	5				

Fig. 3 – One-way ANOVA results of ADAM12 mean gray area comparing control and experimental means.

SUMMARY – SINGLE FACTOR ANOVA, ADAM 17

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	100.247	33.41567	2.481157
Experimental	3	99.445	33.14833	3.266532

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.107201	1	0.107201	0.037302	0.856261	7.708647
Within Groups	11.49538	4	2.873845			
Total	11.60258	5				

Fig. 4 – One-way ANOVA results of ADAM17 mean gray area comparing control and experimental means.

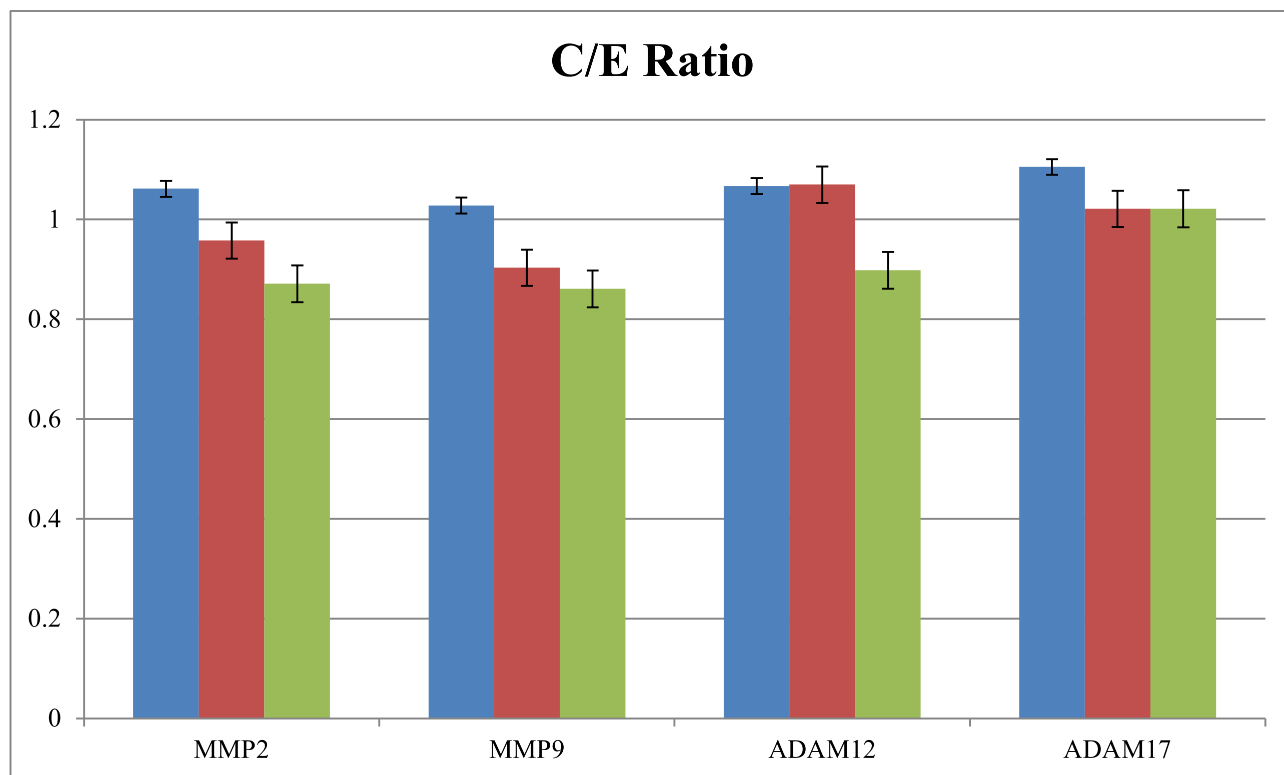

Fig. 5 – Ratio of Control to Experimental mean gray area graphed between each protein analyzed. Blue, red, and green bars represent ratio comparisons between wells 1 and 4, 2 and 5, and 3 and 6 respectively.

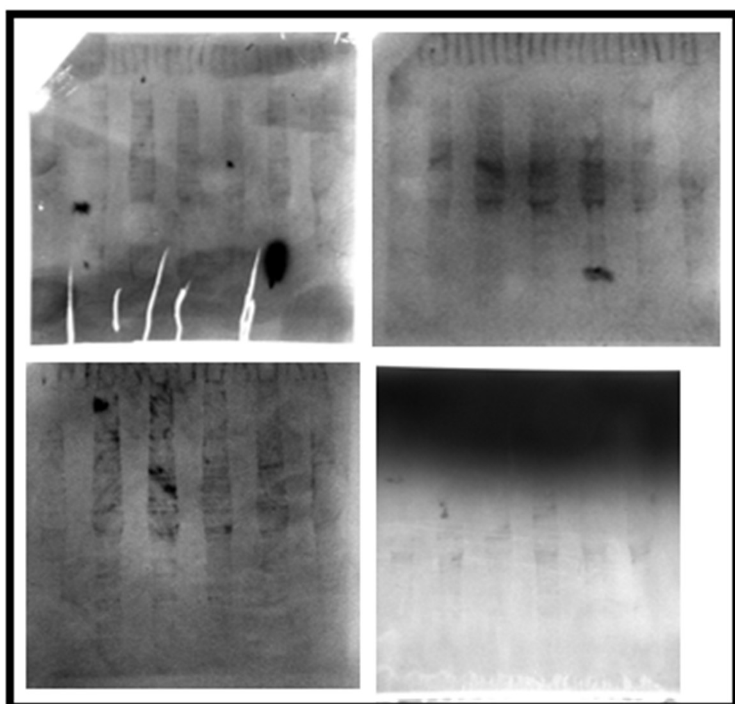

Fig. 6 – Images of Western blot films; brightness and contrast edited. Top left: MMP2; Top Right: MMP9; Bottom Left: ADAM12; Bottom Right: ADAM17