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Exploring the "Cascade Effect" of AEBSF on Reduced Glioblastoma Proliferation

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ABSTRACT: Glioblastomas (GBMs) are one of the deadliest cancers, and yet still lack treatment. In this study, serine protease inhibitor AEBSF is shown to decrease GBM viability and proliferation through a series of intertwined steps. By decreasing the expression of ATP6V1A--the first step of the "cascade effect"--AEBSF creates an unfavorable environment for protease activity. Such "unfavorability" is proven by a decline in expression of proteases MMP9 and ELANE upon GBM treatment with AEBSF. Increasingly higher concentrations of AEBSF are shown to decrease the percentage of cell viability, proliferation, and metastasis. This is explained through biological process enrichment analyses, which indicate that MMP9 and ELANE play a role in the disassembly of the extracellular protein network. This disassembly of the extracellular environment enables tumorigenic spread and viability. The results of the viability and proliferation assays, in combination with those of the indirect ELISAs, enrichment analyses, and scratch assays, underscore an intricate and profound sequence of events that constitute the "cascade effect".

INTRODUCTION

Glioblastomas (GBM) are grade IV gliomas and are the most lethal primary malignancy of the central nervous system (CNS). Despite comprising 54% of all gliomas and 16% of all primary brain tumors, GBM lacks a cure and only has a handful of treatments [1]. The lack of curative treatment for GBM can be attributed to several factors, some of which being high GBM resistance rates, rapid angiogenesis, and vast spread [10].

Studies have demonstrated that, unlike non-tumorigenic cells – which have extracellular pH (pHe) of approximately 7.4 – GBM have acidic pHe ranging between 6.7-7.1 [3]. This acidic extracellular pH has recently been demonstrated to activate proteases (such as serine proteases, plasminogen activators, and metalloproteinases). These proteases degrade the GBM extracellular environment, explaining why they have been indicated to be involved in GBM growth, invasion, angiogenesis and metastasis [2, 4]. Given the importance of pH in GBM progression, this study aimed to focus on pH regulation as a means of GBM treatment.

A major regulator of physiological pH is the vacuolar (H+)-ATPase (V-ATPase). V-ATPases are ubiquitously expressed and can be found in lysosomes,

endosomes, secretory vesicles, clathrin-coated vesicles, and the plasma membrane [4]. Consisting of 13 subunits in mammalian cells, the primary function of these proton pumps is to regulate both intracellular and extracellular acidification [5]. V-ATPases comprise two domains: the V0 and V1 domains. The peripheral V1 domain consists of eight subunits (A–H), all of which assist in ATP hydrolysis. The membrane-embedded V0 domain--consisting of subunits a, c, c″, d, and e--is responsible for proton translocation [6]. In this study, subunit A of the V1 domain (ATP6V1A) is closely examined due to its cytoplasmic location and ubiquitous nature across the body [14, 15].

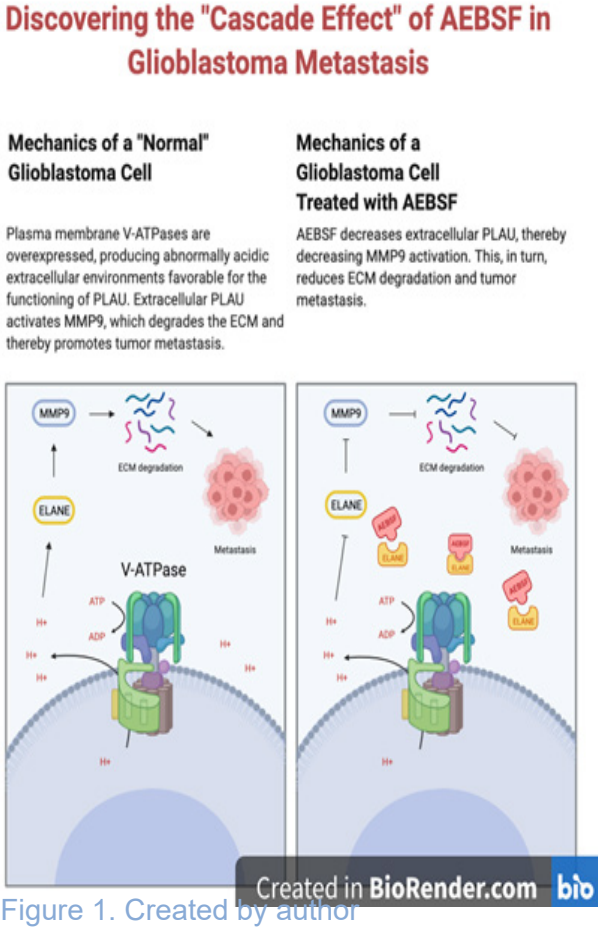


Figure 1. Created by author

This study explores the sequence of events in the “cascade effect” perpetuated by 4-benzenesulfonyl fluoride hydrochloride (AEBSF). AEBSF, also known as AEBSF hydrochloride, is an irreversible serine protease inhibitor that has previously been indicated to inactivate serine proteases such as chymotrypsin and proteinase K [11]. AEBSF has been shown in literature to have a potential regulatory effect on the secretion of antitumor effector molecules in leukemia [12]. AEBSF has also been shown to reduces dengue virus infection via decreased cholesterol synthesis [13].

In this study, AEBSF was studied under the context of GB, proliferation and metastasis. The question sought to be answered is, “What are the chronological events that occur in the reduction of GBM metastasis perpetuated by AEBSF?”

The findings in this study may: 1) provide more insight into the mechanics of GBM metastasis, 2) highlight the role of serine proteases in GBM progression, and 3) provide greater insight into such proteases’ mechanisms of action, thereby clarifying the link between ECM disassembly and GBM metastasis.

METHODS

Cell Culture

T98G human glioblastoma cells were obtained from the American Type Culture Collection (CRL-1690; ATCC, Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 1% 5000 U/mL Penicillin-Streptomycin (ThermoFisher, Waltham, MA, USA) and 10% fetal bovine serum (FBS) (Gibco, Waltham, MA, USA). Cells were incubated at 37 °C with 5% CO2 and 100% humidity. Cell culture images were taken with a Nikon Eclipse TS100 light microscope at 100x magnification.

Reagent preparation

AEBSF (solubility 50mg/mL H2O, molar mass: 239.69 g/mol) was dissolved in H2O.

Treatment of cells with AEBSF

Cells were seeded at ~10^6 cells in T25 flasks for 24 hours (incubated at 37°C with 5% CO2 and 100% humidity). Cells were subsequently treated with the appropriate AEBSF concentrations--125μM, 500μM, or 2000μM--for 48 hours.

Preparation of lysates for enzyme-linked immunosorbent assay (ELISA)

Phosphate buffered saline (PBS) (ThermoFisher, Waltham, MA, USA), 1X cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA), and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) were used to lyse 10^6 cells/mL from each treatment group. Cells were incubated at 37°C with 5% CO2 and 100% humidity with trypsin for 3 minutes until detachment. Cells were subsequently neutralized with DMEM and transferred to 15mL centrifuge tubes. Tubes were gently inverted and cells were counted using ViCell XR (Beckman Coulter, Indianapolis, IN, USA). Tubes were subsequently centrifuged at 1000 rpm for 7 minutes at room temperature, after which the pellets were poured off and the pellet was broken. Chilled PBS was added to the tubes, which were subsequently re-centrifuged at 1000 rpm for 7 minutes at 25°C. The residual supernatant was poured out and the pellet was broken up. The appropriate volume of lysis buffer, calculated using the following equation, was added to each tube and incubated on ice for 10 minutes: [Viable cell count (cells/mL) × volume of cell suspension prior to first round of centrifugation (mL)] ÷ 106 cells/mL

Lysates from the tubes were subsequently transferred to chilled Eppendorf tubes. The tubes were micro-centrifuged at 13000 rpm for 15 minutes at 4°C. The supernatants were stored at -80°C.

Indirect ELISA

100μL/well of lysates were transferred at 4 replicates per treatment group to a 96-well plate. Lysates were left to incubate for 24 hours at 4°C and then removed by blotting. 200μL/well of blocking buffer (ThermoFisher, Waltham, MA, USA) with 1% bovine serum albumin (BSA) (Seracare, Milford, MA, USA) was added and left to incubate for 10 minutes at 25°C.

Cell proliferation assay

Cells were seeded at 62,500 cells/well 24 hours prior to treatment in a 6-well plate. Cells were subsequently treated for 48 hours, supernatants were collected, and cell proliferation was analyzed using a synergy machine.

Trypan Blue Exclusion Viability Assay

Cells were seeded at ~40,000 cells/flask 24 hours prior to treatment. Cells were subsequently treated for 48 hours, supernatants were collected, and cells were trypsinized and counted using ViCell XR (Beckman Coulter, Indianapolis, IN, USA).

Reverse Docking

Reverse docking tool ChemMapper was used to predict the protein targets of AEBSF. The SMILES (Simplified Molecular Input Line Entry System) notation of AEBSF, obtained from PubChem, was inputted into ChemMapper. Subsequently, the “Target Navigator” service was selected and the 3D similarity method SHAFT was chosen. The default similarity threshold of 1.2 was selected, and the Bioactivity Database ChEMBL was then chosen [7-9].

Data Analysis

Statistical and computational biology analyses were performed using Microsoft Excel. An unpaired, two-tailed T-test was performed in Excel, and a p-value < 0.05 was considered statistically significant. Means and standard deviations are taken for all data and compared as % of control.

RESULTS

AEBSF reduces T98G proliferation

AEBSF was first demonstrated to reduce proliferation in T98G cells. As hypothesized, AEBSF reduces proliferation in a statistically significant manner, demonstrating the efficacy of this treatment (Fig. 1A).

AEBSF decreases T98G viability

AEBSF was also demonstrated to reduce viability in T98G cells. Specifically, higher concentrations of AEBSF were shown to decrease the number of viable cells and increase the number of dead cells (Fig. 2). As hypothesized, AEBSF reduces viability in a statistically significant manner, once again demonstrating the efficacy of this treatment (Fig. 2).

Plasma membrane V-ATPase subunit A (ATP6V1A) protein expression is enhanced in glioblastomas and decreased upon AEBSF treatment

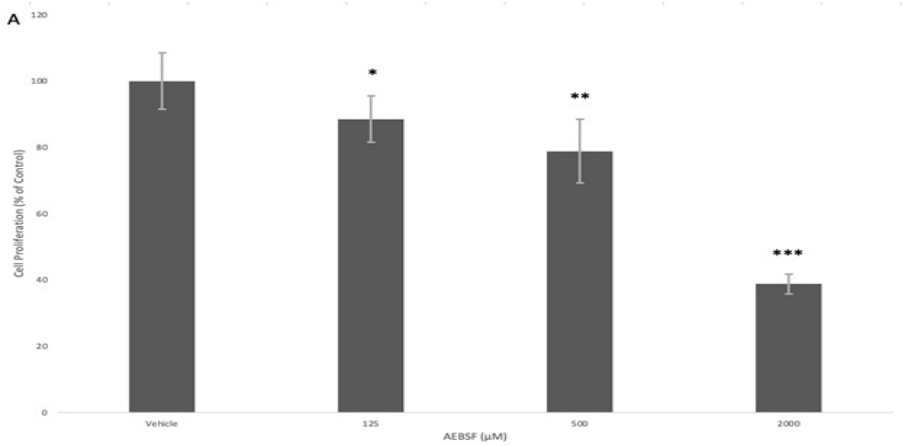
V-ATPase subunit A (ATP6V1A) plays a vital role in the primary function of the overall proton pump--acidification of the extracellular environment. Through an indirect ELISA, it is demonstrated that ATP6V1A protein expression decreases with increasing AEBSF concentration (Fig. 3).

AEBSF targets serine proteases and metalloproteinases

AEBSF is a well known serine protease inhibitor. Although it is known that AEBSF inhibits its serine proteases such as trypsin and chymotrypsin, shape-screening tool ChemMapper was utilized to predict its protein targets with the highest confidence scores. ChemMapper is fundamentally rooted in the “chemical similarity principle”, which states that structurally similar compounds have similar bioactivies [7]. Thus, it can be said that a higher similarity score between the query ligand (AEBSF in this study) and the database ligands indicates a higher likelihood that a given protein target of the database ligand is also a target of AEBSF, the query ligand. Thus, as previously discussed, a similarity threshold of 1.2 (on a scale of 2.0) was set in order to filter out protein targets of database ligands with low-similarity scores. Using ChemMapper, it was predicted that AEBSF targets 6 serine proteases (Fig. 4A) and 2 metalloproteinases (Fig. 4B).

Serine proteases and metalloproteinases targeted by AEBSF primarily play a role in extracellular matrix disassembly and cell migration in glioblastomas

The eight protein targets predicted by ChemMap were plugged into “Search Tool for the Retrieval of Interacting Genes/Proteins” (STRING) to carry out an enrichment analysis of the biological processes that they are involved in. The top five biological processes, along with their false discovery rates (FDRs) and strength values, were analyzed (Fig. 5).



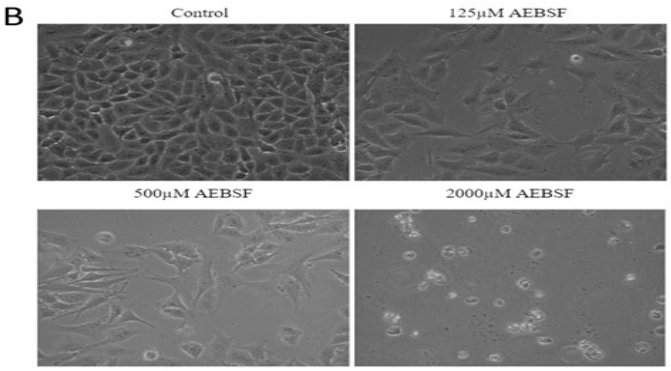
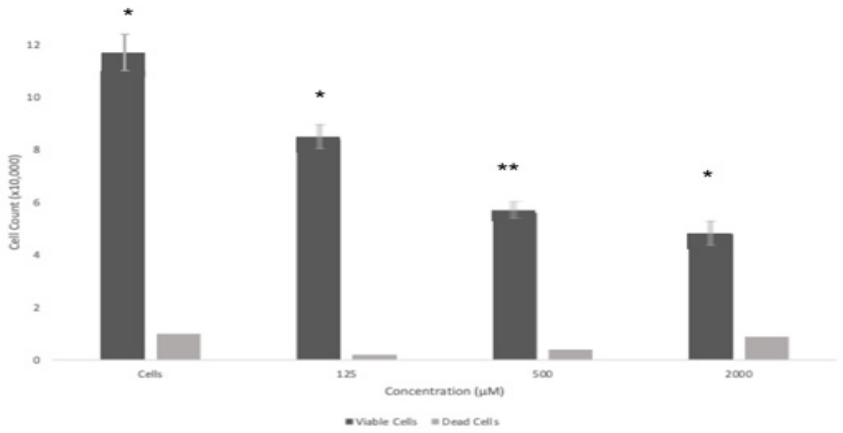


Figure 1. 48-hour treatment with 4-benzenesulfonyl fluoride hydrochloride (AEBSF) reduces proliferation in T98G cells. A: Bars are means ± STDEV (n=4). *Significantly different by t-test at $p<0.05$ comparing data to that at vehicle control (cells and H2O). **Significantly different by t-test at $p<0.01$ comparing data to that at vehicle control. ***Significantly different by t-test at $p<0.001$ comparing data to that at vehicle control. B: Light microscopy images of T98G treated with 125μM, 500μM, or 2000μM AEBSF at 100x.

Figure 2. 48-hour treatment with 4-benzenesulfonyl fluoride hydrochloride (AEBSF) reduces viability in T98G cells.



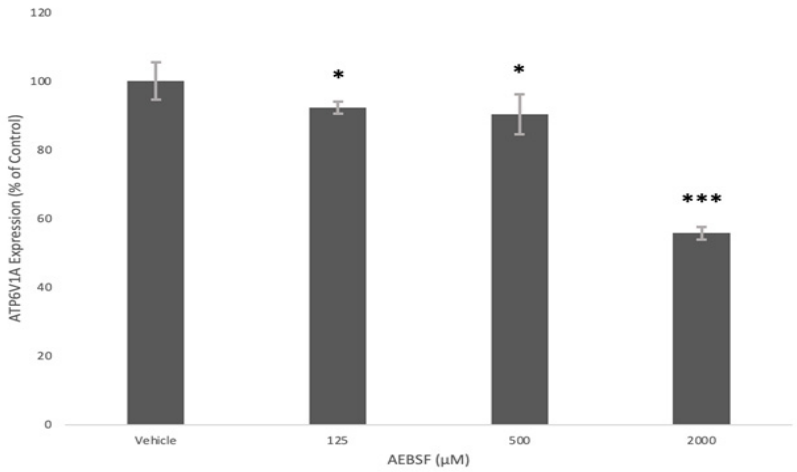


Figure 3. ATP6V1A ELISA on T98G cells treated with 4-benzenesulfonyl fluoride hydrochloride (AEBSF) for 48 hours. 48-hour treatment with AEBSF reduces ATP-6V1A protein expression in T98G.

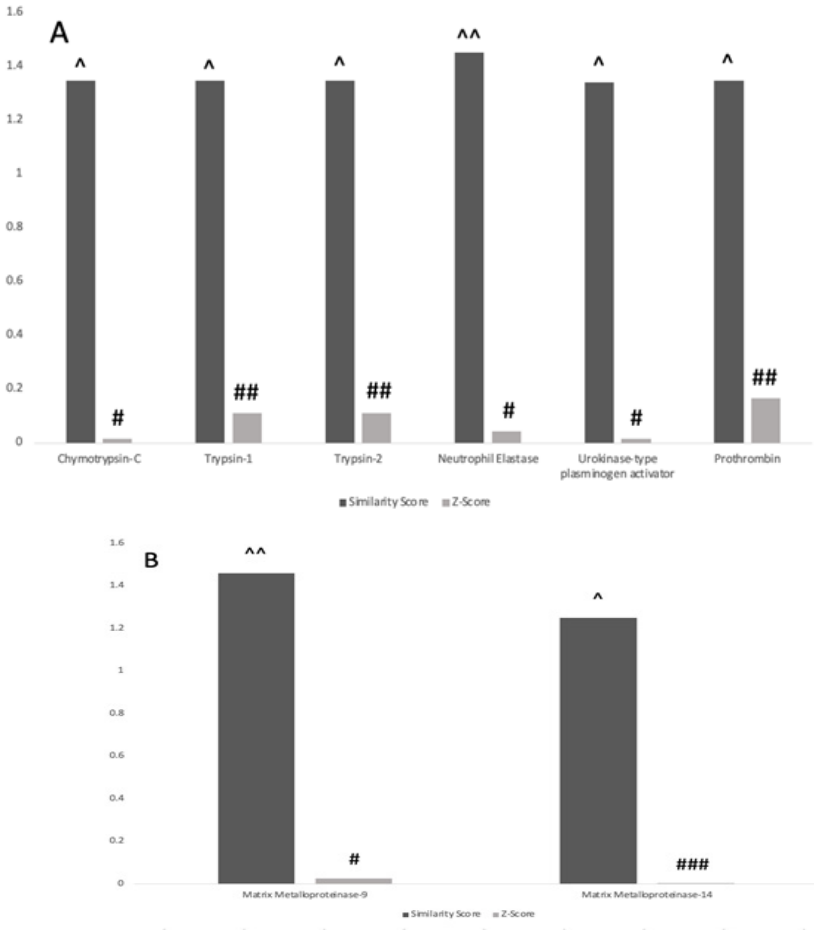


Figure 4. Reverse docking analysis of 4-benzenesulfonyl fluoride hydrochloride (AEBSF) using ChemMapper. Z-scores and similarity scores of each predicted target are displayed. The similarity score ranges from [0, 2]. The closer the score is to 2.0, the higher the potential of pharmacological association there is between AEBSF and the database ligands of the outputted protein targets ^Similarity score ranges between 1.2-1.4 ^^Similarity score ranges between 1.4-1.6 #Z-score<0.5 ##Z-score<0.1 ###Z-score<0.01 A: AEBSF targets serine proteases Chymotrypsin-C, Trypsin-1, Trypsin-2, Neutrophil Elastase, Urokinase-type plasminogen activator, and Prothrombin. B: AEBSF targets matrix metalloproteinase-9 (MMP9) and matrix metalloproteinase-14 (MMP14).

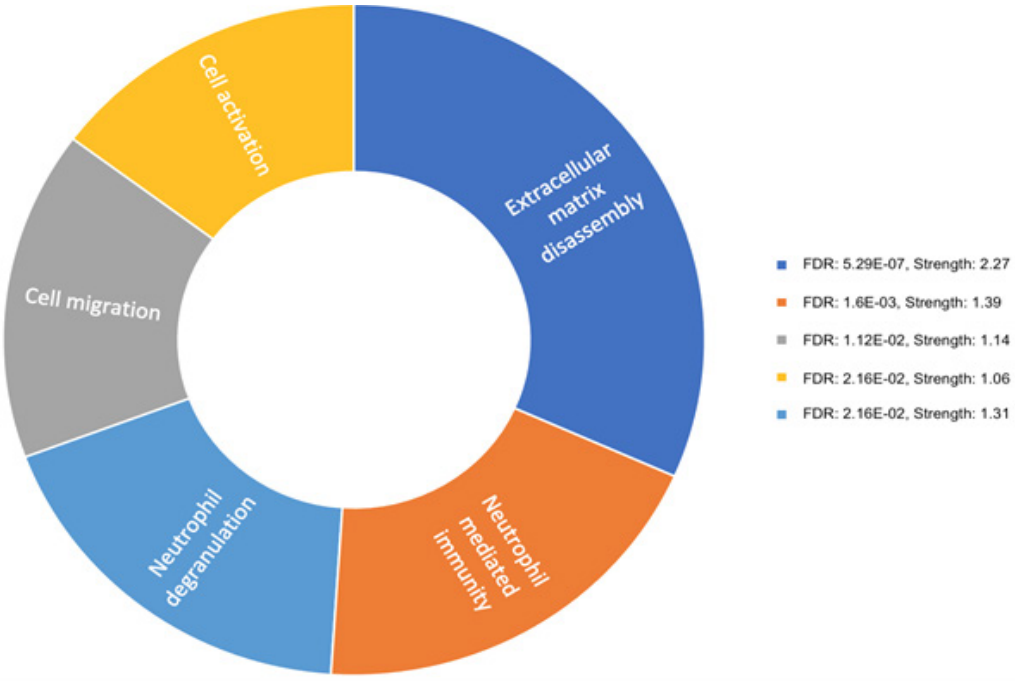


Figure 5. Biological process enrichment analysis of AEBSF serine protease (Chymotrypsin-C, Trypsin-1, Trypsin-2, Neutrophil Elastase, Urokinase-type plasminogen activator, Prothrombin) and metalloproteinase (MMP9, MMP14) targets. Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) was utilized for this analysis. False discovery rates (FDRs) and strength values are displayed; p = 7.61e-1. Five biological processes with the highest strength values are displayed.

AEBSF downregulates ELANE and MMP9 protein expression

Following the reverse docking and biological processes enrichment analyses, neutrophil elastase (ELANE) and matrix metalloproteinase-9 (MMP9) expression were selected to be analyzed. These two proteins were selected to be studied, as in the reverse docking analysis, ELANE and MMP9 were demonstrated to have the highest similarity scores, which was previously established to be a good indicator of the likelihood that a given protein target of the database ligand is also a target of AEBSF, the query ligand (Fig. 4A, 4B). Hence, serine protease ELANE and metalloproteinase MMP9 expression were analyzed through indirect ELISAs. Both ELANE and MMP9 protein expression were demonstrated to decrease with increasing AEBSF concentrations (Fig. 6A, 6B).

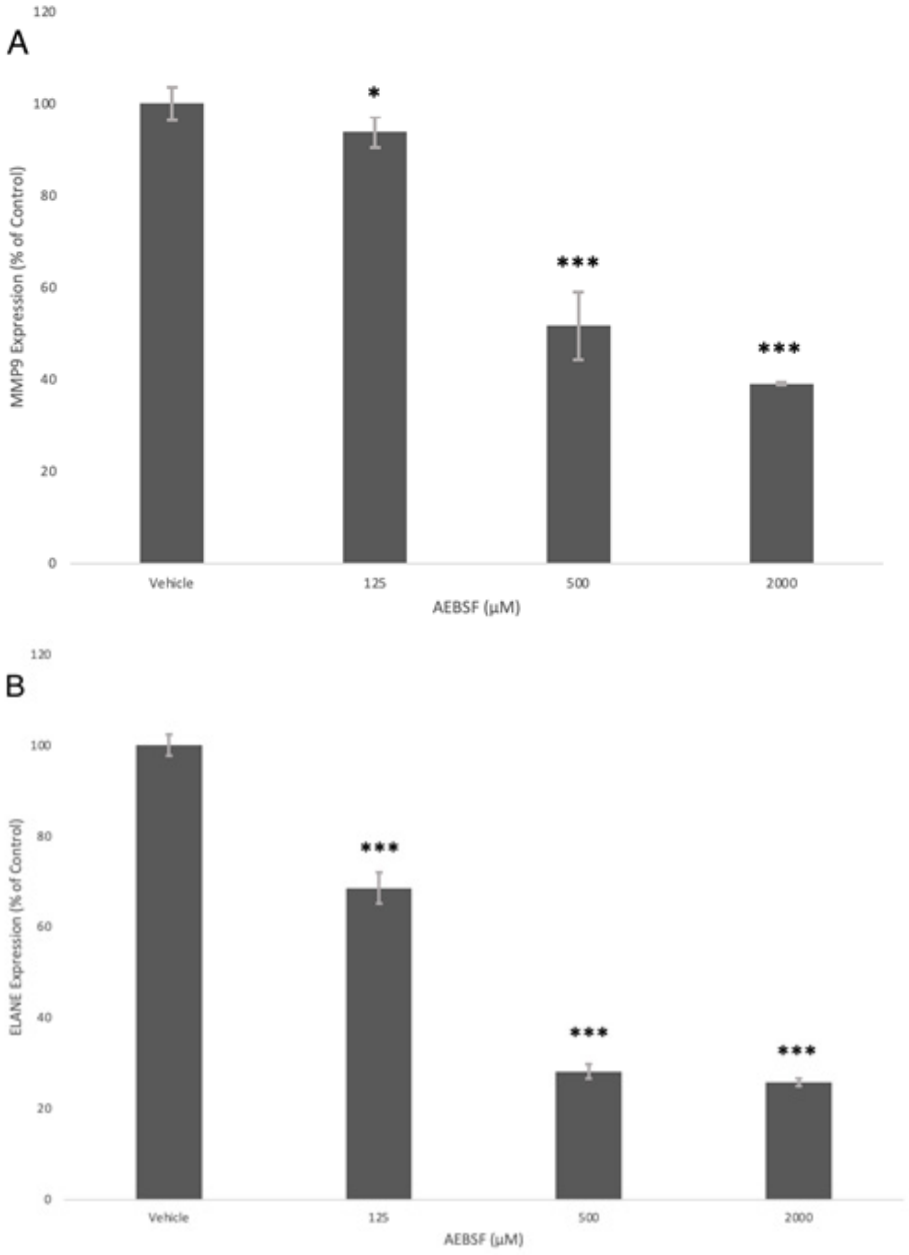


Figure 6. MMP9 and ELANE ELISAs on T98G cells treated with 4-benzenesulfonyl fluoride hydrochloride (AEBSF). Bars are means ± STDEV (n=4). A: MMP9 ELISA on T98G cells treated with AEBSF for 48 hours. 48-hour treatment with AEBSF reduces MMP9 protein expression in T98G. B: ELANE ELISA on T98G cells treated with AEBSF for 48 hours. 48-hour treatment with AEBSF reduces ELANE protein expression in T98G.

DISCUSSION

This project sought to explore the sequence of events of the "cascade effect" perpetuated by AEBSF in GBM. Through an indirect ELISA, it was demonstrated that serine protease inhibitor AEBSF decreases ATP6V1A expression, thereby activating ELANE and MMP9 proteases. This, in turn, was indicated to stimulate ECM degradation and overall GBM metastasis and migration (Fig. 7). It was demonstrated that higher AEBSF concentrations had a greater effect on the studied parameters. However, the optimal AEBSF dosage remains to be determined and will be studied in the future.

In the future, the mechanisms of ELANE and MMP9-mediated ECM disassembly will be studied. This may give greater insight into these proteases' mechanisms of action, and possibly further clarify the link between ECM disassembly and GBM metastasis.

The findings in this study may provide more insight into the mechanics of GBM metastasis, highlighting the role of serine proteases and tumor metastasis.

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Author Contributions

Mehwish Mirza performed the data analysis and produced the figures and manuscript. Dr. German Sabio provided technical assistance with analysis and pipelines. Dr. German Sabio and Donna Leonardi added project direction and provided guidance. Elisa Huang and Christine Chow provided expert feedback for the project.

Competing Interests

The authors declare no competing financial and non-financial interests.

REFERENCES

[1] Tamimi AF, Juweid M. Epidemiology and Outcome of Glioblastoma. In: De Vleeschouwer S, editor. Glioblastoma [Internet]. Brisbane (AU): Codon Publications; 2017 Sep 27. Chapter 8. doi: 10.15586/codon.glioblastoma.2017.ch8. https://www.ncbi.nlm.nih.gov/books/NBK470003/

[2] Kato, Y., Ozawa, S., Miyamoto, C., Maehata, Y., Suzuki, A., Maeda, T., & Baba, Y. (2013). Acidic extracellular microenvironment and cancer. Cancer cell international, 13(1), 89. doi: 10.1186/1475-2867-13-89. https://cancerci.biomedcentral.com/articles/10.1186/1475-2867-13-89

[3] Ward C, Meehan J, Gray ME, Murray AF, Argyle DJ, Kunkler IH, et al. The impact of tumour pH on cancer progression: strategies for clinical intervention. Explor Target Antitumor Ther. 2020;1:71-100. doi: 10.37349/etat.2020.00005. https://www.explorationpub.com/Journals/etat/Article/10025

[4] Park, K. C., Dharmasivam, M., & Richardson, D. R. (2020). The Role of Extracellular Proteases in Tumor Progression and the Development of Innovative Metal Ion Chelators that Inhibit their Activity. International journal of molecular sciences, 21(18), 6805. doi: 10.3390/ijms21186805. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7555822/

[5] L. Shannon Holliday, "Vacuolar H+-ATPase: An Essential Multitasking Enzyme in Physiology and Pathophysiology", New Journal of Science, vol. 2014, Article ID 675430, 21 pages, 2014. doi: 10.1155/2014/675430. https://down-

[6] Cotter, K., Capecci, J., Sennoune, S., Huss, M., Maier, M., Martinez-Zaguilan, R., & Forgac, M. (2015). Activity of plasma membrane V-ATPases is critical for the invasion of MDA-MB231 breast cancer cells. The Journal of biological chemistry, 290(6), 3680–3692. doi: 10.1074/jbc.M114.611210. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4319033/

[7] Jiayu Gong, Chaoqian Cai, Xiaofeng Liu, Xin Ku, Hualiang Jiang, Daqi Gao and Honglin Li. ChemMapper: a Versatile Web Server for Exploring Pharmacology and Chemical Structure Association Based on Molecular 3D Similarity Method. Bioinformatics. 2013, 29 (14), 1827-1829. https://pubmed.ncbi.nlm.nih.gov/23712658/

[8] Xiaofeng Liu, Hualiang Jiang, Honglin Li. SHAFTS: A Hybrid Approach for 3D Molecular Similarity Calculation. 1. Method and Assessment of Virtual Screening. J. Chem. Inf. Model. 2011, 51 (9), 2372-2385. https://pubs.acs.org/doi/abs/10.1021/ci200060s

[9] Weiqiang Lu, Xiaofeng Liu, Xianwen Cao, Mengzhu Xue, Kangdong Liu, Zhenjiang Zhao, Xu Shen, Hualiang Jiang, Yufang Xu, Jin Huang and Honglin Li. SHAFTS: A Hybrid Approach for 3D Molecular Similarity Calculation. 2. Prospective Case Study in the Discovery of Diverse p90 Ribosomal S6 Protein Kinase 2 Inhibitors to Suppress Cell Migration. J. Med. Chem. 2011, 54, 3564-3574. https://pubs.acs.org/doi/10.1021/jm200139j

[10] Calinescu AA, Kauss MC, Sultan Z, Al-Holou WN, O'Shea SK. Stem cells for the treatment of glioblastoma: a 20-year perspective. CNS Oncol. 2021 Jun 1;10(2):CNS73. doi: 10.2217/cns-2020-0026. Epub 2021 May 19. PMID: 34006134; PMCID: PMC8162173. https://pubmed.ncbi.nlm.nih.gov/34006134/

[11] BioChemica. (2022). AEBSF hydrochloride BioChemica. PanReac AppliChem ITW Reagents. Retrieved from https://www.itwreagents.com/italy/en/product/aebsf+hydrochloride+biochemica/A1421

[12] Nakabo Y, Pabst MJ. Lysis of leukemic cells by human macrophages: inhibition by 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), a serine protease inhibitor. J Leukoc Biol. 1996 Sep;60(3):328-36. doi: 10.1002/jlb.60.3.328. PMID: 8830789. https://pubmed.ncbi.nlm.nih.gov/8830789/

[13] Sreelatha L, Malakar S, Songprakhon P, Morchang A, Srisawat C, Noisakran S, Yenchitosomanus PT, Limjindaporn T. Serine protease inhibitor AEBSF reduces dengue virus infection via decreased cholesterol synthesis. Virus Res. 2019 Oct 2;271:197672. doi: 10.1016/j.virusres.2019.197672. Epub 2019 Aug 3. PMID: 31386864. https://pubmed.ncbi.nlm.nih.gov/31386864/

[14] François Jouret, Céline Auzanneau, Huguette Debaix, Ge-Hong Sun Wada, Chrystel Pretto, Etienne Marbaix, Fiona E. Karet, Pierre J. Courtoy, Olivier Devuyst
JASN Nov 2005, 16 (11) 3235-3246; DOI: 10.1681/ASN.2004110935. https://jasn.asnjournals.org/content/16/11/3235#:~:text=The%20vacuolar%20H%2B%2DATPase%20(,of%20intercalated%20cells%20(IC).

[15] Sarkar, J., Wen, X., Simanian, E. J., & Paine, M. L. (2016). V-type ATPase proton pump expression during enamel formation. Matrix biology: journal of the International Society for Matrix Biology, 52-54, 234–245. https://doi.org/10.1016/j.matbio.2015.11.004