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# EVALUATE GAQ3 CHEMOTHERAPY EFFECTS AND MECHANISM OF ACTION IN ESOPHAGEAL CANCER

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# EVALUATE GAQ3 CHEMOTHERAPY EFFECTS AND MECHANISM OF ACTION IN ESOPHAGEAL CANCER

by

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ABSTRACT

EVALUATE GAQ3 CHEMOTHERAPY EFFECTS

AND MECHANISM OF ACTION IN

ESOPHAGEAL CANCER

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According to the American Cancer Society, esophageal cancer is the sixth leading cause of cancer worldwide. With only an 18% five years survival rate, this type of cancer has few symptoms and usually diagnosed at a later stage. Previous studies have shown that multiple gallium compounds have been investigated in both animal studies and clinical trials against various cancer. GaQ3 is an organo-gallium complex that has both anti-tumor and anti-bone resorption activity. The action of GaQ3 can be divided into two phases: acute phase (minutes) involving a steady rise in intracellular $Ca^{2+}$ levels and execution phase (hours) involving calpain activation and consequent calpain-regulated down-regulation of focal adhesion factors (focal adhesion kinase, talin and integrins) and up-regulation of pro-apoptotic factors (Bax, Bim and Bak). How GaQ3 induce intracellular calcium elevation and the primary target (s) of GaQ3 remain unclear. Using our well-established fluorescent-

based method (Pan, et al. 2012), we will quantitatively measure intracellular calcium concentration upon treatment of GaQ3 using live cell imaging in normal esophageal epithelial, insensitive and sensitive cancer cell lines. If intracellular calcium elevation plays an essential role in the anticancer effect of GaQ3, we expect that GaQ3 will induce more intracellular calcium elevation in GaQ3 sensitive cancer cells than those in insensitive cancer or normal cells.

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# CHAPTER 1

# INTRODUCTION

## 1.1 Functions and Regulations

In order for the human body to function correctly, every tiny aspect is closely and strictly regulated. From hormone imbalance to glucose intake, any type of disturbance in functions and regulations starts at the cellular level. Although we cannot see it with our eyes, our cells and their specific functions are the reason why we humans can do everyday tasks.

### *1.1.1 Human Cells: The Power House*

Cells are membrane-bound units that make up every aspect of life. The human body is composed of trillions of cells, with components to absorb nutrients from food, convert energy, and also contain hereditary information to partake in the circle of life. Within the cells are organelles. From the nucleus to the Golgi apparatus, each of the organelles have specific functions and purpose. With permeable membranes, some chemicals can passively diffuse inside the cells. However, when substances are charged or larger in size, membrane bound proteins are used to transport things in and out of the cells. Substances such as calcium, sodium, and potassium, although small, charge on these substances making it difficult to cross by simple diffusion. In order to enter the cells, specialized proteins are used to maintain sufficient concentrations of substances at all times.

*1.1.2 Calcium Within the Cells*

Secretion, gene expression, muscle contraction, and metabolism is controlled by $Ca^{2+}$ levels. (Bagur and Hajnoczky, 2018) It is known that $Ca^{2+}$ is a potential cytotoxic and has been linked to necrotic cell death in the heart and liver. (Orrenius et al., 2003) Concentration of $Ca^{2+}$ within the cells depends on its location or organelles. High intracellular $Ca^{2+}$ are either stored in the endoplasmic reticulum (ER) or within sarcoplasmic reticulum in muscle cells. (Bagur and Hajnoczky, 2018) $Ca^{2+}$ carries a charge; therefore, specialized proteins are used to maintain specific levels. Calcium signaling within the cells are highly regulated and prefers to be at low resting levels. (Bagur and Hajnoczky, 2018) Since $Ca^{2+}$controls the major aspect of cell function, elevation in calcium levels would lead to cell injury or cell death.

## 1.2 Esophageal Cancer

The esophagus is a long, hollow tube that runs from the throat to the stomach, also known to be the site of esophageal cancer. Cancer can occur anywhere along the esophagus and on the different type of cells within it. According to the Mayo Clinic, this type of cancer is more common in men than women. The early stages of this cancer typically lack signs or symptoms; therefore, most cases are diagnosed during the metastatic stage. Due to esophageal cancer being the sixth most common cause of cancer deaths worldwide, the demand for more effective prognosis and treatment is called for. Treatment options depend on the type of esophageal cancer patient has. The first type is adenocarcinoma, typically occur in the lower portion of the esophagus. The second type is squamous cell carcinoma, happening in the upper and middle portions of the esophagus. There are other rare types of

esophageal cancer, such as small cell carcinoma, sarcoma, lymphoma, melanoma, and choriocarcinoma.

*1.2.1 Observation of GaQ3*

Multiple gallium compounds have been investigated in both animal studies and clinical trials against various cancers. GaQ3 (under other names including KP46, NKP-46, and FFC11) is a novel organo-gallium complex, with both anti-tumor and anti-bone resorption activity in the same dose range. Its anticancer mechanism of action (MOA) appears different from gallium nitrate. Previous studies showed that GaQ3 induce G1/S-phase arrest and apoptosis in cancer cells. The action of GaQ3 can be divided into two phases: acute phase (minutes) involving a steady rise in intracellular $Ca^{2+}$ levels and execution phase (hours) involving calpain activation and consequent calpain-regulation of pro-apoptotic factors. Execution phase may also involve p53, and a forward feedback loop is formed in which p53 actives the expression of TRPC6 and further increase intracellular $Ca^{2+}$.

1.2.1.1 IC 50 of GaQ3

In a previous study, we determined the half maximal inhibitory concentration (IC50) to measure the effectiveness of GaQ3. Data supports that a higher concentration of GaQ3 leads to cancer cell apoptosis. Using KYSE-30, 150, 70, and 270 cell lines, multiple measurements were taken to determine the concentration and time the gallium based drugs will lead to low cell viability. Data concluded that as time increases and concentration increases, cell viability treated with GaQ3 would decrease.

Table 1.1: IC50 of KYSE-150, KYSE-70, KYSE-270

| | KYSE-150 | KYSE-70 | KYSE-270 | History (%) |
|---|---|---|---|---|
| IC50 | 8.016 | 21.47 | 25.00 | 30 |

1.2.1.2 Anti-Tumor Activity

Data shows that KYSE-150 cell line is more sensitive to GaQ3; therefore, it was the cell line used to determine cell viability after different time intervals. Figure 1.1 supports that the longer the drug is incubated, the lower the cell's viability. As shown, after 72 hours, cell viability is at its lowest. The previous study concluded that although the mechanism of GaQ3 is unclear, this gallium based drug has anti-tumor activities and able to induce apoptosis in esophageal cancer cells at the correct amount of drug concentration and time of incubation.

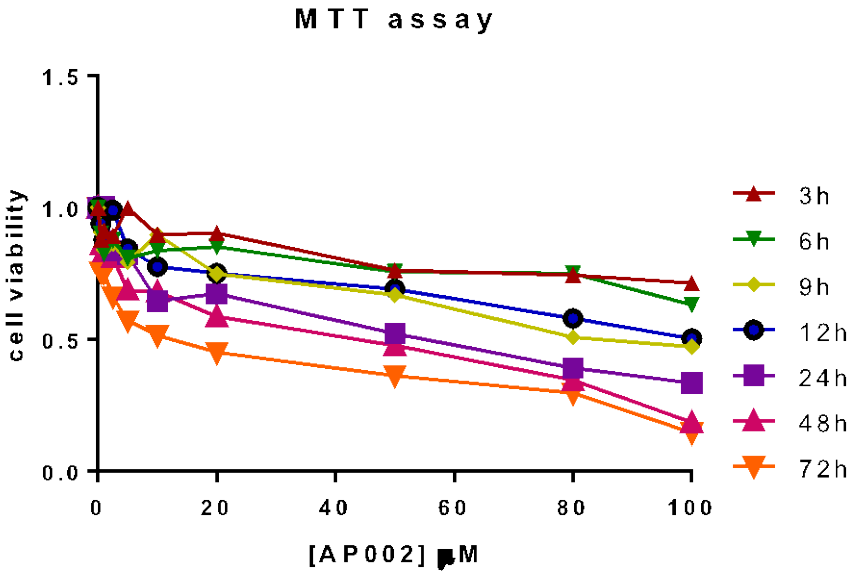


Figure 1.1: Cell Viability After Different Time Intervals

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 GaQ3 Anticancer Mechanism of Action

According to the studies conducted at the national cancer institute (USA), gallium nitrate was found to display the greatest in vivo anti-tumor activity. From non-Hodgkin's lymphoma to bladder cancer, gallium has the ability to inhibits the proliferation of tumor cells. However, the nephrotoxicity due to rapid renal excretion of gallium ions was observed as a side effect of gallium nitrate. Therefore, further investigations was done to evaluate gallium for cell toxicity in animals, while also observing antineoplastic and toxicity activity in humans.

Previous studies showed that GaQ3 induces a slow but steady rise in intracellular calcium levels in the tumor cells. (Cui, et al. 2012) and a slow rise in intracellular calcium induced by GaQ3 causes calpain hyper-activation. In turn, it leads to down-regulation of focal adhesion factors including focal adhesion kinase, talin and, integrins, in particular integrin a5 and B1, B4 & B5. (Jungwirth, et al. 2014) These changes in cell signaling cause tumor cell death.

### *2.1.1 Calcium Signaling Pathways*

There are four possible sources for intracellular calcium in epithelial cells: from ER through IP3R, from extracellular calcium reservoir through voltage-gated calcium channel or SOCE channel, from lysosome through two-pore calcium channels (TPCs) or other channels, from mitochondria through mPTP.

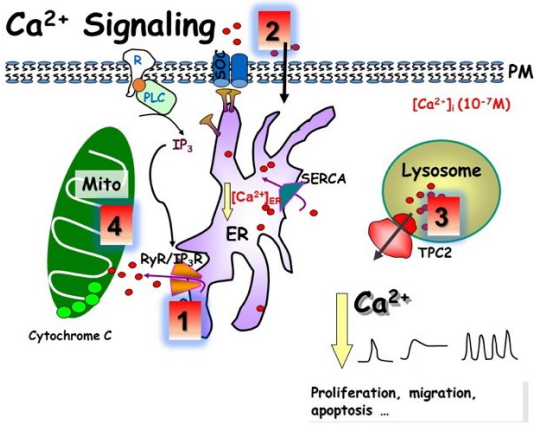


Figure 2.1 Intracellular $Ca^{2+}$ sources:
1. ER/SR Ca release through IP3R or passive release through inhibition of SERCA pump;
2. extracellular Ca entry through voltage gated channels or store-operated Ca channel (SOC);
3. Ca release from other intracellular organelles, such as lysosome;
4. Mitochondrial Ca release, such as through mPTP.

Apoptosis is a highly regulated process that normal and healthy cells do on a normal basis for development and tissue homeostasis. When cells become cancerous, this regular process gets disrupted. Regulations occur in two pathways, extrinsic and internal. Focusing on the ER-mitochondrial $Ca^{2+}$ influx, there is clear evidence that changes in $Ca^{2+}$ concentration can regulate and trigger apoptosis. (Orrenius et at., 2003) As a storage organelle, the ER contains a higher concentration of $Ca^{2+}$ at all time. (Bagur and Hajnoczky, 2018). When apoptosis is stimulated, the mitochondria release a small amount of cytochrome c to bind to inositol trisphosphate (IP3) receptors on the ER. (Orrenius et al., 2003) Using the internal pathway to the mitochondria, $Ca^{2+}$ is released from the ER through the inositol trisphosphate (IP3) receptor. (Orrenius et al., 2003) Once $Ca^{2+}$ gets accumulated in the mitochondria, the mitochondrial permeability transition pore (mPTP) opens and pro-apoptotic factors are released.

*2.1.2 BCL-2 Proteins*

The BCL-2 family of proteins reside in the mitochondria and is an important structure in regulating apoptosis. (Chipuk et al,. 2011) These proteins are divided into anti-apoptotic such as BCL-2 and BCL-$X_L$ and pro-apoptotic such as BAX, BAK, and BAD. (Chipuk et al,. 2011) Anti-apoptotic are also associated with the ER, with the main function to reduce excessive $Ca^{2+}$ to be released. The pro-apoptotic proteins are located in the same organelles but do the opposite by signaling the cell to release $Ca^{2+}$ and induce apoptosis.

# CHAPTER 3

# METHODOLOGY

We first culture KYSE-150 cells for 24 hours and begin treatment of different dose of GaQ3 for the indicated times. Western blot is a commonly used technique to detect protein. To begin the process, cells are lysed with RIPA buffer (150 mM NaCl, 50 mM Tris-Cl, 1 mM EGTA, 1% Triton X-100, 0.1% SDS and 1% sodium deoxycholate, pH 8.0) supplemented with proteinase inhibitor cocktail (Sigma-Aldrich, US). Protein concentration was quantified using a BCA kit (Thermo, US). Primary antibodies that will be used in this study include P-ERK (1:1000, Cell Signaling Technology), ERK (1:1000, Cell Signaling Technology) Bax (1:1000, Cell Signaling Technology, US), Bcl-2 (1:1000, Cell Signaling Technology) and anti-GAPDH (1:1000, GeneTex, US). Secondary antibodies will include HRP-labeled goat anti-rabbit IgG (1:1000, Cell Signaling Technology, US) and anti-mouse IgG (1:1000, Cell Signaling Techonolgy, US) signals will be detected on ChemiDoc (Biorad, USA). After data collection is completed, we will determine what protein was affected. We can also view what signaling pathway was affected and whether this drug performs up-regulating or down-regulating in genes that are responsible for cell apoptosis or cell proliferation.

## 3.1 Calcium Signaling

During the same time, we will determine if cell calcium signaling was affected by GaQ3. KYSE-150 cells were loaded with 3μM Fluo-4 in 96-well imaging plates (BD Falcon, NJ) at 37° C for 30 min. After washing, cells were kept in culture medium without

phenol red. The intensity of fluorescent signals were recorded by Hamamatsu digital camera C11440 complemented with DMi8 inverted microscope (Leica, Germany) with 20x objective (dry lens, NA 0.75) Time lapse live cell images were recorded every 4s for 3 min and analyzed using imaging.

# CHAPTER 4

# DISCUSSION

## 4.1 Calcium Influx

Time lapses were recorded to observe calcium influx. There were a total of two 96 wells plate, one control and one treated with GaQ3. Figure 4.1 is the fluorescent image of cells from the control sample. Time laps shows $Ca^{2+}$ influx over time intervals of 10 seconds. Figure 4.2 is the fluorescent image of cells after 72 hours of GaQ3 treatment. There were no $Ca^{2+}$ influx over the 10 seconds intervals.

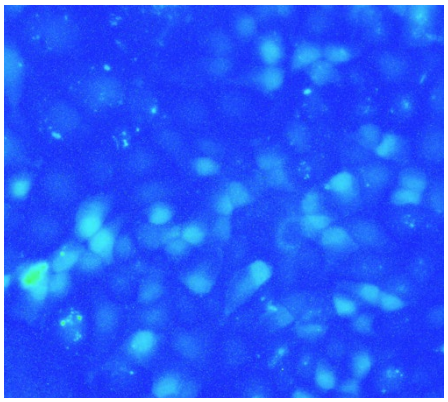

Figure 4.1: Fluorescent Image of Cells in Control Sample

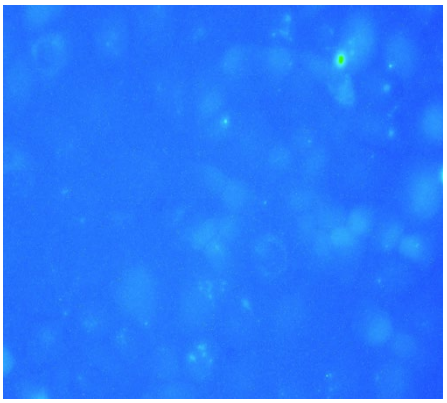

Figure 4.2: Fluorescent Image of Cells with GaQ3 Treatment

*4.1.1 Observing Cells in Control Sample*

Using the computer program to analyze the cells, a graph was populated to shows the influx of $Ca^{2+}$. About 30 cells were observed; all have an oscillations in signals. Figure 4.3 shows the signal intensity of the 61 seconds interval. Some cells within the 96 well plates have stronger intensity compared to another. However, all cells show sinusoidal oscillations.

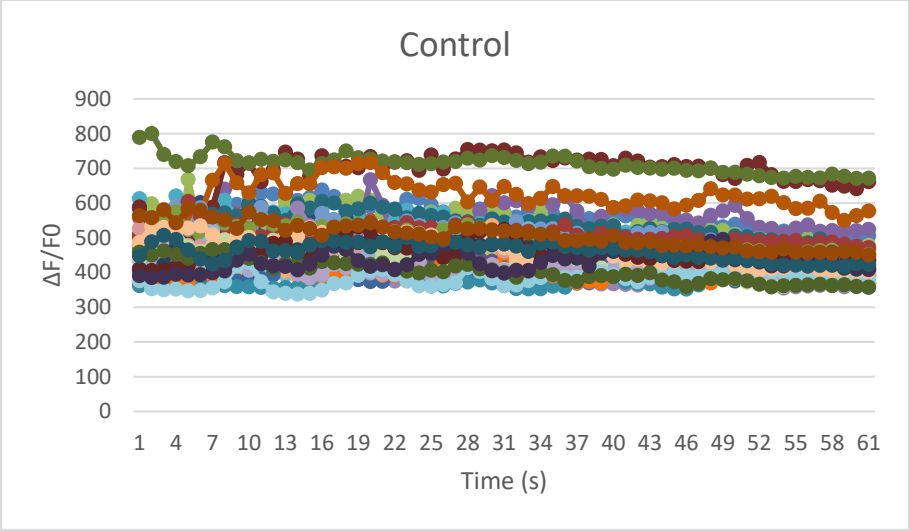


Figure 4.3: $Ca^{2+}$ Signaling of About 30 Cells in Control Sample

4.1.1.1 Strongest Signal in Control Sample

Shown in figure 4.4, one strongest signal was isolated from the control group. The oscillation in $Ca^{2+}$ signaling had strong intensity throughout the whole time interval being observed. The highest peak represents the strong intensity, and the lowest peak shows the weakest intensity. Sinusoidal waves were also observed through the whole time interval.

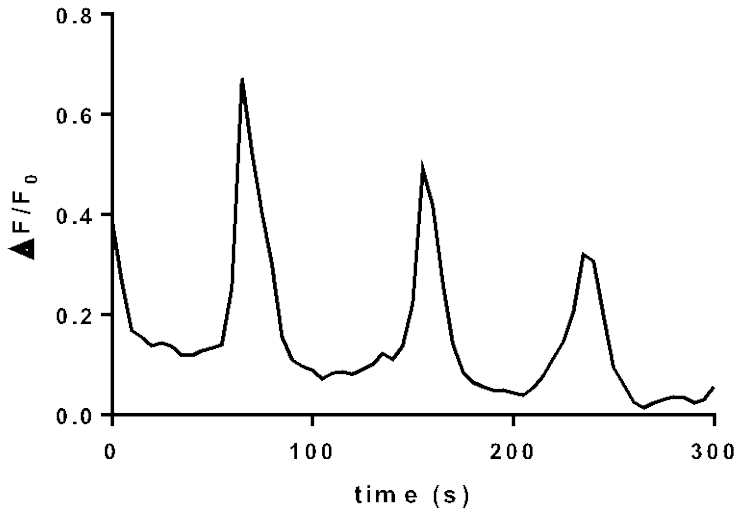


Figure 4.4: One Strongest Signal from Control Sample

*4.1.2 Observing Cells with GaQ3 Treatment*

Using the same technique to analyze the data, the cells were observed for $Ca^{2+}$ signaling after 72 hours of GaQ3 treatment. About 30 cells were evaluated and represented in Figure 4.5. Data shows a steady rise in oscillation for the first few seconds but plateau quickly afterward.

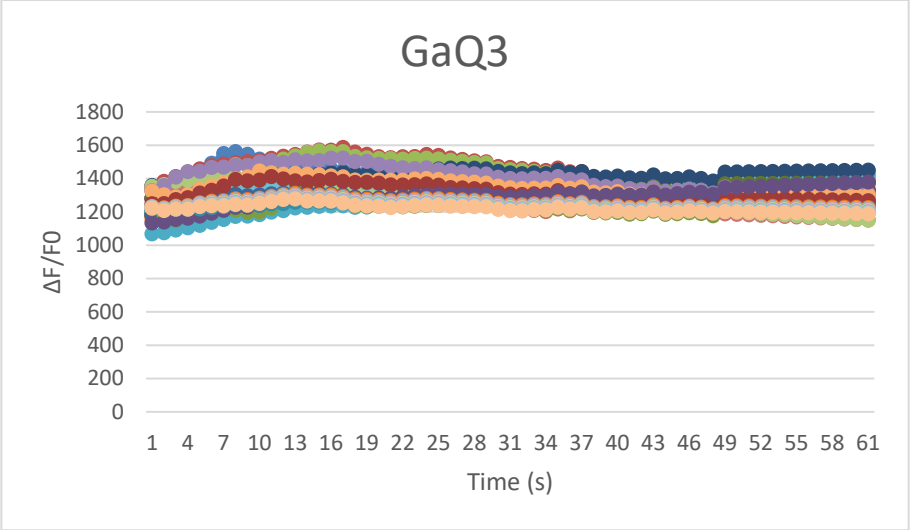


Figure 4.5: $Ca^{2+}$ Signaling of About 30 Cells with GaQ3 Treatment

4.1.2.1 Strongest Signal with GaQ3 Treatment

Isolating the strongest signal, Figure 4.6 shows that the calcium signaling in cells treated with GaQ3 did not have sinusoidal oscillations compared to the control group. During the first few seconds, the line shows a peak intensity and continued to decline as time continued.

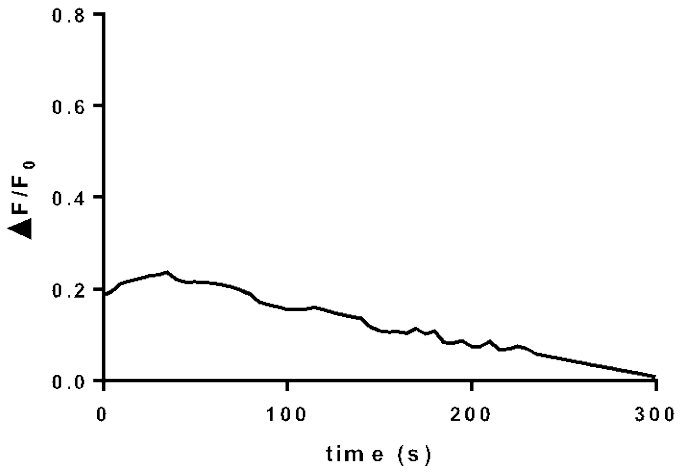


Figure 4.6: One Strongest Signal from GaQ3 Treatment Sample

# CHAPTER 5

# CONCLUSION

After the determination that Gallium have anti-tumor activities in our previous experiment, this experiment expands on how GaQ3 induces intracellular $Ca^{2+}$ elevation in acute phase.

## 5.1 Fluorescent Images

Within the fluorescent images, two time lapses were collected to observe $Ca^{2+}$ oscillations. The control data, with no GaQ3 treatment, had strong oscillations through the whole interval of time being observed. Within the image, each light blue circles represent a cell, and most cells are still in a circular shape. The oscillation in $Ca^{2+}$ signaling and circular shape show that cells not treated with GaQ3 are still alive and functioning. The image of the cells treated with GaQ3 shows no active cells. $Ca^{2+}$ signaling in Figure 4.2 show very little to zero cell viability after GaQ3 treatment. This data lead to the conclusion that $Ca^{2+}$ signaling is down-regulated.

## 5.2 Calcium Oscillations

Using the computer program to analyze the data, two graphs populated to show the intensity of $Ca^{2+}$ oscillation. About 30 cells were analyzed and graphed for both variables. With the control variable, sinusoidal waves were observed. Which shows that there are strong $Ca^{2+}$ signaling. Comparing to the GaQ3 graph, cells did not oscillate in calcium signaling. There was a decrease in $Ca^{2+}$, which confirms that $Ca^{2+}$ signaling is in fact down-regulated.

## 5.3 Future Studies

This experiment evaluated GaQ3 and focused on the mechanism of GaQ3 in the acute phase. For future experiments, further investigation should be done to focus on the executive phase of GaQ3. Once more data is collected, future studies should pinpoint the exact targets of GaQ3.

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## BIOGRAPHICAL INFORMATION

Ms. Quynh T. Tran is a senior at The University of Texas at Arlington (UTA). She is pursuing a degree in Honors Bachelor of Science in Biology, with a minor in Biochemistry. She completed grade-school through the Mansfield Independent School District and graduated early with her high school diploma in 2016. She started her college journey right away and spent all four years at UTA. During her undergrad, she completed all pre-requisite courses and laboratories before joining Dr. Zui Pan's BL-2 lab. Under the sponsorship of the UTA Honors College, Ms. Tran was funded to complete a summer research fellowship in 2019. The fellowship was completed in Dr. Zui Pan's lab, to conduct an experiment to find the IC50 of an organo-gallium compound drug on esophageal cancer cells. For future plans, Ms. Tran will continue her journey to pursue medicine. Within the medical field, Ms. Tran aims to become a Radiologist one day, specialized in the diagnosis of cancer.