ABSTRACT

Synergistic Effects of Hydroxychloroquine on the Activity of Thiomaltol against Melanoma Cancer Cells

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Although melanoma accounts for a very small percentage of all skin cancer cases, it is the most responsible for skin cancer related deaths. Therapeutic agents that induce apoptosis in the cancer cells have been heavily researched. Some recent research has focused on enhancing the effects of apoptotic agents by blocking autophagy, a cancer survival mechanism where the cells digest themselves. Research has shown that inhibiting this pathway with certain drugs can lead to cell death at a greater rate than by using apoptosis inducing agents alone. However, the universal application of blocking autophagy is debated by most researchers. The main question this study seeks to answer is whether the effects of an autophagy blocking agent used alongside an apoptosis inducing drug will increase cell death at levels higher than using the drug alone. The apoptotic drug used is thiomaltol (Htma). The autophagy blocking agent used is hydroxychloroquine (HCQ). The two compounds are tested against B16F1 melanoma cells from mice. Overall, data indicates that although the activity of Htma in these cells is not as high compared to other cell lines, there is an increase in cell death when both Htma and HCQ are administered. This increase in activity is higher when the compounds are administered over an extended period of time.

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SYNERGISTIC EFFECTS OF HYDROXYCHLOROQUINE ON THE ACTIVITY OF THIOMALTOL AGAINST MELANOMA CANCER CELLS

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By

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CHAPTER ONE

Introduction

The Scope of the Melanoma Problem

Melanoma has received a good deal of attention over the past two decades. Despite having a low incidence rate, 1 in 51 skin cancer cases, it is the leading cause of skin cancer-related deaths (1). Rise in incidence in recent years has been attributed to global climate changes and the depletion of the ozone layer (2). Treatment of the disease is limited to surgery, a process which is only effective during early stages of pathological development. The high risk nature of melanoma has thus caused increased research attention focused on non-invasive chemotherapy treatment.

The cancerous cells arise from mutated melanocytes which are responsible for producing the pigment melanin. Melanoma cells are inherently drug resistant even more so than other cancers, so developing effective treatment proves problematic. Drug resistance is attributed to the redox chemistry of the cells. This principle is best understood by examining normal function of the melanocyte with particular attention to the role of melanin and then comparing this physiology with the aberrant nature of melanoma.

The Role of Melanin

Melanin in melanocytes is contained within organelles called melanosomes. Melanin acts as a UV filter for the body. The compartmentalization of the melanin from other cellular contents separates harmful species that arise as the byproducts of melanin

production from the cytoplasm and the extracellular space. Melanosomes also sequester metal ions which are neutralized via redox pathways involving melanin in an attempt to mediate chemical stress (3). In this regard, melanin acts as an antioxidant, reducing potential oxidative processes that would otherwise disrupt normal cell physiology. However, the primary role of melanin is UV blockage, not antioxidant activity.

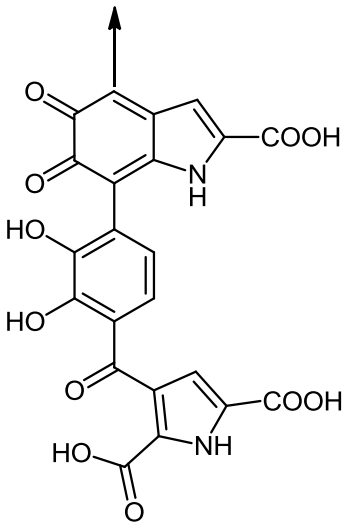


Figure 1- Structure of Melanin

Reactive oxygen species (ROS) are at higher than normal concentrations in melanocytes as a result of the cell's role in pigment generation. As long as antioxidants in the cells can keep pace with ROS production, the cell will function normally. However, when the antioxidant to ROS balance is disturbed, ROS accumulation will occur. This is a hallmark characteristic of melanoma further aggravated by the unique chemistry of melanin which can potentially act as a pro-oxidant capable of producing excess ROS (4). Increased level of metal uptake promotes this pro-oxidant role and can lead to redox cycling. Essentially, the cell is trapped in a continuous cycle where melanin oxidizes chemical species to produce ROS, is in turn oxidized, then starts the cycle again. The excess ROS will not be

neutralized because production outpaces antioxidant capacity. Thus ROS is left to react with DNA and other important cellular substrates. Moreover, increased levels of ROS activate proto-oncogenes which cause unmediated cell growth and contribute to drug resistance (5). In conjunction with UV radiation and genetic disposition, an increase in ROS levels is thought to be one of the contributing factors that lead to the conversion of melanocytes to melanoma.

The comparison between normal melanocytes and melanoma cells can be simply defined by melanosome function. Normal melanosomes scavenge ROS while cancerous melanosomes generate ROS (5). Thus, most drug treatment research has focused on targeting ROS. Early efforts focused on restoring normal ROS scavenging in deviant melanocytes by increasing antioxidant levels (5). This would eliminate high levels of ROS and return the cell to a normal redox state. However, recent studies have attempted deplete ROS scavenging. This causes an extreme amount of oxidative stress in the cell and thus triggers the intrinsic apoptotic pathway.

Apoptosis and the Role of the Mitochondria

Apoptosis can be defined as programmed cell death (PCD), although the two are not necessarily synonymous. The intrinsic apoptotic pathway features what is general termed the "caspase cascade." Caspases are enzymes which cleave cellular substrates when activated. In mammalian cells, mitochondria play a significant role in initiating the caspases that commit the cell to the apoptotic pathway.

The mitochondrion, which consists of two bilayer membranes, contains several important proteins, known as apoptotic factors, that control caspase activation. One of

these apoptotic factors is cytochrome c. The cytochrome c protein binds to apoptotic protease activating factor 1 (APAF1) in the presence of deoxyadenosine triphosphate (dATP) to form an apoptosome (6). The apoptosome activates caspase 9 which in turn activates caspase 3. Caspase 3 activates enzymes responsible for substrate and DNA cleavage. The inner membrane space also contains apoptotic factors that block the activity of inhibitor of apoptosis proteins (IAP's) and activate endonucleases which are independent of the caspase cascade. The variety of apoptotic factors present in the mitochondrion has led some to call the inner membrane space the "Pandora's Box" of apoptosis.

In non-apoptotic cells, the contents of the inner membrane space are kept separated from the cytoplasm. When the cell commits to apoptosis, the contents of the mitochondria are emptied due to an increase in membrane permeability. This increase in permeability is attributed to the activity of caspase 2 (7). One method that is used to quantify increased permeability is to measure the loss of mitochondrial membrane polarization (MMP) (8). The loss of polarization is a result of increasing permeability of the outer membrane. The inner membrane space is filled with protons which are used in oxidative phosphorylation. Thus, once the membrane becomes permeable, the protons travel down their electrochemical gradient and flow out of the inner membrane space causing a drop in membrane potential. Measuring the loss of polarization allows researchers to predict the relative level of apoptosis efficiencies in cells they are drugging.

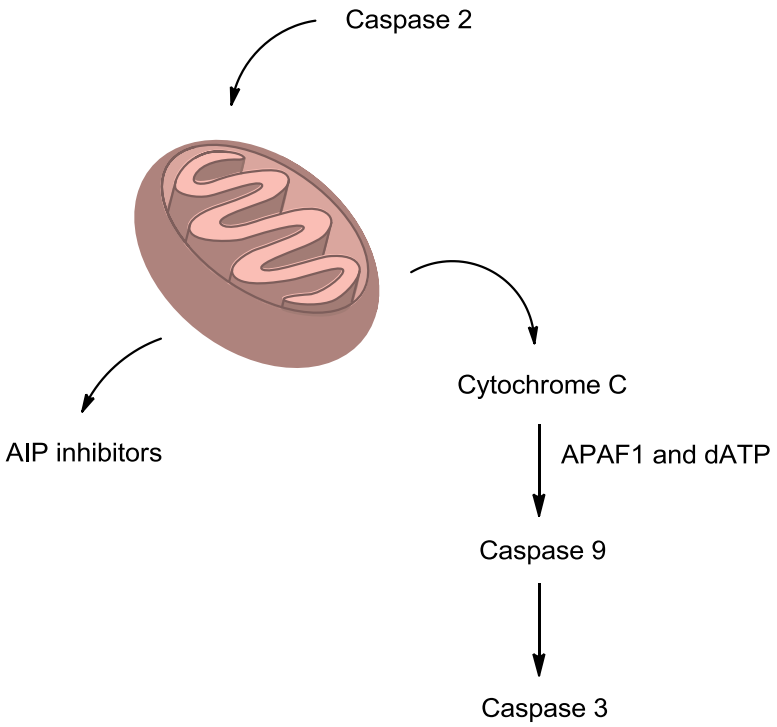


Figure 2- Intrinsic Apoptosis (Caspase Cascade)

Antioxidants in Melanoma

A brief mention of antioxidant function is necessary in order to understand early research as well as place current studies in perspective. Normal melanocytes have good safeguards in place to handle the increased levels of ROS associated with melanosomes function. The cells typically have an abundance of antioxidants that help mediate redox activity. These species react with ROS, metal ions, and other radicals, thus alleviating chemical stress. Two antioxidants that are important in cellular physiology are N-acetyl-cysteine (NAC) and glutathione (GSH). Of the two, GSH has attracted more attention because of its important role in maintaining a functional redox state in melanocytes. One measure that has been developed in order to quantify the redox status of cells is to

measure the ratio of GSH to its oxidized analog glutathione disulfide (GSSG), or in mathematical terms, $\frac{[GSH]}{[GSSG]}$ (9). As the ratio decreases, the concentration of ROS increases. Research studies have sought to eliminate GSH in melanoma in order to achieve ROS levels capable of inducing apoptosis.

The Disulfiram Discovery

One of the earliest compounds whose ability to increase ROS levels in melanoma was disulfiram. Disulfiram (DSF) is a member of the dithiocarbamate family. Its potential for use as an anti-cancer agent stems from its long time use as an alcohol deterrent (10). When administered to cell cultures, DSF induces apoptosis. Thus, it has received a great deal of attention as a potential melanoma drug.

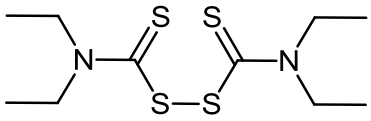


Figure 3- Disulfiram

The mechanism of action for DSF is not known definitively. However, there seem to be two main lines of thought. First, DSF induces apoptosis by oxidizing GSH which then oxidizes a thiol residue integral in the mitochondrial membrane (11). This oxidation causes apoptosis repressors to be released from the membrane and the membrane to increase in permeability thus initiating the caspase cascade of intrinsic apoptosis. Second, DSF competes with GSH for the active site of GSH reductase (5). This diminishes the

antioxidant abilities of GSH in the cells, causing increased ROS levels and ultimately apoptosis.

The success of DSF as an anti-cancer drug was very promising. Studies showed the small doses 0.09-0.17 μM DSF was enough to induce cell death (a combination of apoptosis and necrosis) in levels of 400-600% of control groups (11). However, as studies developed, new methodology was used in order to maximize the apoptotic effects of DSF.

DSF Coordination with Copper II

As mentioned previously, melanocytes tend to sequester metal ions. The increase in metal ion concentration increases melanin promotes oxidation and redox cycling of melanin (4, 12). Extracellular trace metals, which tend to be divalent, such as zinc, iron, and copper therefore could be used to alter the redox status of melanoma if they were transported into the cytoplasm.

DSF has four terminal sulfurs which are highly polarizable. Therefore, DSF is a good ligand for soft metals, such as copper (II) (Cu^{2+}). DSF will tend to form coordination complexes in the presence of metal ions. These coordination complexes tend to be lipophilic and can passively diffuse through the cell membrane (13).

Divalent copper has proven to be the most effective metal ion with regards to chelating DSF, so it has been the most studied. The coordination complex that DSF forms with Cu^{II} is copper (II) diethyl-dithiocarbamate, or $Cu^{II}(deDTC)_2$.

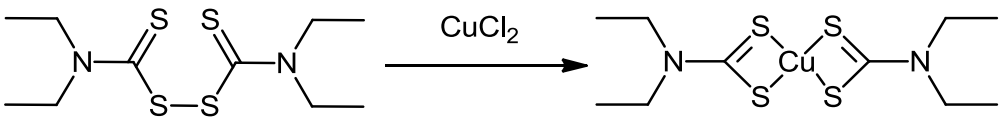


Figure 4- $Cu^{II}(deDTC)_2$ Formation

Research shows that adding $CuCl_2$ and DSF to cell media causes higher rates of apoptosis than DSF acting independently. In one such study, melanoma cultures drugged with DSF exhibited viability at 25% of the control while cultures drugged with DSF and 1 μM or $CuCl_2$ exhibited viability at 4.2% of the control (14). The study demonstrated that there is a linear trend between DSF-Cu^{II} complex formation and cell death verifying that Cu^{II}(deDTC) is the active complex involved in stimulating apoptosis and necrosis. Typical cycles for these studies last 72 hours.

It is worth mentioning one point here that has been contested. One recent study has shown that the activity of $Cu^{II}(deDTC)_2$ does in fact stimulate apoptosis but not through the intrinsic pathway; rather it has been suggested that the complex stimulates the extrinsic pathway of apoptosis by activating caspase 8 (15). In the experiment, the addition of Z-IED-FMK, which inhibits caspase 8, protected melanoma against apoptosis. However, given the newness of this finding and the evidence to suggest that apoptotic death in $Cu^{II}(deDTC)_2$ drugged melanoma cells occurs via intrinsic pathways as discussed in the next section, it seems as though more studies will be needed to corroborate this claim.

Other Thione Drugs

The positive results of DSF research led to an increase in search for similar thione containing drugs with similar activity. One such compound is elesclomol. When elesclomol (STA-4783) is administered with paclitaxel it has been shown to increase levels of ROS in melanoma (16) as well as in other cancer lines, such as breast cancer (17). The increased levels of ROS have been observed at levels high enough to induce

apoptosis. Moreover, the addition of NAC has inhibited cell death, proving that the ROS generation by elesclomol is responsible for initiating apoptosis (16). Elesclomol is currently in phase III clinical drug trials.

Another class of compound that has received attention for anti-melanoma activity are derivatives of 3-hydroxy-2-methyl-4-pyrone, commonly known as maltol or Hma. It has been shown that reaction with P_4S_{10} can form thiomaltol (Htma) and dithiomaltol (Httma) through a Michael addition reaction pathway (18).

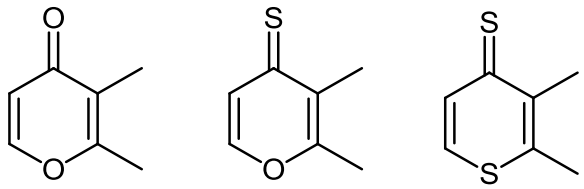


Figure 5- Maltol, Thiomaltol, and Dithiomaltol

Both Htma and Httma can coordinate with divalent copper in a two-to-one ratio like DSF (9, 13). This gives complexes of the form $Cu^{II}(tma)_2$ and $Cu^{II}(ttma)_2$ respectively for Htma and Httma. However, the use of maltol derivatives has intrinsic benefits that are not afforded by DSF.

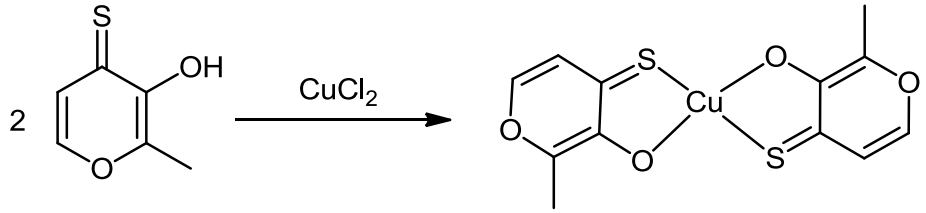


Figure 6- $Cu^{II}(tma)_2$ Formation

Thiomaltol, Dithiomaltol, and Ligand Cycling

These two compounds are the most studied thus far in the realm of maltol derivatives. The IC_{50} value for $Cu^{II}(tma)_2$ is equal to 1.45 μM while the IC_{50} for

$Cu^{II}(ttma)_2$ is 0.20 μM (9,13). These values were obtained by administering the drugs to A135 melanoma cells. There are several key differences that distinguish Htma and Httma from DSF, the most important being that toxicity is directly proportional to the concentration of free metal (9). It is most toxic in the presence of Cu^{II} having the lowest IC_{50} compared to the values for other divalent metals complexes (13). The ligand is ideal because it forms a neutral coordination complex at physiological pH and can passively diffuse through the cell membrane.

It seems thus far that DSF and the subsequent $Cu^{II}(deDTC)_2$ complex would be more effective than the analogous complexes of Htma and Httma based on comparison of IC_{50} data. However, Htma and Httma have a faster response time than does DSF. While DSF can take 72 hours to achieve maximum apoptotic effect, Htma and Httma take between 6 and 24 hours (9, 13). This can be attributed to ligand cycling. The postulate for ligand cycling is based upon the difference between extracellular and cytoplasmic copper. Free copper in mammalian cells is usually Cu^{I} (19). Moreover, extracellular Cu^{II} must be reduced to Cu^{I} in order to enter the cell (20). When Htma coordinates with Cu^{II} in the extracellular space and crosses the cell membrane, the Cu^{II} is reduced to Cu^{1} and loses its affinity for the thione chelators (9). This allows the ligand to leave the cell and continue the process of metal trafficking.

Autophagy: A Survival Mechanism

Under normal circumstances, cancer cells receive nutrition from circulatory supply. However, once cell division outpaces the supply, the cells no longer have the necessary materials to continue growing. One process that has proven to be important to

the survival of the cells in such situations is autophagy. The autophagic process involves the digestion of cytosolic substrates in autolysosomes which are lysosomes fused to autophagosomes (21). These organelles sequester and subsequently digest intracellular proteins, making essential amino acids available for cellular function. This process mediates metabolic stress and contributes to drug resistance.

The role autophagy plays in cancer cells is unique, so it is a targetable characteristic that researchers can use. The scope of such studies lies in blocking the pathway in order to increase the potential for cell death. Research shows agents that block autophagy increase the likelihood of tumor death.

Hydroxychloroquine

Chloroquine (CQ) and hydroxychloroquine (HCQ) have been two very well-studied modulators of autophagy. While these compounds are widely used as anti-malarial drugs, they have been effectively used to block autophagy and increase the rates of cell death when administered alongside an apoptotic inducing drug in several different types of cancer (22). Of the two, this study deals with HCQ.

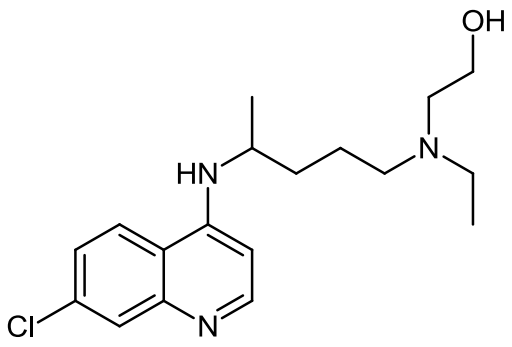


Figure 7- Hydroxychloroquine

Cells tend to sequester HCQ in lysosomes, so the compound acidifies the organelle's contents, thus denaturing the essential enzymes necessary for autophagic digestion (22). While there is evidence that this drug increases the rate of apoptosis, there is limited evidence of its effects when used synergistically with the thione drugs discussed previously. Thus this study seeks to gain insight into the potential syngergism in using both HCQ and one of the derivatives of the thione apoptosis inducing agents, Htma.

CHAPTER TWO

Methods and Procedures

Scope of the Study and Procedures

The scope of the methodology is two-fold. First, Htma is administered to B16F1 cells in order to study the compounds's therapeutic effects. Second, the syngersim of hydroxychloroquine is analyzed. The procedures and general cell drugging procedures are listed herein.

Synthesis of 3-Hydroxy-2-methyl-4H-pyran-4-thione (Htma)

A 4.0 g sample of maltol (Hma) and a 3.5 g sample of P_4S_{10} were weighed out and combined in a round bottom flask in a dry box. The flask was brought out of the glove box and put under an N_2 atmosphere. The solids were dissolved in 25 mL of dry 1,4-dioxane. The solution was stirred and then brought to reflux for 30 min. Over the course of reflux, the solution changed from lime green to orange. The solution was filtered leaving a lime green solid in the reaction flask. The solid was then dissolved in water (200 mL) and allowed to precipitate to yield an orange solid. The solid was collected by filtration, and precipitated again from $CHCl_3$ to give 500 mg of final product (18% yield).

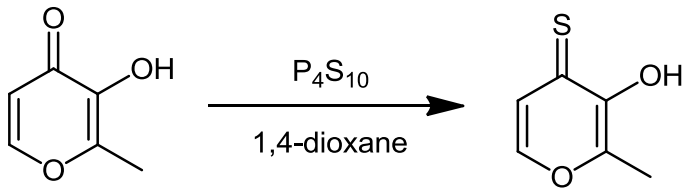


Figure 8- Htma Synthesis

Cell Preparation and General Procedures

The melanoma cells used in the studies were from a B16F-1 cell line. The cells were cultured in a 5% fetal bovine serum (FBS). The cells were pelleted out and suspended in phosphate buffered solution (PBS) for counting purposes as well as dividing cells for treatment. The PBS suspended solutions were added to FBS for incubation and drug administration.

Drug Administration

The cells were divided in 3 mL wells so that there were 15,000 cells per well. The compounds tested were delivered to the cells in the medium using DMSO as solvent. After administration, the cells were incubated at 37°C, 40% humidity, and 5% CO_2.

Cell Counting Assays

Live cell concentration and viability were obtained using a cellometer using AOPI as stain. A sample of 20 μL of the PBS suspended cell solution was mixed with 20 μL of AOPI for counting. After drugging, the cells were counted using a FD Canto II flow cytometer using PI as stain. The cells were pelleted out of solution and then suspended in 600 μL of PBS. The solution was then divided into two; one was stained with 3 μL of PI. The data was analyzed using FloJo software. The data for viability was used for analysis.

Htma and HCQ 12 Hour Synergistic Studies

The first experiment seeks to identify the effectiveness of the Htma against B16F1 and the potential synergy over the course of a 12 hour drugging cycle. From the data, IC_{50}

values for the Htma can be calculated. Concentrations of Htma of 0.4, 0.8, and 1.2 μM were chosen in conjunction with 10 μM HCQ. Cells were drugged with the various dosages of Htma, HCQ, and then a combination of the two. The viability of the control groups prior to division for each trial ranged from 52.5% to 62.5%. Three trials were performed.

Htma and HCQ 72 Hour Synergistic Studies

A previous experiment done over the course of 72 hours showed a significant increase in synergism. Therefore, two additional studies were performed that closely mirrored the initial study. The concentrations of Htma were 0.8 μM and 1.6 μM. The concentration of HCQ was 10 μM again. The viability of the control group prior to division for each trial ranged from 52.5% to 65%. Overall, data from the initial trial and the two additional trials were used to qualitatively observe the synergism over an extended period of time.

CHAPTER THREE

Results

Htma and HCQ 12 Hour Studies Results

The data from the 12 hour study is presented below. Table 1 shows the trial data as well as averages. The standard deviations for each of the trial groups are given as well. The values are given as percentages.

Table 1- Htma and HCQ 12 Hour Study Trial Data

Viability (percentage)					
	1	2	3	AVG	STD
No Teatment	59.3	62.9	50.2	57.5	5.3
DMSO	50.9	54.1	44	49.7	4.2
10 uM HCQ	62.9	51.2	none	57.1	5.9
0.4 uM Htma	56.7	59.8	51.6	56.0	3.4
0.8 uM Htma	53.6	55.8	47.6	52.3	3.5
1.2 uM Htma	40.3	51.9	49.7	47.3	5.0
0.4 uM Htma + HCQ	42.8	59.7	45.5	49.3	7.4
0.8 uM Htma + HCQ	51.9	47	42.1	47.0	4.0
1.2 uM Htma + HCQ	37.7	47.5	40.8	42.0	4.1

Linear regression of the data for the cell groups treated with Htma alone give a best fit line of the form given below. The IC_{50} using this equation gives a value of 2.9 μM.

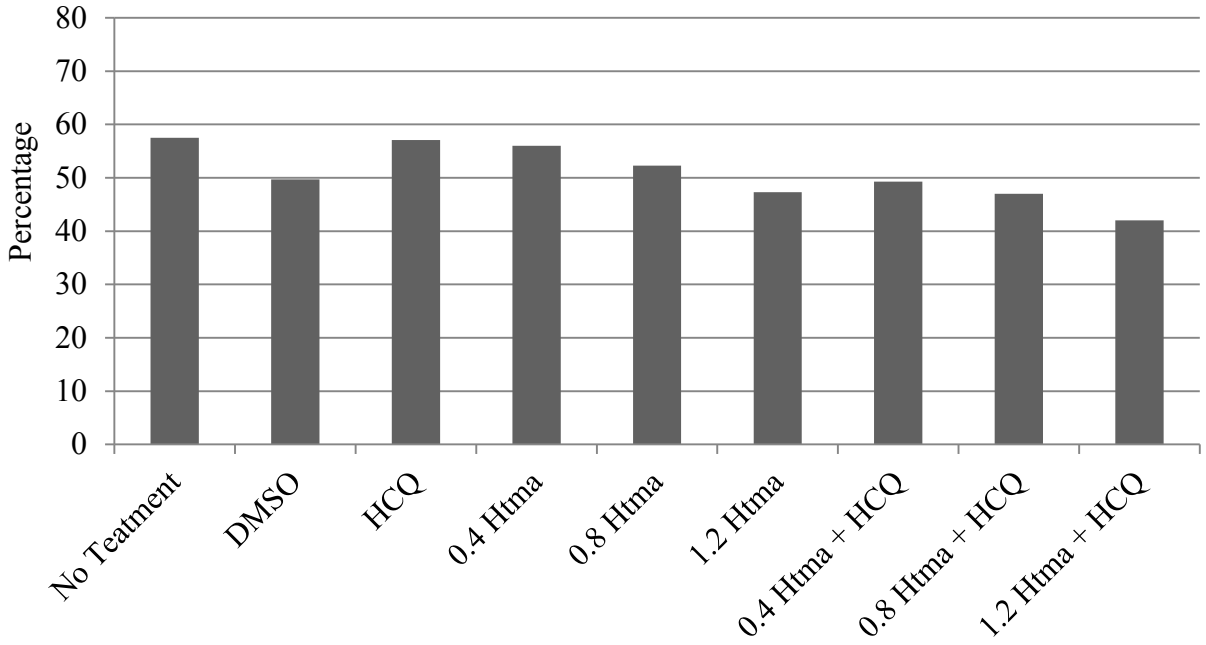
$$y = -10.875x + 60.567 \quad R^2=0.996$$

Linear regression of data for the cell groups treated with Htma and HCQ give a best fit line of the form below. The IC_{50} using this equation gives a value of 2.7 μM.

$$y = -9.125x + 53.4 \quad R^2= 0.956$$

The viability and cell death of the average of the trials is represented graphically as is shown in Figure 9.

Figure 9- Htma and HCQ 12 Hour Study



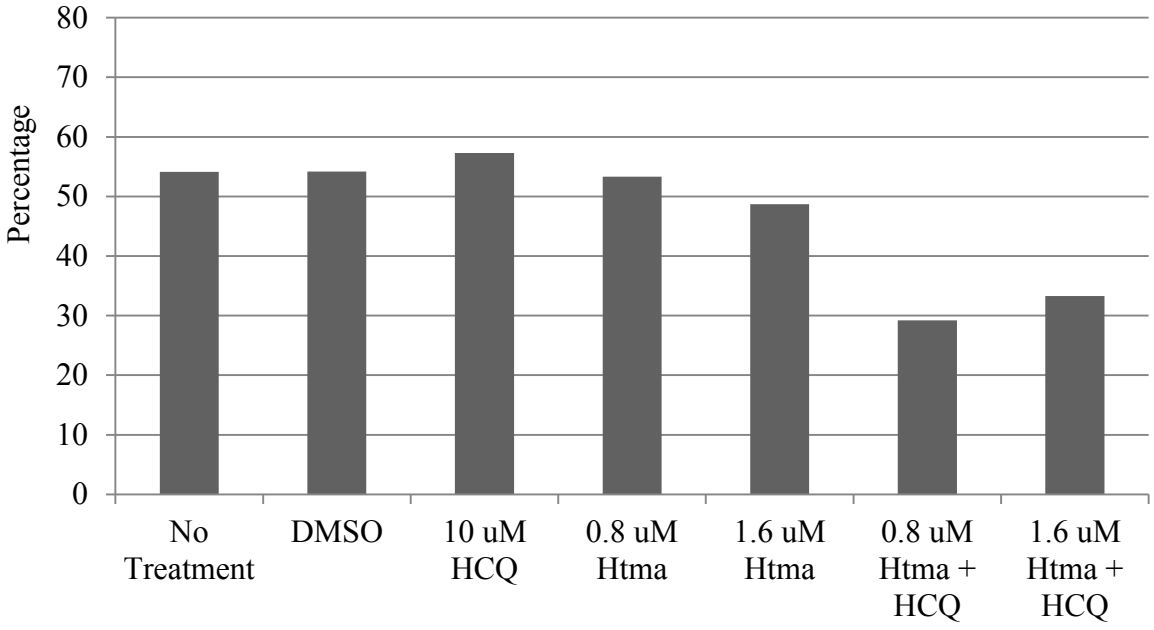
Htma and HCQ 72 Hour Studies Results

The data from the 72 hour study is presented on the next page. Table 2 shows the trial data as well as averages. The standard deviations for each of the trial groups are given as well. The values are given as percentages.

Table 2- Htma and HCQ 72 Hour Study Trial Data					
Viabile (percentages)					
	1	2	3	AVG	STD
No Treatment	33.7	63.2	65.3	54.1	14.4
DMSO	45.5	59.6	57.5	54.2	6.2
10 uM HCQ	54.2	52.7	65.1	57.3	5.5
0.8 uM Htma	47.1	57.8	55.7	53.5	4.6
1.6 uM Htma	26.3	58.4	61.3	48.7	15.9
0.8 uM Htma + HCQ	8.9	38.3	40.3	29.2	14.4
1.6 uM Htma + HCQ	14	45.5	40.3	33.3	13.8

The data can again be represented graphically as is shown in Figure 10.

Figure 10- Htma and HCQ 72 Hour Study



CHAPTER FOUR

Discussion

In order to make sense of the data and to effectively characterize the synergy between the Htma and the HCQ, the therapeutic effects of the Htma should first be examined independent of the HCQ. This discussion draws largely from the linear regression analysis from the 12 hour study. The IC_{50} value of thiomaltol in the B16F1 cells is greater than what the literature discusses. While the value established is 1.45 μM, that concentration in this study produces cell death at only 78% of the control group. Addition data at concentrations higher than 1.45 μM are needed in order to establish a correct IC_{50} value. However, a best fit line for the data presented predicts that the IC_{50} for the Htma in B16F1 cells to be close to 2.9 μM. This demonstrates that the Htma drug is not as effective against this cell line compared to other cell lines.

For the 12 hour study, the effects of the HCQ alone did not induce cell death in any appreciable manner. However, it is clear that is does increase cell death when administered with Htma as is shown by the decrease in IC_{50} from 2.9 μM to 2.7 μM.

The 12 hour study shows that there is promise in blocking autophagy in order to increase the activity of Htma. However, the effect is not particularly pronounced. The main factor that could affect the results is the time line for drugging. While the study was done over enough time for the Htma to affect the cells fully, the HCQ could be slower. Longer incubation times for the cells could be an effective way to observe the effects of inhibiting autophagy. This can be tested using the data from the 72 hour study.

The data from the 72 hour study show qualitatively that cell death for the Htma and HCQ treated cells versus those treated with Htma alone. The 0.8 μM Htma and HCQ drugged cells exhibit viability at 55% that of the 0.8 μM Htma treated cells. Likewise, the 1.6 μM Htma and HCQ drugged cells exhibit viability at 68% that of the 1.6 μM Htma treated cells. Therefore a synergy is evident in the data for the 72 hour study. However, the synergism is not linear and does not produce cell death at a predictable level. Despite this, the important point is that death rates were greater in the cells treated with the autophagy blocking agent meaning that the potential for HCQ to increase the activity of apoptotic inducing agents is qualitatively appreciable. Additionally, data for more dosage concentrations and more trials could produce results that are linear which would be useful quantitative analysis.

Although the 72 hour time frame effectively shows synergism, this amount of time is not usual for thionated maltol drugs like Htma and Httma. Therefore, preloading the HCQ for 72 hours proceeded by drugging the cells over a 12 hour time period could produce excellent results. This time line would take into consideration that HCQ takes more time to take effect than do the apoptosis inducing drugs.

Overall, the results seem to indicate that the autophagy blocking effects of HCQ can be used to increase the activity of thione drugs. Further studies taking the aforementioned parameter adjustments into consideration will very likely produce results that prove blocking autophagy is an effective means to increase the therapeutic effects of Htma.

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