Investigating the Cellular Response of Copper (II) Ions on both Cancerous and Noncancerous Cell Lines: A Closer Look into MCF-7, A375, and HFF Cells

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Abstract

Copper compounds are known for their toxicity toward bacteria and a limited number of viruses. This project investigated the effects of copper compounds on breast cancer, skin cancer and noncancerous cell lines. These metal ions may enter the cell through the Na^+/K^+ pump, disturb chemical processes, and, therefore, could lead to cell death. These cell lines were treated with various concentrations of $CuCl_2 \cdot 2H_2O$. A Sulforhodamine B assay monitored overall cell death. The antiproliferative effects of the cancerous cells were compared to that for the noncancerous cells and allowed for unique projects for two undergraduate nursing majors and one honors nursing major. This work enhanced the students' understanding and critical thinking in inorganic chemistry and its real-life applications. Interestingly, the data support that Copper (II) ions possess anticancer properties and have the ability to kill cancer cells, in vitro.

Key Words: A375 cells, Cancer, Copper (II) ions, MCF-7 cells

1. *Introduction*

One critical aspect of the mission of Walsh University is to aid our students to become confident scholars in their chosen field and apply their talents to benefit today's society. Therefore, this project was created to allow for the opportunity for undergraduate nursing majors to enhance their technical scientific skills and, thereby, becoming curious about the applications of chemistry outside the classroom. CHEM 411-412 (Introduction to Research) is open for all students, regardless of class rank or major. This course provided the students the opportunity to appreciate scientific detail to a greater extent and helped students to become responsible scientists. Many research projects in chemistry require continuous hours of dedication, creating a challenge for nursing majors who are also completing clinical courses at local hospitals.

This project was designed with many stopping points as a way to maximize the students' success and also further developed into a significant project, a senior honors thesis. Another focus was to ensure safety so the students always worked in teams of 2 or 3 students and exposure to harmful reagents and toxins was limited. Prior to the start of this work, several detailed training sessions were conducted to ensure the students were both comfortable with the protocol and proficient at sterile techniques.

The overall goal was to enhance the education of Walsh University's nursing majors by guiding them through this undergraduate research course. Copper is an effective antimicrobial agent and, therefore, may possess the ability to kill cancer cells. The focus of this work was to investigate the impact of these ions on cancerous cells using various concentrations and treatment times. Copper has been found to also possess important health benefits and copper is one essential dietary mineral that plays an important role in enzyme activity and oxygen transport. (Yin, *et al.* 2012) In previous reports, it was stated that copper found in drinking water and dietary supplements, also called inorganic copper, is processed differently in our bodies than copper found in food, also called organically complexed copper. (Brewer, 2017) (Brewer, 2010) It was discovered that inorganic copper can bypasses the liver and settles in the bloodstream and, therefore, adds to the free copper pool. (Brewer, 2010) Today's vitamins consist of primarily inorganic copper and can increase these free copper concentrations and, thereby, intensify the risk of free radical formation. Due to these current concerns, it would be interesting to see how aqueous copper salts may impact cellular growth and its implications as a possible anticancer system.

This undergraduate research project explored the antiproliferative effects of copper compounds on biological cells, specifically MCF-7 (breast cancer), A375 (skin cancer) and HFF (human foreskin fibroblasts) cells. MCF-7 cells are breast cancer cells that were chosen for this study in order to learn more about the mechanism of their cell death. It is hypothesized that Copper (II) ions, Cu^{2+}, can enter the cell through the Na^+/K^+ pump. Once the ions are present inside a cell, they can disrupt normal cellular processes, potentially interfere with oxidative phosphorylation, and disrupt the mitochondrial membrane potential. (Denning, *et al.* 2002) This observation could lead to mitochondrial membrane break down and the release of Cytochrome C. The release of Cytochrome C will result in the activation of the intrinsic apoptotic pathway and cellular death. All three cell lines were treated with 1-100 μM $CuCl_2$•$2H_2O$ and 150-500 μM $CuCl_2$•$2H_2O$ and Sulforhodamine B (SRB) assays monitored overall cell death. The antiproliferative effects of the cancerous cells were compared to that for the noncancerous cells and provided unique projects for two undergraduate nursing majors and one honors nursing major. Interestingly, the data support that Copper (II) ions possess anticancer properties and have the ability to kill cancer cells, *in vitro.*

2. Experimental

2.1 Cell Culture and Reagents

At Walsh University, students work in a laboratory designed for tissue culture research and they received several weeks of training so they understand safety procedures and become proficient in sterile technique. They learned how to conduct manipulations carefully in order to effectively maintain a sterile environment. 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% CO_2 at 37°C. Ranin pipettes (L10, L20, L200) were used in this work. Cells were counted using a disposable hemocytometer (incyto, C-chip Neubauer improved). The cell lines were treated with metal ions and the growth inhibition was monitored using SRB assays. Each assay took approximately three weeks to complete, depending on the cellular growth. Walsh University supported the ethics of this work because our focus was to continue the advancement in the area of science as we sustained high standards for our students and the quality of their results. This project was completed without any limitations.

2.1 SRB Assay and Project Design

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 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 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.

In 1973, Herbert Soule and co-workers discovered this stable and viable breast cancer cell line, MCF-7. (Soule, *et. al*, 1973) MCF-7 stands for Michigan Cancer Foundation-7, the center in Detroit where they conducted these initial studies. MCF-7 cells are adherent epithelial cells of the mammary gland, a human adenocarcinoma cell line. Studies with this cell line initially began in 1970, originating from Frances Mallon, a 69 year-old female. (Soule, *et. al*, 1973) (Levenson, *et. al*, 1997) This research was pivotal to the advancement of cancer research due to limited knowledge of this disease and its complications. Before the exploration of MCF-7 cells, challenges arose due to the instability of breast cancer cells. It was impossible for research groups to acquire a mammary gland cell line that was able to survive longer than a few months. (Levenson, *et. al*, 1997) (Cass, 1990) Therefore, the discovery of the MCF-7 cell line has been critical to the advancement in breast cancer research. (Soule, *et. al*, 1973) (Levenson, *et. al*, 1997) Fig. 1 shows a photograph of the MCF-7 cells used in this work.

There are many general risk factors for developing melanoma including: excessive exposure to the sun including blistering sunburns, fair and or freckled skin that easily burns, family history of skin cancers, previous melanoma, previous nonmelanoma skin cancer (squamous cell carcinoma and/or basal cell carcinoma), and a significant quantity moles and abnormal moles on the skin. (Cheng, et. al, 2007) A375 cells, as shown in Fig. 1, are adherent malignant melanoma cells from the surface of the skin that originated from a 54 year-old female. (Cheng, *et. al*, 2007) HFF cells are adherent cells that originated from a male newborn's foreskin following circumcision. These cells are an optimal choice because they can serve as an experimental control in research projects as well as to aid in comparisons due to their noncancerous characteristics. (Usuki, *et. al*, 1988) (Hovatta, 2003) In 2003, Hovatta and coworkers conducted a study using human foreskin fibroblasts as feeder cells allowing for the production of human embryonic stem cells. (Usuki, *et. al*, 1988) (Hovatta, 2003) Current research is continuing in this area to further understand how these cells may benefit the advancement of cancer research. A photograph of HFF cells used in this work is as shown in Fig. 1.

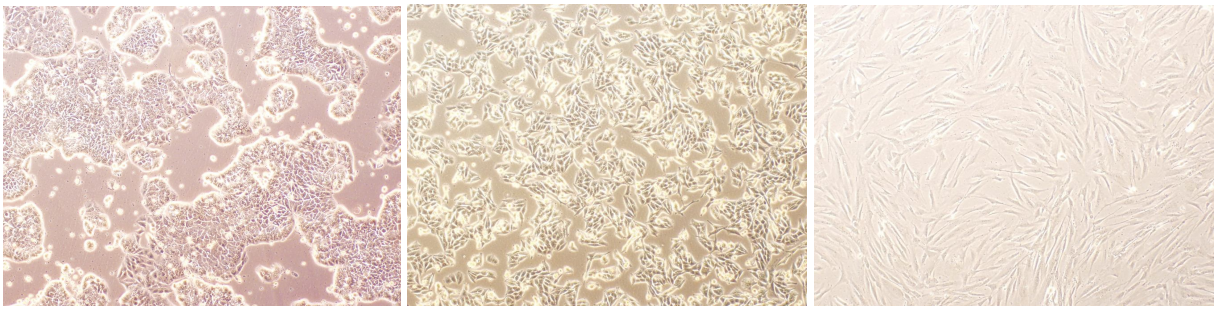

Figure 1. Cell Morphology of MCF-7 (left), A375 (middle), and HFF (right) Cells.

3. Results

3.1 SRB Assay Utilizing Copper (II) Chloride Dihydrate

For the first study, the cells were treated with 1-100 μM $CuCl_2 \cdot 2H_2O$. The cells were treated with the compound until the cells in each well were 80% confluent. All these cell lines were able to reasonably tolerate this concentration with little growth inhibition. The antiproliferative effects of $CuCl_2 \cdot 2H_2O$ on these cells lines are illustrated in Fig. 2-4. This data showed that these cells lines had a high tolerance for this metal compound and so further studies at high concentrations were conducted. This was a necessary step to further understand the sensitivity of these cells.

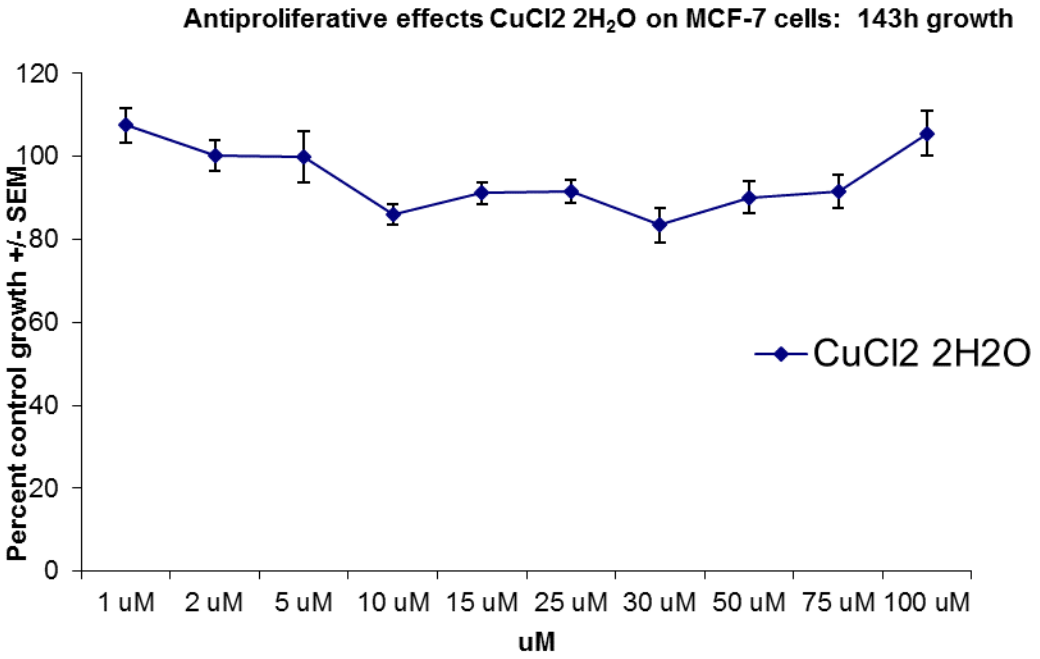

Figure 2. MCF-7 Cells & $CuCl_2 \cdot 2H_2O$.

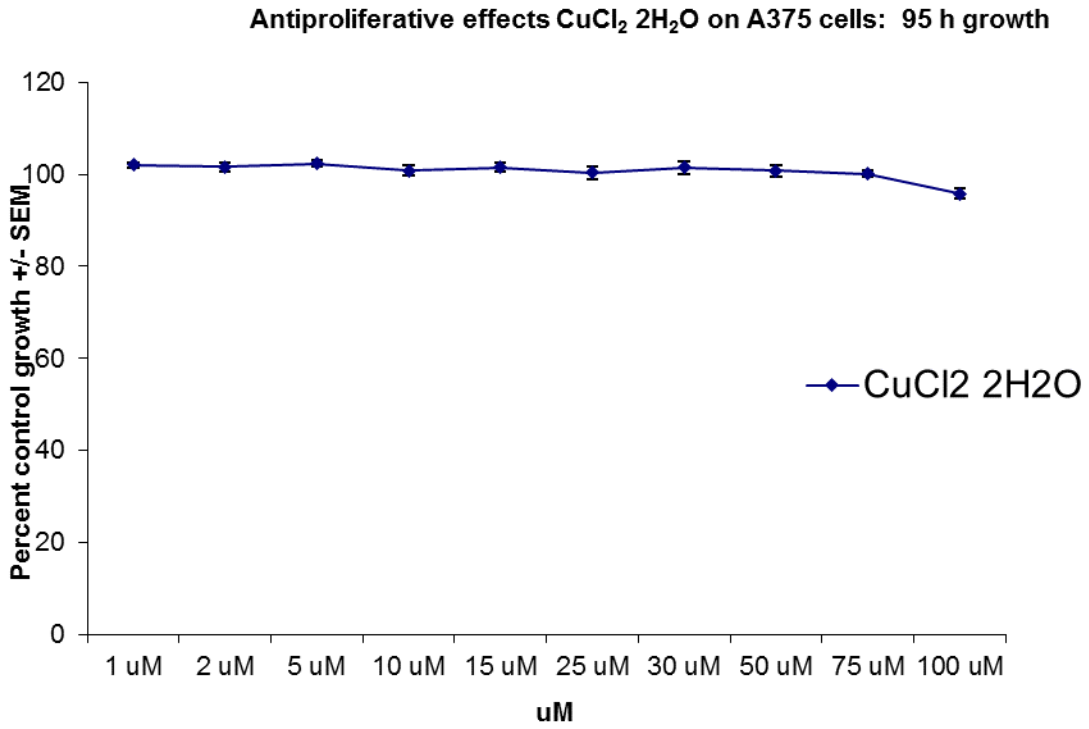

Figure 3: A375 Cells & $CuCl_2 \cdot 2H_2O$.

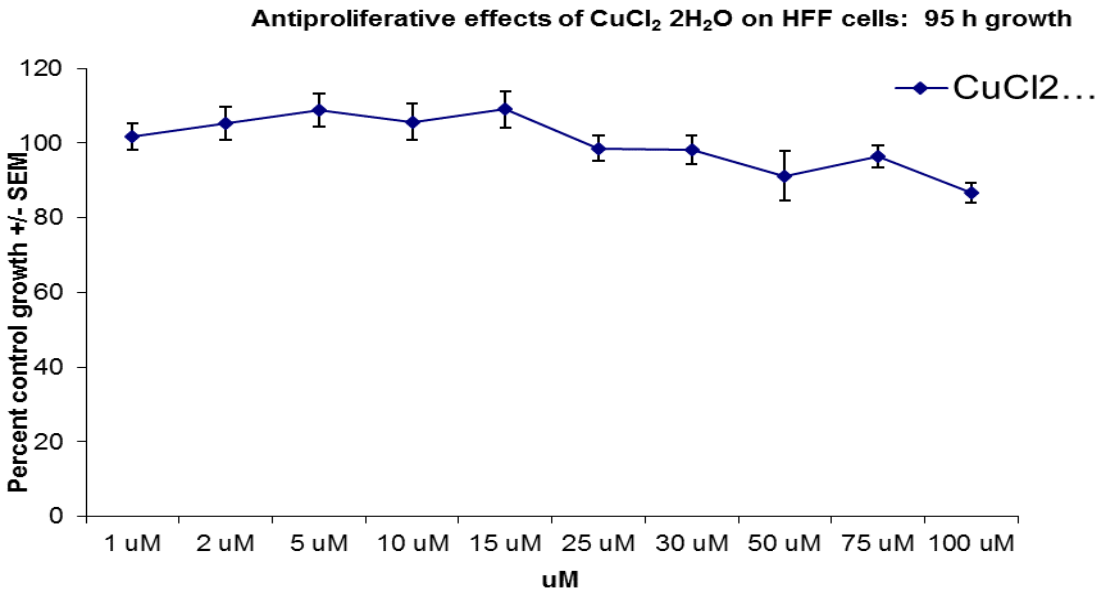

Figure 4: HFF Cells & $CuCl_2 \cdot 2H_2O$.

3.2 SRB Assay Utilizing Copper (II) Chloride Dihydrate-Higher Concentrations

The second study involved further studies at higher concentrations. For consistency, the cells were treated with this compound until the cells in each well were 80% confluent. At 150 µM $CuCl_2 \cdot 2H_2O$, the cancer cells showed antiproliferative effects, but HFF cells were unaffected at this concentration. At 200 uM $CuCl_2 \cdot 2H_2O$, both MCF-7 cells and A375 cells had significant growth inhibition and complete cell death occurred at 300 µM $CuCl_2 \cdot 2H_2O$ for both cancerous cell lines. Interestingly, the HFF cells were able to tolerate this concentration and complete cell death was observed at 500 µM $CuCl_2 \cdot 2H_2O$. The antiproliferative effects of $CuCl_2 \cdot 2H_2O$ at higher concentrations are illustrated in Fig. 5-7.

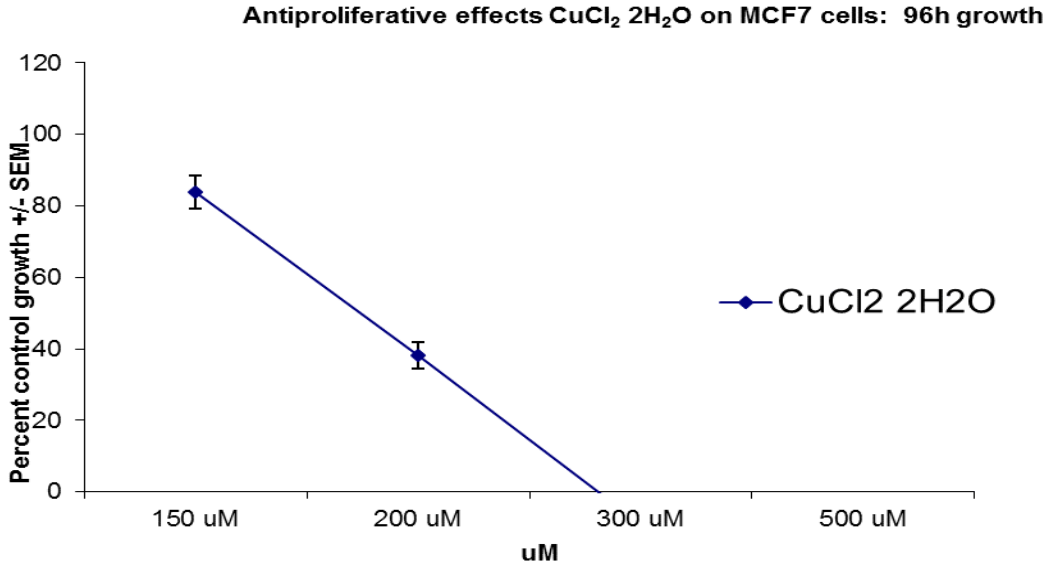

Figure 5: MCF-7 Cells & $CuCl_2 \cdot 2H_2O$.

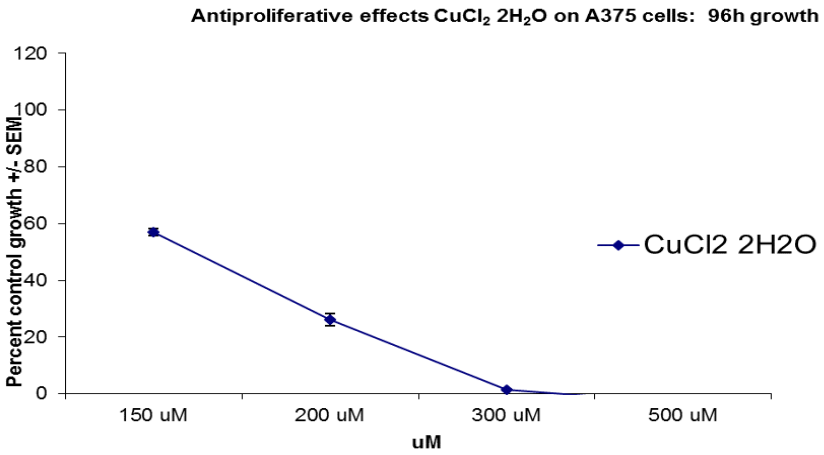

Figure 6: A375 Cells & $CuCl_2 \cdot 2H_2O$.

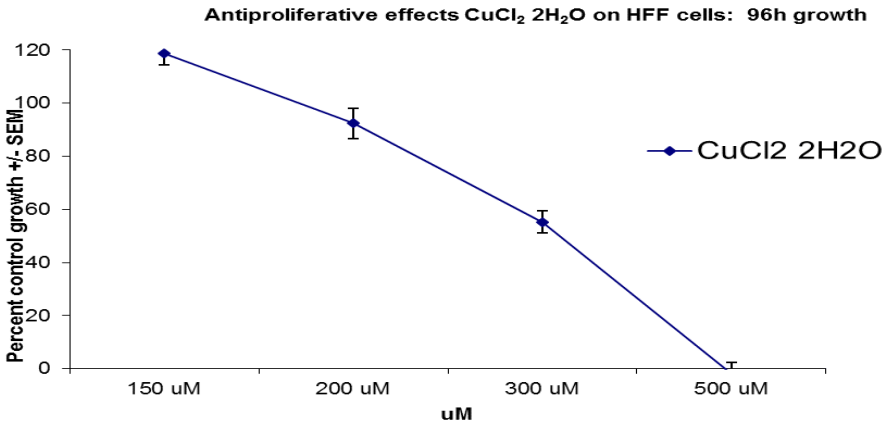

Figure 7: HFF Cells & $CuCl_2 \cdot 2H_2O$.

4. Discussion

Under these conditions, this project illustrated the antiproliferative effects for all three cell lines. It was found that all three cell lines can tolerate 1-100 µM $CuCl_2 \cdot 2H_2O$. However, at higher concentrations including 150-500 µM $CuCl_2 \cdot 2H_2O$, the cancerous cells are more susceptible to growth inhibition as compared to the noncancerous cell lines. This was an important discovery because this finding indicated that the cancer cells are more susceptible to this metal compound than the noncancerous cells, a critical step to minimizing the cell death of healthy cells. These results led to an idea that future student projects could investigate other copper salts to compare how changing the ion could impact cellular growth. In order to obtain a greater understanding into the cells' sensitivity, studies at high concentrations may be necessary or varying the treatment time may also provide valuable insight. These results were interesting for the students to observe as they became increasingly curious about future outcomes. The results of the SRB assays in this project clearly demonstrated the antiproliferative effects of Cu^{2+} on MCF-7, A375, and HFF cells, *in vitro*.

5. Conclusions

Cu^{2+} served as a poison for the cancerous cell lines and could possess anticancer properties by inhibiting cellular processes and growth. At the lower concentrations, the assay proved that growth inhibition was not significant. At higher concentrations, it was found the breast cancer cell possessed the greatest sensitivity to this metal compound.

Both breast cancer and skin cancer cell lines were more sensitive than the noncancerous cells, a positive result supporting that Cu^{2+} could be further studied as an anticancer option. Because aqueous solutions with Cu^{2+} are soluble in DMEM media, it will be convenient to continue using a variety of Cu(II) salts with varying concentrations. Future work including new honors projects may include exploring treatments with other metal ions and conducting experiments with shorter treatment intervals. Another future step would be to consider Western blot analyses to further understand the mechanism of cell death of these cells. The continued use of "super media", including fetal calf serum, would optimize the rate of cell growth and maintaining sterile technique is essential to avoid contamination. Another interesting and important feature of further experiments would be to include a comparison of these results to other cancer cell lines. This would allow for the opportunity for students to compare results to this project and also to explore the sensitivities of new cell lines.

This project provided nursing students with the unique opportunity to acquire knowledge in biological applications of inorganic compounds, tissue culture procedures, and sterile techniques. Consequently, this work enhanced the undergraduate students' understanding and critical thinking in chemistry and allowed for the application of inorganic chemistry in real-life situations. Future projects will continue to enhance their academic experience and increase student self-efficacy in chemistry as well as contribute to the advancement of this area of science.

6. Acknowledgements

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