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Using α-Mangostin from Garcinia Mangostana to Block Cell Death Caused by Paclitaxel in Proliferating BHK Cells

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USING α-MANGOSTIN FROM *Garcinia mangostana* TO BLOCK CELL DEATH CAUSED BY PACLITAXEL IN PROLIFERATING BHK CELLS

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ABSTRACT

One of the most commonly found mutations in cancers is a mutation in p53. A mutation in p53 does not allow the cell to correct DNA damage or mutations properly, leading to uncontrolled growth and a tumor. α-Mangostin is a p53 activator found in a fruit from Southeast Asia, and when applied to cells, it will arrest them in the S phase. Paclitaxel is a chemotherapy that kills cells as they enter mitosis. Arrested cells will not enter mitosis and therefore will not be killed by paclitaxel. Because of mutated dysfunctional p53, cancer cells are not susceptible to arrest by the p53 activator and therefore can still be killed by paclitaxel. This technique of selectively killing cancer cells while protecting the healthy cells is called chemoprotection. It allows doctors to use higher dosages of chemotherapy without the side effects because of damage to the healthy cells. The purpose of this study is to determine if α-mangostin, a potential chemoprotectant, can be used to protect BHK cells from the cytotoxic effects of paclitaxel.

Because α-mangostin is an anti-proliferative, treating the cells with α-mangostin decreases total cell number in a dose dependent manner. Interestingly, I found that pretreating cells with increasing concentrations of α-mangostin before treating them with paclitaxel increased the total cell number per well when compared to cells treated with paclitaxel alone until the toxic concentration of α-mangostin. This finding indicates that α-mangostin may be a good candidate for a chemoprotectant to be used when treating cancer patients.

INTRODUCTION

The purpose of this research is to determine if the α-mangostin from *Garcinia mangostana* can be used as a natural chemoprotective agent to protect normal cells from being killed by Paclitaxel, a chemotherapeutic agent. α-Mangostin, a small molecule found in the pericarp of mangosteen fruit, is known to bind to a protein called MDM2 to disrupt the p53-MDM2 interaction, an interaction important for cell cycle regulation and tumor suppression (Leão, 2013). Without p53-MDM2 binding, free p53 levels increase which induces cell cycle arrest in healthy cells. (Alberts et al, 2008). In cancer cells with a mutant form of p53, the cells continue to proliferate, regardless of the levels of p53 in the cell (Vogelstein, Lane, & Levine, 2000). Cancer cells with mutant p53 will not arrest and will be susceptible to certain chemotherapeutic drugs. However, cells with wildtype p53 that are pretreated with α-mangostin will arrest and avoid the cytotoxic effects of paclitaxel.

In this study, BHK cells will be treated with varying concentrations of α-mangostin ranging from 0 uM to 20 uM before being treated with 50 nM paclitaxel. The cells will then be counted to compare the cell number at the varying concentrations. Protein will also be collected to perform a BCA total protein assay to supplement the total cell number. This data will show the effects of α-mangostin concentration on the chemoprotective ability. If normal cells can successfully be protected from chemotherapy, oncologists can safely use higher doses of chemotherapeutic agents on cancer patients.

LITERARY REVIEW

Cancer and p53

According the Center for Disease Control, cancer is the second leading cause of death in the United States (Center for Disease Control, 2015). Over half of all cases of cancer involve a

mutant form of protein p53 (Vogelstein, Lane, & Levine, 2000). This biochemical difference allows researchers and doctors to distinguish between normal cells and certain tumor cells. In normal cells, p53 serves as a checkpoint in the cell division process. Tumor suppressor p53 is a transcription factor that responds to various cell stressors by preventing progression through the cell cycle or initiating apoptosis (Vogelstein, Lane, & Levine, 2000). In normal functioning cells, free p53 binds to another protein called MDM2. MDM2 helps catalyze the addition of ubiquitin groups to p53 which tags it for degradation in order to maintain normal p53 levels. When DNA damage occurs, p53 and MDM2 do not bind because amino acids in the binding domain of MDM2 are phosphorylated (Vogelstein, Lane, & Levine, 2000). This results in an increased concentration of free p53 and MDM2 and a decreased concentration of the bound form. The increased concentration of p53 signals another protein, p21, which arrests the cell in the G_1 or S phase and prevents progress in the cell cycle giving the cell an opportunity to repair its DNA. If the DNA is not repaired, apoptosis occurs, also called programmed cell death (Alberts et al, 2008). In many cancer cells, this pathway does not work properly because p53 is mutated. Despite damage to DNA, the cell continues to replicate, resulting in a tumor (Vogelstein, Lane, & Levine, 2000).

Chemoprotection and Nutlins

A promising method for improved cancer treatment is known as chemoprotection or cyclotherapy. The purpose of this method is to treat the cells with a small molecule that allows the chemotherapy to selectively kill cancer cells without harming normal cells. This is beneficial because it will allow doctors to use higher concentrations of chemotherapy on cancer patients because the healthy cells are protected (Van Leeuwin, 2012). An effective category of chemoprotectants disrupts the p53-MDM2 interaction. A small molecule can be used to selectively inhibit the interaction of MDM2-p53 interaction in cells with wildtype p53 resulting in

cell cycle arrest. Cells with mutant p53, a characteristic of the cells in over 50% of cancers cases, continue normal cell cycle progression and are susceptible to the cytotoxic effects of paclitaxel (Blagosklonny, 2003). This is because the tumor cell does not have the correct form of the p53 domain to bind to the DNA and activate tumor suppressor activity, the first step being cell cycle arrest. Tumor cells with mutant p53 should act the same with and without a p53-MDM2 small molecule inhibitor, in this case α-mangostin.

Nutlin was the first discovered molecule to effectively inhibit the p53-MDM2 interaction (Hoe, 2014). Nutlins prevent the interaction of p53 and MDM2 by binding to the p53-binding pocket on MDM2 which activates the p53 pathway by stabilizing the protein (Vasilev, 2004). A study was done using the isoform Nutlin-3a which explored ways to protect normal proliferating cells from cell-cycle specific chemotherapeutic agents such as Paclitaxel and Nocodazole without protecting the mutant cancer cells (Apontes et al., 2011). Other small-molecule inhibitors that have been recognized as potential chemoprotectants include low dose doxorubicin, low dose actinomycin D, tenovin-6, and leptomycin B (Van Leeuwin et. al., 2012).

***Garcinia mangostana* and its properties**

In the tropical environment in Southeast Asia, one can find a fruit commonly known as mangosteen from the tree *Garcinia mangostana.* It is known as the "queen of all fruits" because of its incredible taste (Pedraza-Chaverri, 2008). The pericarp of this fruit contains a family of compounds called xanthones, mainly α-mangostin and ɣ-mangostin. The extract is known to possess a wide variety of pharmacologic properties including anti-tumor, anti-allergic, anti-bacterial, anti-inflammatory and many more (Shan et. al., 2011). α-Mangostin has been found to inhibit p53-MDM2 interaction in yeast cells, specifically at concentrations of approximately 10 uM (Leão, 2013). It is believed that α-mangostin binds to the p53 binding site on MDM2 preventing p53 from binding, a mechanism similar to the known positive, Nutlin-3a. This

inhibition up-regulates free p53 and induces cycle cell arrest by allowing the transcription of tumor suppressing genes (Leão, 2013). This group also showed that α-mangostin did not impact the growth of cells expressing a mutant form of p53 (Leão, 2013). This is important because it indicates that cancer cells with a mutant from of p53 will not be susceptible to the cell cycle arrest typically initiated by disruption of the p53-MDM2 interaction. This property makes it a good chemoprotectant canidate.

Paclitaxel

Cell cycle arrest is essential for selectively targeting cells for death via Paclitaxel. Paclitaxel (PTX) is one of the most widely used chemotherapy agents (Blagosklonny, 2004). PTX is a microtubule-active drug so it kills cells as they enter mitosis (Blagosklonny, 2002). Cells that are arrested due to chemoprotectants such as Nutlin-3 or the α-mangostin will not enter mitosis and therefore will not be killed by PTX. However, the cells cannot survive in the arrested state indefinitely. Eventually, the small molecule and the PTX must be removed and the cell can return to normal function.

MATERIALS AND METHODS

Cells and Reagents

BHK-21 cells were cultured in Dulbecco's Modified Eagle's medium supplemented with 5% Fetal Bovine Serum (FBS), 4 mM L-Glutamine, and 1% penicillin-streptomycin. Hanks Balanced Salt Solution (HBSS) buffered with HEPES buffer was used to rinse cells before lifting. 0.25% Trypsin-EDTA solution was used to lifts cells. Nutlin-3, consisting of a racemic mixture both the 3a and 3b enantiomers with only 3a being active, was stored as 20 mM in DMSO with only 10 mM of active 3a. All Nutlin-3 concentrations are given as concentration of the active form only rather than total Nutlin-3 concentration. α-Mangostin was stored as 10 mM in DMSO.

Paclitaxel was stored as 25 μM in DMSO. In order to determine the appropriate LD_{50} concentration of Paclitaxel to use for treatment, a dose-response toxicity curve was created with concentrations of 0 nM, 25 nM, 50 nM, and 100 nM.

Chemoprotection Cell Culture Assay

Cells were seeded on 12-well plates on day 0. On day 1, the cells were pretreated with Nutiln-3 or α-mangostin at varying concentrations (0 μM, 5 μM, 10 μM, or 20 μM) or vehicle for pretreatment. On day 2, the cells were treated with 50 nM Paclitaxel in addition to the α-mangostin or vehicle. After a day, the cells were examined using an inverted phase contrast microscope and photographed. The cells were then rinsed with HBSS, lifted using Tryspin, and counted using a hemocytometer to determine total cells per well. Once a working protocol was established using Nutlin-3 as the known positive, further experiments were completed using only α-mangostin.

In addition to total cell counts, total protein per well was determined as another indicator of cell abundance. To do this, Cells were washed with HBSS and lysed using 2 N NaOH for 15 minutes at 37°C then the wells were scraped from the well into microcentrifuge tubes. Total protein was measured using the Pierce™ BCA Protein Assay Kit.

RESULTS

Establishing a Working Paclitaxel Concentration

It is important to establish an appropriate concentration of Paclitaxel to treat the cells that will demonstrate paclitaxel toxicity without exhibiting complete cell death leaving. An approximate working concentration of Paclitaxel was gathered from the literature (Apontes et. al., 2011) as 50 nM. A toxicity curve, shown in Figure 1, was created to determine the toxicity of

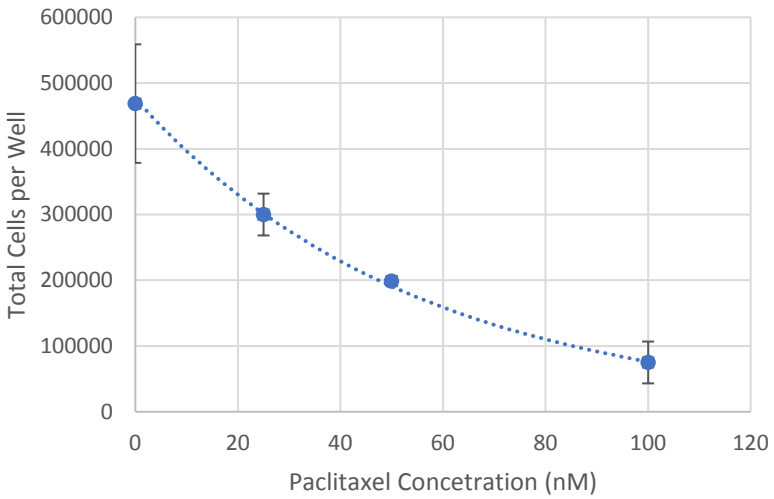

Figure 1: Toxicity Curve for establishing a working concentration of Paclitaxel. Cells are treated with varying concentrations of paclitaxel then counted to determine the number of cells per well.

PXT for the BHK-21 cell line. In the BHK cell line, the 50 nM paclitaxel treatment concentration killed approximately 57% of the cells compared to the control. The 50 nM concentration was selected for treatment in the experiments with chemoprotectants as it exhibited some cell survival despite 57% death. This also aligned with the concentration used in previous studies (Apontes et. al., 2011).

Analysis of α-Mangostin Pretreated Cells

When making observations of the pretreated cells, control cells had significantly more cells than the cells pretreated with α-mangostin. This was expected because α-mangostin arrests the cells and prevents proliferation. Figure 2 shows photographs which display both relative cell number and phenotype of both the control and cells pretreated with 10 uM α-mangostin.

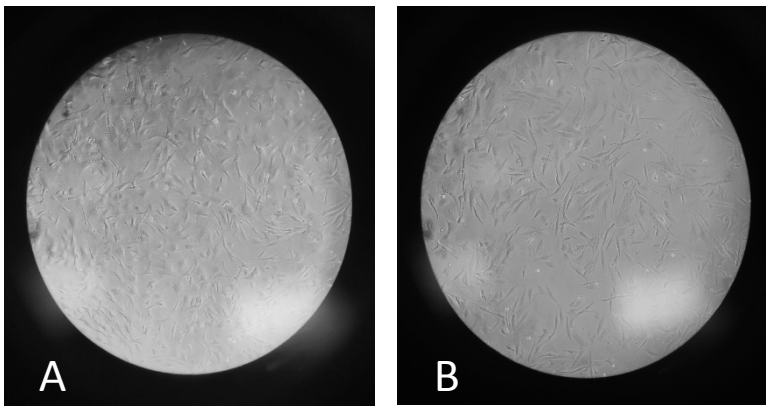

Figure 2: Untreated and 10 uM α-mangostin pretreated cells after 1 day of incubation (100x). Cells were treated with DMSO (A) or 10 uM α-mangostin (B) then photographed under a phase contrast microscope after 1 day of incubation. Both treatments resulted in high cell vitality of cells but the cells treated with α-mangostin had a lower cell number.

Additionally, examining cell numbers of

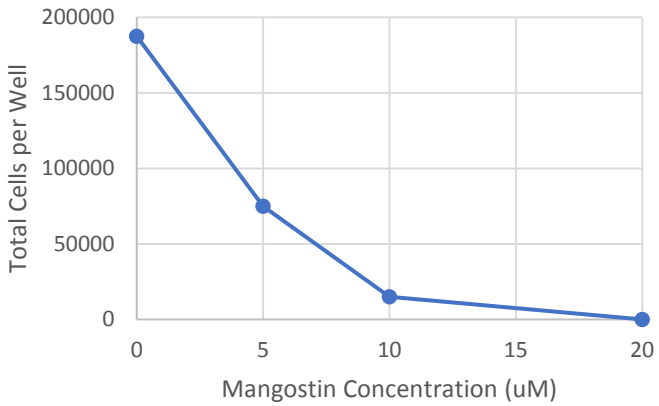

Figure 3: Dose Response Curve for α-mangostin. Cells were treated with varying concentrations of α-mangostin then counted for total cell number. The decrease in cell number with increasing concentrations could be due to inhibition of proliferation or due to cell death.

cells treated with α-mangostin only, Figure 3 shows a dose response curve for cells treated with varying concentration of α-mangostin only. Treatment with 0 uM α-mangostin served as a control and yielded almost 200,000 cells during the one day treatment period. At 5 uM, there were approximately half as many cells as the control, and the 20 uM concentration killed all of the cells. As expected, with increasing α-mangostin concentration, the total cells per well decreases. α-Mangostin is an anti-proliferative agent which explains the decrease in cell replication when exposed to α-mangostin.

Observations of α-Mangostin Pretreated and Paclitaxel Treated Cells

Cells were treated with varying concentration of α-mangostin pretreatments before treatment with 50 nM paclitaxel. When examining the phenotype of the cells receiving no α-mangostin or 5 uM α-mangostin as a pretreatment, there were lots of rounded floating cells indicating a high incidence of death. However, at α-mangostin concentration of 10 uM, the cells were still attached to the well but appeared very granular. Finally, at the highest α-mangostin concentration, 20 uM, there were very few attached cells with many dead floating cells. This data, in addition to the α-mangostin dose response curve from Figure 2 indicate that 20 uM concentration of α-mangostin is toxic to the cells.

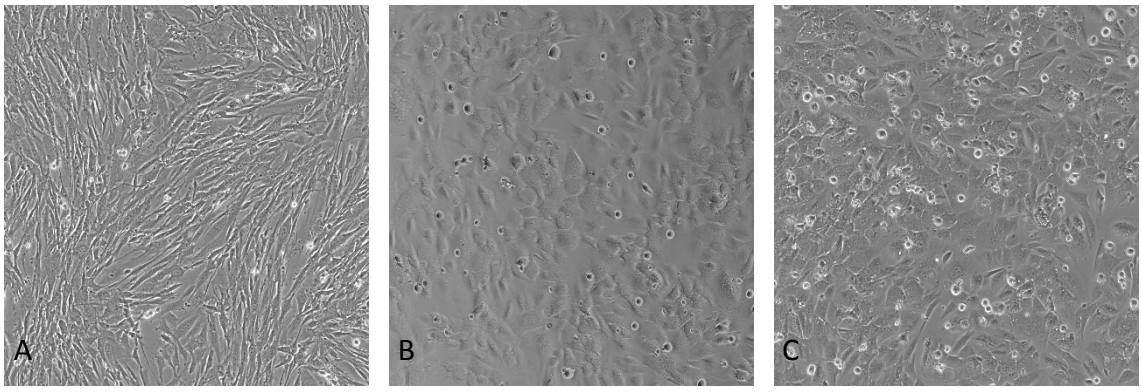

Figure 4: Cells treated with DMSO control, Paclitaxel, and 10 uM α-mangostin and Paclitaxel (100x). Cells were treated with DMSO only (A) or paclitaxel only (B) for 1 day or treated with α-mangostin on day 1 with the addition of paclitaxel on day 2 (C) then photographed using a phase contrast microscope.

Figure 4 displays a representative image of the DMSO control, cells treated with paclitaxel only, and cells treated with 10 uM α-mangostin before paclitaxel. The control appears to have the greatest number of live cells with very few dead in this field of view. While there are many dead cells in both the paclitaxel treated and the 10 uM α-mangostin pretreated before paclitaxel treatment, the paclitaxel only treatment appeared to yield many fewer attached cells than the cells that received the pretreatment. The cells treated with α-mangostin before paclitaxel treatment (C) appear more round and granular than the DMSO control (A). The rounded appearance is typical of arrested cells (Apontes et. Al., 2011).

Cell Counts of α-Mangostin Pretreatment and Paclitaxel Treatment

Cells were treated with varying concentrations of α-mangostin then 50 nM paclitaxel to create the representative dose response curves displayed in Figure 5. Treatment with 0 uM α-mangostin averaged approximately 250,000 cells per well which serves as a control for cell numbers from paclitaxel alone. As α-mangostin concentration increased, the cell numbers also

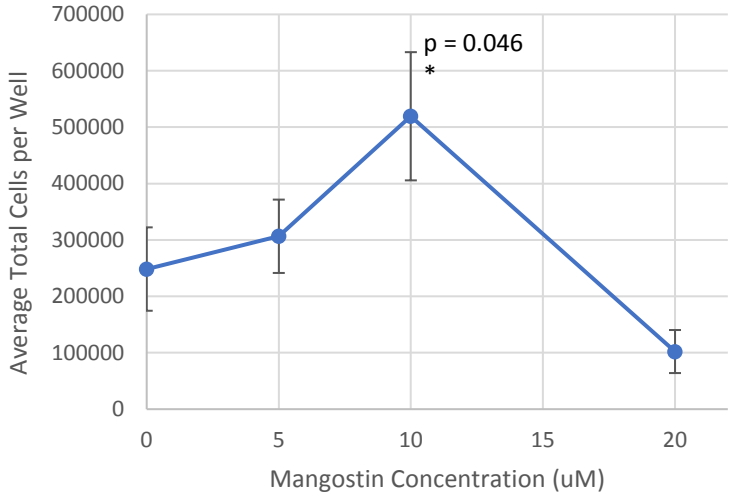

Figure 5: Dose response curve for cells pretreated with α-mangostin and paclitaxel. Cells were pretreated with varying concentrations of α-mangostin for 1 day then treated with both α-mangostin and paclitaxel for an additional day. The cells were lifted and counted to determine total cells per well. The protocol was replicated 4 times and plotted as average total cells per well as a function of α-mangostin concentration.

increased. However, at 20 uM α-mangostin, there was a sharp decrease in cell numbers indicating toxicity due to the α-mangostin rather than paclitaxel. The average total cells per well demonstrated a significant increase in cell number when compared to the paclitaxel control.

In order to obtain a closer approximation of the toxic concentration of α-mangostin, a more extensive dose response curve, shown in Figure 6, was created using additional concentrations of α-mangostin. Although not perfect, the dose response curve in Figure 6 had a shape that mimicked the dose response curve in Figure 5 but shows a decrease in cell number after 17.5 uM rather than 10 uM. The α-mangostin was effective in protecting cells from paclitaxel induced death in a dose dependent manner up to 17.5 uM.

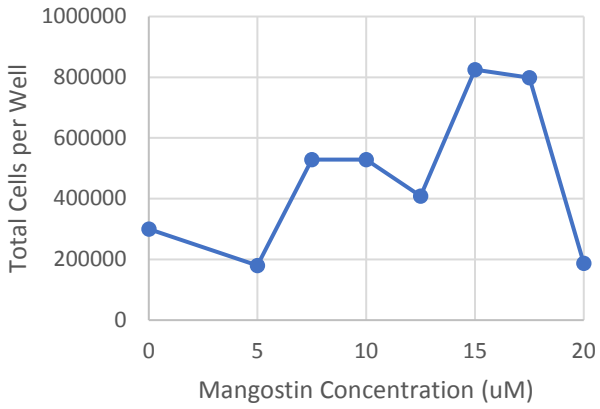

Figure 6: Extended dose response curve for cells pretreated with α-mangostin and paclitaxel. Cells were pretreated with a more extensive array of α-mangostin concentrations for 1 day then treated with both α-mangostin and paclitaxel for an additional day. The cells were lifted and counted and plotted as total cells per well as a function of α-mangostin concentration. The pattern resembles the pattern in Figure 5.

Comparison of Nutlin-3a and α-Mangostin Pretreatment before Treatment with Paclitaxel

Nutlin-3a was used as a known positive to confirm that α-mangostin is an effective chemoprotectant. It has previously been established that active isomer of Nutlin-3, specifically Nutlin-3a, is a wildtype p53 inducer and can therefore arrest the cells and prevent lethality due to paclitaxel (Apontes et. al., 2011). In order to see if the assay protocol including pretreatment and paclitaxel concentrations as well as treatment time, Nutlin-3a and α-mangostin were run side-by-side and their results were compared to see if they resemble one another. Figure 7 shows both a Nutlin-3a and α-mangostin dose-response curves. They have comparable responses including the greatest fold induction at the 10 uM concentration. In addition, they had similar cell numbers as both treatment regiments generating close to 600,000 cells per well at 10 uM concentrations. This data gives reason to believe that α-mangostin protects the cells from the lethal effects of paclitaxel similar to Nutlin-3a.

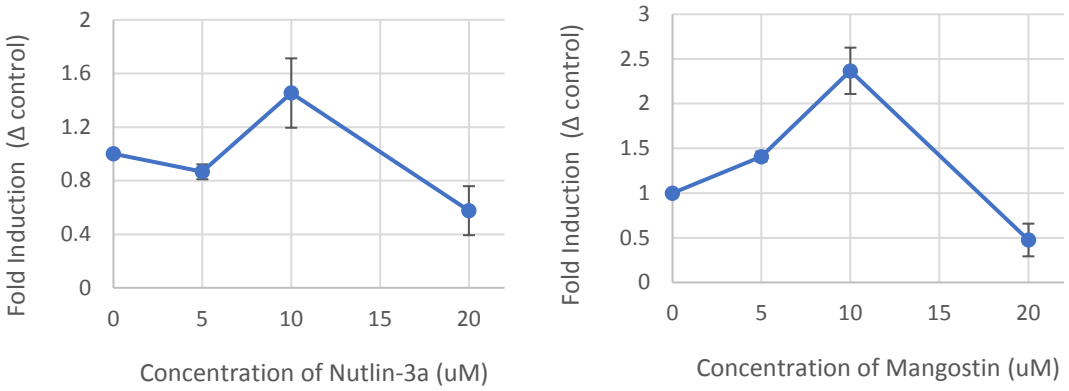

Figure 7: Comparison of Nutlin-3a and α-mangostin dose response curves. Cells were treated with varying concentrations of chemoprotectants (Nutlin-3a (A) or α-mangostin (B)) for one day then treated with chemoprotectant and 50nM paclitaxel for an additional day. The cells were lifted and counted then compared to the control and plotted in response to changing chemoprotectant concentration. Results are shown as fold induction relative to control treated with paclitaxel only.

Total Protein of Cells Receiving α-Mangostin Pretreatment and Paclitaxel Treatment

Total protein concentrations were gathered using the BCA Assay to supplement the total cell count. Total protein often mimics the total cell count dose response curve but can

provide insight on the size of the cell as well. A relatively high total protein concentration but a relatively low total cell count can indicate that the cells are growing and increasing size and

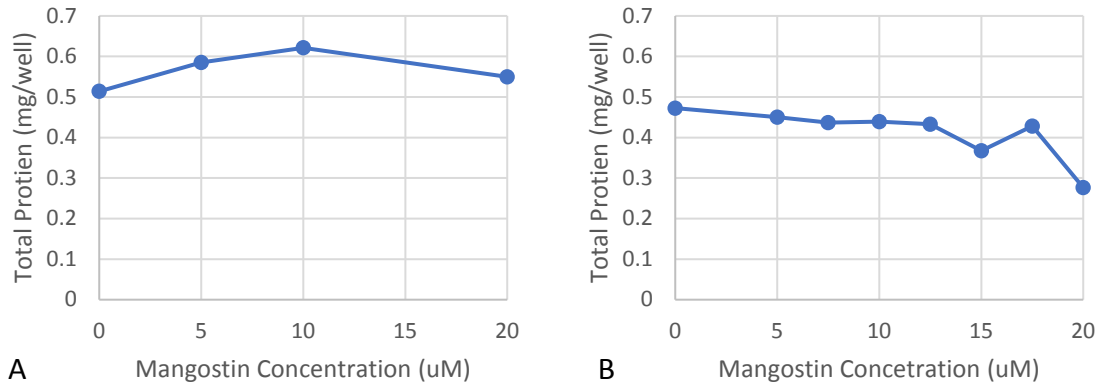

Figure 8: Total protein dose response curves for cells pretreated with varying α-mangostin concentrations before treatment with paclitaxel. Cells were pretreated with α-mangostin for one day and then treated with α-mangostin and paclitaxel for an additional day. Cells were lysed using NaOH to obtain total protein. The total protein was analyzed using the BCA method. The total protein per well is represented as a function of the α-mangostin concentration.

number of proteins without dividing. Figure 8 uses the total protein to measure the dose response of cells to changing α-mangostin concentration. In Figure 8A, the shape of the curve mimicks the dose response curve shown in Figure 5 but less dramatic. Both curves demonstrate a relatively constant total protein concentration which is expected because the cells can continue to produce proteins at the same rate even if they cannot divide. Figure 8 also shows a decrease in protein after 10 uM in 8A and after 17.5 uM in 8B which is the same concentrations that demonstrated a decrease in cell counts in Figure 6, further confirming the fact that the cells are experiencing toxic effects from the α-mangostin at these concentrations.

DISCUSSION

The goal of chemoprotectants is to find a way to selectively kill cancerous cells while keeping normal healthy cells intact. This will allow doctors to use high dosages of chemotherapies because the chemoprotectant pretreatment can attenuate the side effects

associated with chemotherapeutic drugs. The purpose of this study was to determine whether α-mangostin can be used to protect normal proliferating cells from paclitaxel induced cell death by inducing cell cycle arrest. Pretreatment with α-mangostin before paclitaxel treatment increased total cell numbers compared to cells treated with paclitaxel alone in a dose dependent manner until reaching a toxic concentration. The increase in cell number per well was significant at a concentration of 10 uM α-mangostin. This is demonstrated in Figure 5 as the p-value at 10 uM was 0.046 when compared to control. There was also trend indicating a toxic dosage as there were much fewer cells at the 20 uM concentration.

The interesting thing to note when examining total cell numbers throughout this study is that α-mangostin is an anti-proliferative agent because it binds MDM2 and induces p53 which arrests the cell (Vogelstein, Lane, & Levine, 2000). Cells exposed to higher doses of α-mangostin should have fewer cells because they are not actively growing and dividing. This makes the increase in total cell number with the increase in α-mangostin concentration even more interesting. Additionally, the total protein concentration remained relatively constant but did increase slightly as the α-mangostin concentration increased in Figure 8A. This indicates that the cells continue to produce proteins, regardless of whether they have been exposed to α-mangostin.

Comparison with the known positive, Nutlin-3a, provided insight into the effectiveness of α-mangostin in this study. It has previously been shown that Nutlin-3a can protect cells from the cytotoxic effects of chemotherapies (Apontes et. Al., 2011). Nutlin-3a also appears to demonstrate some adverse effects at effective concentrations above 20 uM and effective concentrations below 4 uM demonstrating no significant antiproliferative effects (Van Leeuwin et. al., 2012). This adds validity to the effectiveness of α-mangostin to accomplish the same goal

because the fold induction increased at the 10 uM concentration in both the Nutlin-3a and α-mangostin as demonstrated in Figure 7.

This study has important clinical implications. If α-mangostin can be an effective chemoprotectant and allow normal cells to proliferate without protecting cancerous cells with a mutant form of p53, it can be a very significant pharmacological tool for treatment of cancer patients. The concentration of paclitaxel used in this study approximates the dose of paclitaxel that a cancer patient would receive during treatment so this findings in this study can easily translate to a clinical setting. However, this study is not complete. The same chemoprotection cell culture assay must be performed on cancerous cells with a p53 mutation to see if α-mangostin is ineffective in protecting them. In order for α-mangostin to work as an effective chemoprotectant, it must not protect the cancerous cells because they still need to be susceptible to paclitaxel induced cell death. Nutiln-3a is an already known chemoprotectant but α-mangostin would provide a natural option to accomplish the same goal. With as many drugs and chemicals that are administered to cancer patients for treatment, a natural option may provide some ease of mind and avoid some unwanted side effects associated with synthetically-made chemicals such as Nutlin-3a. α-Mangostin is already found in many multi-vitamins and dietary supplements because of its other beneficial properties (Pedraza-Chaverri, 2008). Drug developers are constantly looking to repurpose compounds that have already been approved by the FDA because it allows for a shorter clinical trial period. α-Mangostin is a promising, relatively unexplored compound that should be further pursued for its chemoprotective properties.

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