

Investigating Anticancer Properties of Thallium Ions: An Innovative Project with GBM, HTB-5, and Canine TCC Cells

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Abstract Heavy metals, such as thallium, are both toxic to humans and hazardous to society. The focus of this undergraduate project was to continue a unique research experience that was tailored for nursing students and to assess its effectiveness while exploring new directions in undergraduate medicinal chemistry. Two honors nursing students, who had only two semesters of chemistry laboratory experience, investigated the antiproliferative effects of thallium salts on glioblastoma multiforme cells (GBM), human bladder cancer cells (HTB-5), and canine bladder cancer cells (canine TCC). Critical thinking in chemistry and its application to real-life situations were key learning objectives in investigating the toxic effects of thallium ions on biological cells. Thallium and potassium possess very similar atomic radii, resulting in the ability of Tl^+ to easily enter the cell via the Na^+/K^+ pump. Cytotoxicity was monitored utilizing a Sulforhodamine B (SRB) assay. Results indicated growth inhibition for all cell lines was observed as the concentration was increased to $25 \mu M TlNO_3$. An in-depth study revealed that cell death was found for GBM ($100 \mu M TlNO_3$) and HTB-5 ($150 \mu M TlNO_3$). Remarkably, the Canine TCC cells tolerated $800 \mu M TlNO_3$. The procedure and techniques were designed to limit exposure to toxins and enhance the education of Walsh University's nursing majors in the honors program.

Keywords: GBM cells, thallium, cancer, medicinal chemistry, HTB-5 cells, Canine TCC cells, undergraduate research

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1. Introduction

Walsh University is a mid-size liberal arts institution located in North Canton, Ohio. Student success, including undergraduate research, is one of our top priorities that aligns with Walsh's mission. Our focus is to provide learning experiences so our students can become responsible scientists and ethical researchers. Research in chemistry can be a challenge to complete for those students having program requirements including both clinical rotations and night classes. One example of this situation includes students in the Honors program who are seeking a bachelor's degree in nursing (BSN). In order to solve this problem, it was decided that this project should be centered on creating safe research techniques and protocols for undergraduate nursing majors who had only two semesters of chemistry laboratory experience. One challenge that we hoped to address was to implement strategies that limit exposure to hazardous substances and to design a protocol that would fit into nursing students' schedules. Furthermore, this learning experience should not negatively impact student achievement of the learning

outcomes or the long-term productivity of the project. A "hidden gem" we discovered was that mentoring, by the senior student, allowed the junior student to gain momentum on performing the lab techniques and collecting data for conference presentation. The main goal was to enhance our BSN students' training by leading them through undergraduate research, CHEM 411-412. Additionally, the vision for this undergraduate project was to create a unique research experience that was modified for BSN students and to assess its effectiveness while exploring new directions in undergraduate medicinal chemistry. Some key measurable learning outcomes were critical thinking in chemistry and its application to the clinical setting by investigating the toxic effects of thallium ions on biological cells. [1]

Heavy metals, such as thallium, are typically avoided in projects due to its toxicity to humans and its potential hazards for the environment and society. [2,3,4] Specifically, regarding environmental concerns, Zitko reported that thallium is marginally more poisonous to humans compared to mercury and thallium has a similar level of toxicity as copper for those studies including fish. [2] One overarching theme to explore is how thallium affects the citizens and the environment and how it might

be incorporated into society. Thallium has been found to be a toxin in both chemical and forensic sciences. [2,4] Additionally, arsenic has been studied in recent years and its possible application as a toxin to kill cancer cells and, therefore, designated as the “king of poisons”. [1,5,6] For more than twenty-five centuries, the Chinese have been using compounds containing this poisonous metal for medical interventions as well as exploring its reactivity. Arsenic trioxide, As_2O_3 , has been found to be effective for patients having acute promyelocytic leukemia (APL) with many studies including those in the age range of 18-70 years. [1,6] After extensive study with much success, it was concluded that this compound is a successful treatment for APL. Essentially, arsenic trioxide is considered a good option because it is a safe and secure option as well as a successful treatment consideration for those patients suffering with leukemia. [1,6] In addition, studies indicate its effectiveness for patients with other malignancies including myeloid neoplasms, but more investigation is needed due to the mixed results for myeloid neoplasms. [6,7]

Diving deeper into the mechanism of action, further work showed that anti-carcinogenic effects of Arsenic trioxide might be associated with the initiation of apoptosis [8]. More recently, it was suggested that analogous stimuli can prompt apoptosis, autophagy or both, perhaps due to the fact that apoptosis and autophagy have mutual effector proteins and upstream pathways. [9]. In another study, researchers concluded that apoptosis and autophagy may be linked or even controlled by the identical trigger in the anti-glioma mechanism of Arsenic trioxide. [10,11]

Our vision for this study was to investigate the antiproliferative effects of thallium salts on glioblastoma multiforme cells (GBM), human bladder cancer cells (HTB-5), and canine bladder cancer cells (canine TCC). Subsequently, this work provided a greater understanding to the sensitivity of each cell line and the comparison of the results of heavy metals upon other cell lines as studies previously. [1] The rationale for these cell lines is that we hoped to widen our range within the cancer studies and the GBM cells were the focal point for a senior nursing student and other two cell lines were the focus for the junior student. The investigations incorporating these cell lines, in turn, were the core component for the two Honors theses. Another hidden benefit of this work was that the senior served as a valuable mentor for the junior student. Essentially, this support gave rise to additional momentum that allowed the study to be a comprehensive study for such a limited time in their academic career at Walsh.

Currently, there is a wide range of cells studied in cancer research, but the mechanism is only partially understood. However, because thallium and potassium have very comparable atomic radii, we predict that this size similarity can result in the capability of Tl^+ to straightforwardly enter the cell via the Na^+/K^+ pump. Once inside the cell, Tl^+ binds to riboflavin and could potentially interfere with oxidative phosphorylation, causing disruption of the mitochondrial membrane potential. [12] This disruption is expected to cause mitochondrial membrane dysfunction and the release of Cytochrome C, an early initiating step of the intrinsic apoptotic pathway. Apoptosis, otherwise known as

programmed cell death, was a focus of this work in order to understand more about the sensitivity of these cells. [1] One main goal was to determine if thallium's toxicity could be utilized beyond its former use as a forensic poison and ultimately used to fight and destroy cancer cells. From this initiative, this could lead to further evaluations of the heavy metal's effectiveness in cell death and its capacity as a new cancer treatment option. [1]

Thallium-201 is categorized as one FDA-approved diagnostic metalloradiopharmaceutical for diagnostic imaging of the heart and thyroid gland, but this metal has traditionally been deemed a forensic poison. [1] [13,14,15,16] In recent years, thallium was confirmed as a concern and threat to public safety due to its simplicity of exposure including through the skin. [17,18,19] Thallium poisoning can cause a variety and wide range of symptoms including flu-like symptoms, hair loss, dystrophy of nails, tachycardia, low blood pressure, infection and inflammation of the digestive system, and simultaneous malfunction of many peripheral nerves in a victim's body. [20,21] It is important to consider thallium's risks to those in various stages of life, too. For example, during pregnancy, thallium is considered a significant threat to the unborn child because it can pass the placental barrier and it can be responsible for birth defects and/or fetal toxicity. [22] The most widely observed side effects upon exposure to thallium include hair loss, infection and inflammation of the digestive system, and simultaneous malfunction of many peripheral nerves and, therefore, have become the main indicators of thallium poisoning. [23] Researchers also found that thallium exposure can present itself through regular everyday activities such as through contaminated food, drink, soil, and air as it is linked to industrial accidents or occupational exposure throughout the world. [24,25,26,27,28]

This project was designed to help students gain new knowledge and apply concepts to the clinical setting, particularly the hazards, toxicity, and impact on biological cells. Lyczko and coworkers studied thallium's antimicrobial properties and reported that this metal has a wide range of properties including physicochemical, insecticidal, and fungicidal activity. [29] Consequently, this work is relevant and beneficial for student careers in healthcare. More specifically, this project gave BSN students studying in the Honors program, having only two semesters of chemical laboratory experience, the special opportunity to not only reinforce content from other courses, but to apply their skills to real-life applications in chemistry. [1] These Honors projects demonstrated the antiproliferative effects of thallium ions on cancer cells, *in vitro*.

2. Experimental

2.1. Cell Culture and Reagents

This work was conducted in the university's tissue culture research laboratory following the same protocol as previously reported. [1] Due to previous successes in student training, we followed a similar routine and the students received several weeks of training sessions regarding safety and how to complete manipulations

without compromising the sterile technique. As required under our protocol, the tissue culture hood was exposed to UV light for 15 min. prior to manipulations and further treatment with 70% ethanol ensured a sterile environment. Experimental procedures and manipulations were conducted under sterile conditions and cells were allowed to grow in the following media to optimize growth: Dulbecco's Modified Eagle's Medium (DMEM, cellgro/mediatech catalog #10-013-CV), 50 mL of 10% Fetal Bovine Serum (FBS), 5 mL of 1% PSF antibiotic/antimycotic (cellgro/mediatech), 10 mL of 50X GlutaGro (L-alanyl-L-glutamine), and 1 mL of 500X Plasmocin (InVivoGen). Cells were grown for several days in a humidified cell incubator held constant at an atmosphere of 5% CO₂ at 37°C. Ranin pipettes (L10, L20, L200) were used in this work. The cells were counted using a disposable hemocytometer (incyto, C-chip Neubauer improved). Cells were treated with thallium ions and the effects were monitored using SRB assays. Each assay took about three weeks to complete depending on the rate of cellular growth. Walsh University supported the ethics of this study to improve the advancement in the area of science as we upheld high standards for our students and their results. This study was completed without any limitations. [1]

2.2. SRB Assay and Project Design

Due to our previous success with growing healthy MCF-7, A-375, and HFF cell lines, we decided to follow a similar protocol. [1] Healthy cells, grown under sterile conditions, were counted using a hemocytometer, and transferred into a 96-well plate (1500 cells per well). Drug treatments were conducted for several days and the cytotoxicity was monitored via the Sulforhodamine B assay also known as SRB assay. There were several advantages for using the SRB assay including: 1) the reagents were safe and inexpensive, 2) the step-by-step protocol became ideal for nursing students who also were registered for hospital clinical courses that required long shifts, 3) the several start/stop points allowed for flexibility so work could be done between classes or exams, 4) SRB assays demonstrated nice visuals for the students to monitor progress (color changes), 5) our procedures were compatible with our current equipment at Walsh University. [1]

For safety, the undergraduate students did not conduct any of the drug treatments (Thallium nitrate) and, therefore, eliminated the potential exposure to toxic reagents. As an appropriate alternative, the drug treatments were completed by the research advisor having extensive experience with inorganic compounds and their toxicity. Furthermore, this project provided students with the unique opportunity to acquire knowledge in biological applications of inorganic compounds, tissue culture procedures, and sterile techniques. [1]

GBM cells are known as glioblastomas due to their origin from glioblastoma multiforme. This type of brain cancer has become a prevalent central nervous system malignancy, but it is very difficult to cure. [30,31] Therefore, there is an increased need for new anticancer therapies. Additionally, the discovery of the GBM cell line has been critical to the advancement in cancer research.

[30,31] Figure 1 shows a photograph of the GBM cells used in this work. All photographs associated with this work were taken in the laboratory at Walsh.

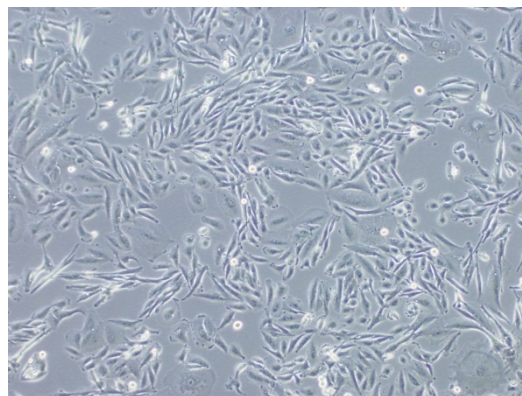


Figure 1. GBM cells

HTB-5 is a cell line having epithelial morphology that can be studied through applications in cancer research. These adherent cells, as shown in Figure 2, are urinary bladder cancer cells that originated from a 67 year-old female in 1974 who had grade IV transitional cell carcinoma. [32,33]

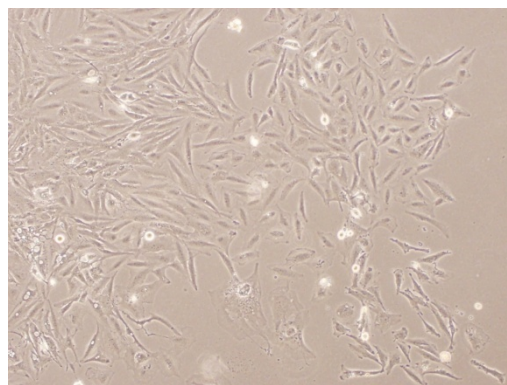


Figure 2. HTB-5 cells

Canine TCC cells are adherent cells that are studied as a unique model for some human cancers with BRAF signaling activation in studies focusing on comparative oncology. [34] Because dogs share lifestyles, routines, and similar environments with humans, naturally occurring cancers in dogs shine new light into cancer development and disease progression in humans. [34,35] A photograph of Canine TCC cells used in this work is shown in Figure 3.

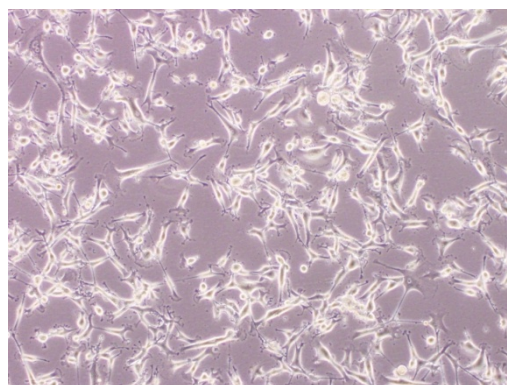


Figure 3. Canine TCC cells

3. Results

3.1. SRB Assay Utilizing Thallium Nitrate at Lower Concentrations

The initial study involved treating the cells with thallium(I) ions using 1-100 μM TlNO_3 . The cells were treated with the compound until the cells in each well were 80% confluent. Significant growth inhibition was apparent at 100 μM TlNO_3 having the following percentages: 97% (GBM cells), 83% (HTB-5 cells), and 83% (Canine TCC cells). The antiproliferative effect of TlNO_3 on these cell lines is illustrated in Figure 4-6.

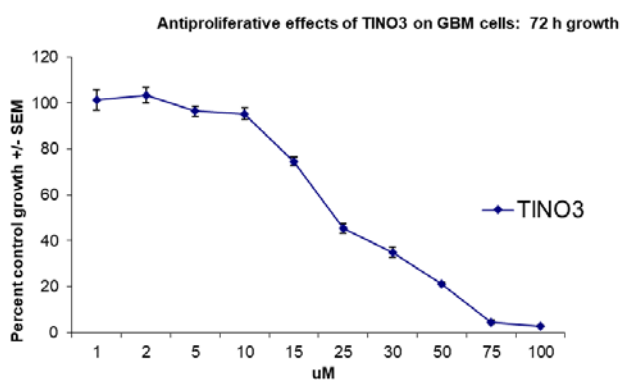


Figure 4. GBM cells & Thallium nitrate

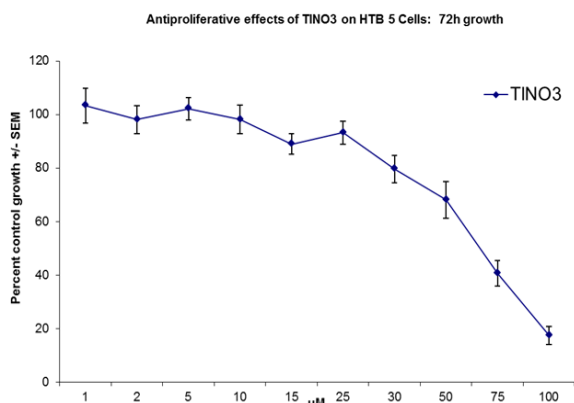


Figure 5. HTB-5 Cells & Thallium nitrate

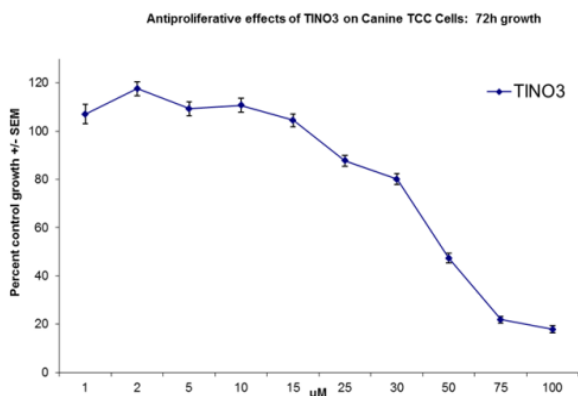


Figure 6. Canine TCC Cells & Thallium nitrate

3.2. SRB Assay Utilizing Thallium Nitrate at Higher Concentrations

Due to the nearly complete cell death for GBM cells, only the other two cell lines were studied at higher concentrations. The second study involved treatments with 150-800 μM TlNO_3 . The cells were treated with this compound until the cells in each well were 80% confluent. Complete cell death was apparent at the following concentration: 150 μM TlNO_3 (HTB-5 cells), but 91% growth inhibition was observed for Canine TCC cells at 800 μM TlNO_3 . The antiproliferative effect of TlNO_3 on these cell lines is illustrated in Figure 7-8.

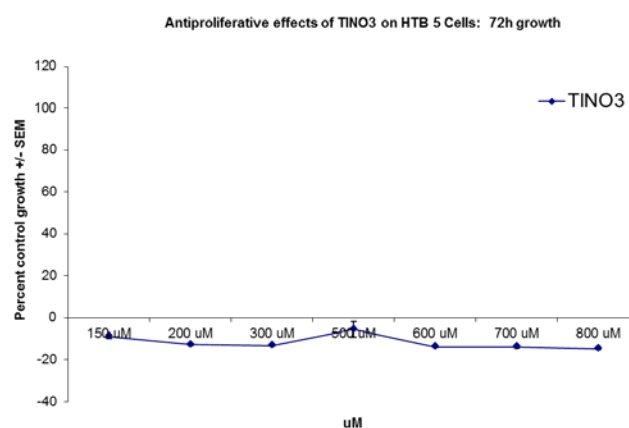


Figure 7. HTB-5 Cells & Thallium nitrate

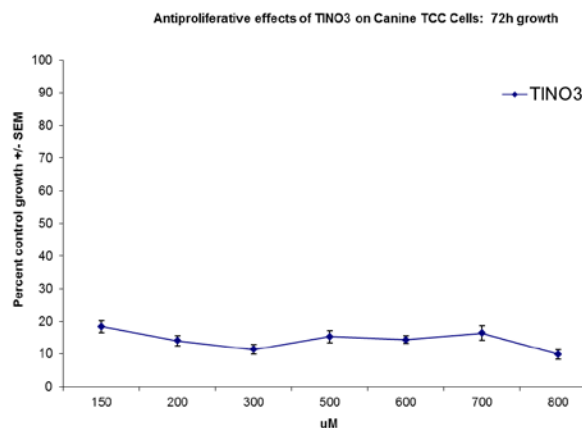


Figure 8. Canine TCC Cells & Thallium nitrate

4. Discussion

The data obtained from this work shows that the GBM cells were not able to tolerate the higher concentrations of TlNO_3 as compared to HTB-5 and Canine TCC cells. In a 72 hour study, the GBM cells had a steady decline toward cell death resulting in 97% cell death at 100 μM TlNO_3 as shown in Figure 4. In comparison, parallel experiments under the same conditions showed that the other cell line exhibited more tolerance to thallium nitrate with cell death for HTB-5 (83%) and Canine TCC (83%), as shown in Figure 5 and 6 respectively. Considering content application to the clinical setting, this observation was beneficial for students to see as an initial study and they

became increasingly curious about further outcomes. In a 2016 study by Kortz and coworkers, it was found that thallium is relevant for today's clinical setting as an antibacterial agent effective against Gram-positive bacteria, specifically *Bacillus aquimaris* and *Bacillus subtilis*. [36,37] Considering the results of this project thus far, the data further support that thallium's toxicity can also make a positive impact science, to kill cancer cells.

For the subsequent study, HTB-5 and Canine TCC cells were exposed to higher concentrations of TlNO_3 for 72 hours. As shown in Figure 7, complete cell death was observed for HTB-5 cells at 150 μM TlNO_3 , whereas the Canine TCC cells showed much more tolerance at these higher concentrations. Remarkably, the cancerous cells tolerated 800 μM TlNO_3 with 91% cell death, as shown in Figure 8. Complete cell death was not observed at this concentration leading to the conclusion that this cell line was more tolerant to the toxic effects of thallium ions. This led to the conclusion that the canine cell lines were not as sensitive to these metal ions when compared to the human cell lines under the same conditions. Moreover, the GBM and HTB-5, having an origin from human cells lines, possessed antiproliferative effects of the thallium in the range of 100-150 μM TlNO_3 . This is what one would predict considering if, in fact, the metal ion is entering the cell through the sodium-potassium pump and responsible for cellular apoptosis. Collectively, the SRB assays from this work confirmed the range of antiproliferative effects of thallium ions on GBM, HTB-5, and Canine TCC cells, *in vitro*.

The ultimate goal for this undergraduate experience was to provide students with an effective learning experience that explored new cells lines compared to the student cohort from the year prior and ensure students could relate the importance of this work to the field of chemistry. Therefore, the goal was to make sure students could positively contribute to relevant research topics in chemistry and supplement current advances in clinical chemistry. Overall, this undergraduate research project and its contributions achieved this goal. Our findings shined new light on the effectiveness of thallium toxicity and its antiproliferative effects on these cell lines. Consequently, our work supported the efforts published by other research groups that investigated thallium's potential in applications for clinical work, nuclear medicine, antibacterial studies, and environmental concerns. [13,16,17] [36,37] Upon reflection of this undergraduate research experience, Honors students have acquired real-life applications in inorganic chemistry as well as increased self-efficacy in clinical chemistry applications.

5. Conclusions

Based on the success of this laboratory experience as evidenced by student achievement of the learning outcomes, it would be beneficial if this work continued with another cohort of students in either the Honors program or traditional track. Moreover, this set of experiments and the results obtained in this project proved it to be a valuable and effective learning

experience for any student focusing on a major in chemistry, biology, or nursing.

Looking ahead to the next experimental design, a new avenue to explore could include investigating the antiproliferative effects of other heavy metal compounds on these cells as well as looking closer into the thresholds of sensitivity via increasing treatment time or ion concentration. A Western blot analysis could be used to aid in students' understanding of the mechanism of cell death for the chosen cell lines. Students could also explore other metal ions in aqueous solutions that have similar atomic radii to potassium and thallium. This would make a nice comparison study to the details described in this paper. Additionally, exploring different cancer cell lines or different noncancerous cell lines could serve as a beneficial project for undergraduates so that they can learn more about the effectiveness of these compounds. Considering the success of this work, it would be necessary and appropriate to continue using fetal calf serum to strive for optimal growth and to continue performing sterile techniques to avoid contamination.

In the future, new students joining the research group can serve as peer mentors and acquire leadership skills, both of which align with the mission of Walsh University. The main objective of this undergraduate research experience should be to generate students' curiosity about the impact of science innovation to the world and to reinforce previous course content. Another important consideration includes the fact that this work will help students to apply scientific applications to real-life situations, improve critical thinking skills in qualitative problem solving, and increase student self-efficacy in chemistry and related topics.

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