4-20-2020

Effect of Amodiaquine on Gene Expression and Apoptosis in Colorectal Cancer Cells

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EFFECT OF AMODIAQUINE ON GENE EXPRESSION AND APOPTOSIS IN COLORECTAL CANCER CELLS

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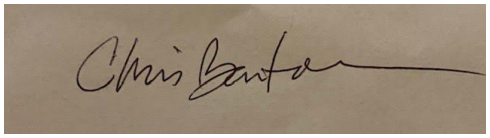
A Senior Honors Thesis project submitted to the Honors Program
in partial fulfillment of the requirements for the degree

Bachelor of Science

Belmont University Honors Program

2020



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Introduction:

Cancer is the tiny fragment of us that found out how to live forever: "a more perfect version of ourselves" (Mukherjee, 2010). This collection of diseases is characterized by uncontrollable cell growth that can move throughout the body, making it difficult to detect, and even harder to kill. Each cancer is unique, as these insuppressible tumors may originate in different areas of the body, growing and spreading at different rates. On a cellular level, each cancer cell mutates as it rapidly divides, resulting in a tumor that is even better equipped for survival. Cancer is specific to every individual it consumes, making a magic bullet nearly impossible. Bray et al. (2018) states that "cancer is expected to rank as the leading cause of death and the single most important barrier to increasing life expectancy in every country of the world in the 21st century." Treatments for cancer have come a long way, with radiation, chemotherapy, and surgery being the most common. These treatment options are usually integrated, with most patients receiving a combination of the three; however, these treatments often have serious side effects and none of these treatments comes close to a cure. According to the U.S. Department of Health and Human Services (2018), the survival rate for all cancers in the United States is around 68%. Researchers have made great strides in the improvement of treatments, as survival rates have generally increased over the past few decades. These improvements can be seen in cancers such as skin and testicular, where five year survival rates are extremely high in the United States at 94% and 97%, respectively (American Cancer Society [ACS], 2019). However, other cancers, such as pancreatic, lung, liver, and colorectal, prove to be a real challenge. Conventional treatments are not nearly as effective for these cancers, which leaves them with relatively low survival rates. For example, the lowest of these is pancreatic cancer with a five

year survival rate of 9% in the United States (ACS, 2019). Despite the major gains in cancer survival rates, there remains a demand for new and different types of drug therapies, and exploring new treatments for cancer is one solution that will bring us closer to closing the gap between survival rates and a cure. One approach that is commonly used for discovering new treatment options is a strategy called drug repurposing.

Drug repurposing can be described as finding a new use for a drug that has been previously approved to treat something else. Repurposing is a very effective way to discover and implement new drug therapies, as it is often quicker and at a lower cost than creating a drug from scratch, which can often take more than 14 years to gain approval (National Institutes of Health, 2019). A repurposed drug not only has approval for most, if not all, of the molecule, but it is also built on a foundation of previous research and will most likely proceed to clinical trials quickly. The power of successful drug repurposing can be seen through the drug sildenafil, more commonly known as Viagra. Originally created to treat coronary artery disease, sildenafil was repurposed to treat erectile dysfunction after participants found themselves experiencing spontaneous erections during Phase 1 clinical trials (Shim & Liu, 2014). An example of drug repurposing related to the field of oncology is a molecule known as chloroquine. At one point in history, chloroquine was a top of the line treatment for malaria. Malaria is caused by Plasmodium, a group of single-celled parasites that are transmitted to humans through bites from infected mosquitos. These parasites first travel to the liver where they mature and multiply, then move to the bloodstream, where they infiltrate, multiply in, and destroy red blood cells (Centers for Disease Control and Prevention [CDC], 2018). By the end of World War II, chloroquine was overwhelmingly preferred and so incredibly successful that many thought the disease could be

wiped out in its wake (Wellems & Plowe, 2001). The mechanism that made chloroquine so effective was the interaction it had with hemoglobin inside of the malaria parasite's lysosome, a cellular vacuole that allows for destruction and recycling of unnecessary cellular molecules. This process is cellular autophagy. While the globin-derived proteins of hemoglobin are useful to the malarial parasite, the heme portion must be destroyed. Normally, the heme is oxidized and converted to hemozoin, a nontoxic pigment. Chloroquine, however, is able to bind to the heme, forming adducts that do not allow the heme to be converted. This leads to a buildup of free hemes in the parasite, which is thought to cause oxidative stress and create reactive oxygen species (ROS) that can damage DNA, RNA, and proteins. This essentially poisons the parasite, ultimately resulting in death (Monti et al., 2002).

Chloroquine was cheap to make and available on a global scale, which led to it being heavily used for many years. An accumulation of genetic mutations over time resulted in two species of parasites, primarily accountable for the spread of malaria, to become completely resistant to the drug (Wellems & Plowe, 2001). Chloroquine resistance left many malaria cases untreatable, so researchers raced to synthesize analogs of the drug that could target the resistant strains. Chloroquine was such a successful drug that both it and its analogs have since been investigated for use as repurposed drugs. Most recently, chloroquine has been explored as a possible treatment option to treat coronavirus disease 2019 (COVID-19), which is a novel strain of coronavirus that emerged in China in December 2019 and has since spread rapidly, causing a pandemic (Gao et al., 2020). Prior to the COVID-19 outbreak, chloroquine was primarily investigated for use as a possible cancer therapy, and the results were full of promise.

There are six core hallmarks of cancer that allow for uncontrollable growth and the development of tumors, and they include sustaining proliferative signaling, evading growth suppressors, activating invasion metastasis, enabling replicative immortality, inducing angiogenesis, and resisting cell death (Hanahan & Weinberg, 2011). Along with these hallmarks, apoptosis-deficient cancer cells rely on the process of autophagy for prolonged survival in long term metabolic stress. As previously mentioned, autophagy is a cellular degradation pathway where proteins, organelles, and cytoplasm are engulfed, digested, and recycled to provide an alternate energy source. The autophagy pathway is often activated in times of cellular stress or starvation, and is essential to the survival of cancer cells (Mathew et al., 2007). Cancer cells use autophagy to maintain homeostasis and continue growth in adverse conditions, and this ability to tolerate long term stress may also allow for the survival of cancer cells following treatment, leaving those individuals receiving cancer therapies worried and wondering. Blocking autophagy is an effective strategy used to kill cancer cells, and as researchers explored new treatment options for cancers that do not respond relatively well to conventional therapies alone, they looked to previously approved drugs that could inhibit autophagy. Because chloroquine interferes with the autophagy function in antimalarial parasites, researchers decided to test the effect of chloroquine exposure on cancer cells. The results were encouraging, as Verbaanderd et al. (2017) discusses how chloroquine inhibits autophagic function in cancer cells, which is its primary anticancer mechanism. They emphasize that chloroquine as a combination treatment with conventional therapies has proven to be successful, as chloroquine can make cancers more susceptible to chemotherapy and radiation. These results are favorable and warrant further

research on chloroquine and its analogs as cancer therapeutics. One analog in particular yields extremely promising results, and that would be amodiaquine.

Amodiaquine is a derivative of chloroquine that was primarily used to treat the strains of malaria that had built up a resistance to chloroquine. Amodiaquine works to attack malaria in a fashion similar to chloroquine, as it does not allow the breaking down of hemoglobin to occur in the parasite. However, amodiaquine is different in that it is metabolized in the liver before it executes the antimalarial mechanism (Whirl-Carrillo et al., 2012). Amodiaquine is correlated with poisoning liver cells, and because much of this toxicity is unknown, it is still not as widely used as chloroquine once was. Research done by Tafazoli and Brien (2009) explores the mechanism by which amodiaquine is toxic to liver cells. One pathway of the drug that results in toxicity suggests that amodiaquine induces oxidative stress by increasing ROS in the cell, leading to apoptosis, while the other pathway was non-oxidative and involved protein carbonylation and mitochondrial membrane collapse. Amodiaquine has proven to be not only destructive to liver cells, but to cancer cells as well. Amodiaquine shows more evidence of anticancer properties than other antimalarial drugs, and its mechanism leads researchers to believe it is a promising drug. A study done by Qiao et al. (2013) reveals hopeful results, as amodiaquine is shown to be highly effective at preventing the growth of melanoma cells. The authors found that amodiaquine targets the lysosome in melanoma cells, similar to the mechanism for malarial parasites. They found that in melanoma cells, amodiaquine blocks autophagy function in the lysosome and causes a buildup of proteins, as well as weakening the mitochondria by depleting the energy source of the cell. These effects lead to the melanoma cells being more susceptible to the toxins of chemotherapy, which has potential to increase the

efficacy of chemotherapy when used as a combination treatment. This study also shows that when melanoma cells are consistently treated with amodiaquine, there is a decline in cell proliferation. This research demonstrates that amodiaquine is very effective against melanoma cells and has the potential to be a successful treatment for cancer.

Previously unpublished work performed by Bailey Bergmann and Dr. Chris Barton at Belmont University suggests that amodiaquine is effective not only against melanoma cells, but also colorectal cancer cells. While Bergmann provides supporting evidence that amodiaquine successfully reduces the viability of colorectal cancer cells, we still do not know the mechanism by which this is done. A foundational step in determining the mechanism of action of amodiaquine is evaluating gene expression. Analyzing gene expression in treated cells identifies modified cellular pathways and networks, and investigating how gene expression is affected by amodiaquine is essential, as it will lead to a better understanding of the anticancer mechanisms being executed by the drug (Bai, 2013). Research on melanoma cells done by Qiao et al. (2013), as mentioned above, explored how gene expression was affected by amodiaquine treatment. It was determined in this study that genes expressed in response to amodiaquine treatment differ from those normally expressed in melanoma cells, and that DNA damage repair genes were among the genes upregulated in the cells treated with amodiaquine. DNA damage also often triggers apoptosis, which may be a possible explanation for reduced cell viability (Roos, 2006). While gene expression was successfully analyzed for melanoma cells, amodiaquine induced gene expression in colorectal cells has yet to be identified.

My research investigates a new treatment option for colorectal cancer, as it will analyze the effect of amodiaquine on gene expression in colorectal cancer cell line HCT116. I hypothesize that amodiaquine will significantly affect gene expression in HCT116 cells, and that the changes in gene expression may provide insight to how these cells are responding to the drug. The results of my research will provide a better understanding of how this drug works in cancer cells and may suggest new treatment options for cancers, like colorectal, that can be resistant to current treatments. With this knowledge, new hope could be given to those who face devastating survival rates.

Methods:

Cell Culture

HCT116 colorectal cancer cells were grown in DMEM with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were incubated at 37ºC and 5% CO_2.

Gene Expression Analysis

HCT116 cells were grown to 60% confluency and treated with either amodiaquine (AMQ, LD50 concentration) or control media for 24 hours. The cells were then harvested and total RNA was purified. The isolated RNA from these cells were then used to generate cDNA for control and AMQ-treated cells. The cDNA was used to analyze gene expression with qPCR via the Qiagen RT_2 Profiler PCR Human Stress/Toxicity Array per the manufacturer's protocols.

Western Blot Analysis

4 plates of HCT116 cells were treated with the corresponding conditions: control (no treatment), Etoposide (71μM), AMQ (20μM), and AMQ (80μM). Each plate was incubated at 37ºC for 48 hours. The cells from each plate were trypsinized and harvested, and protein was isolated using RIPA buffer supplemented with the protease inhibitor PMSF. A Bradford assay was completed and read with a Biotek Epoch plate reader to measure the protein concentrations of each sample. A ladder and protein samples (30ug) were run through a 10% polyacrylamide gel at 50V for 15 minutes and 100V for approximately an hour and a half. The gel was then transferred onto a PVDF membrane using a 40 minute transfer run in a BioRad Trans-Blot machine. The membrane was blocked overnight on a rocker in 5% NFDM. The membrane was then incubated in 1º antibody overnight on a rocker (PARP, Actin, both at 1:1,000 dilution). The membrane was then incubated in 2º antibody on a rocker for 45 minutes (goat anti-rabbit, 1:10,000 dilution). The membrane was imaged with Invitrogen iBright CL1000.

Results:

Control vs. AMQ Gene Expression

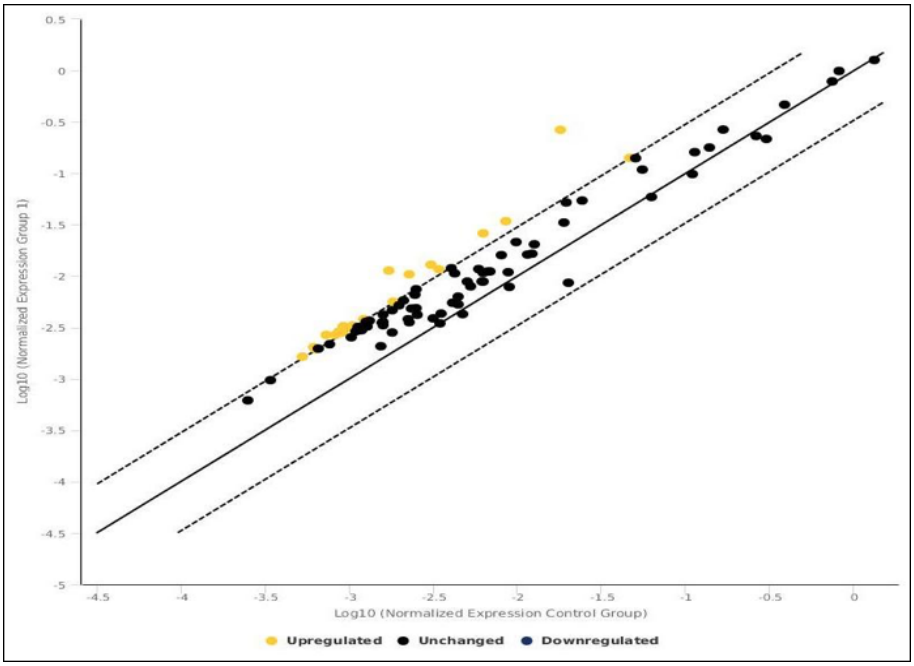

Figure 1: Analysis of gene expression following AMQ exposure in HCT116 cells. Cells were exposed to AMQ (or control) for 24 h and gene expression analyzed using the Qiagen Human Stress and Toxicity Assay. 20 genes (yellow points) are upregulated by at least 3 fold following AMQ exposure.

Amodiaquine has been shown to reduce colorectal cancer cell viability, but the mechanism of action and the effect of amodiaquine on gene expression in these cells is unknown. Gene expression in AMQ-treated HCT116 cells was analyzed by exposing HCT116 cells to amodiaquine (LD50 concentration) for 24 hours and using the Qiagen RT_2 Profiler PCR Human Stress/Toxicity Array to measure the transcript levels of multiple genes. The qPCR results were run through a corresponding Qiagen program that organized the raw data into the graph seen in Figure 1. These results revealed that following AMQ treatment for 24 hours, 20 human stress and toxicity genes were upregulated by at least 3 fold in HCT116 cells (Figure 1). The fold change and basic function of each of these genes can be seen in Figure 2. Of these 20 upregulated genes, it was determined that 9 were pro-apoptotic genes and 2 were DNA damage

repair genes (Figure 2). It was interesting to find that so many apoptotic-associated genes were significantly upregulated following exposure to amodiaquine. Because the majority of upregulated genes were pro-apoptotic, we chose to further explore whether HCT116 cells responded to amodiaquine through the induction of an apoptotic signaling pathway.

Gene Symbol	Fold Change	Gene Description
AQP4	3.16	encodes a member of the aquaporin family
BBC3	3.04	encodes a member of the BCL-2 family, BH3-only pro-apoptotic subclass
CASP1	3.05	encodes a member of the caspase family; activation of caspases play a key role in apoptosis
CD40LG	3.13	encodes a protein expressed on the surface of T cells
CDKN1A	14.69	encodes a cyclin-dependent kinase inhibitor, regulates cell cycle progression at G1
CFTR	3.28	encodes a protein that functions as a chloride channel
CRP	3.49	encodes a member of the pentaxin family; involved in several host defense functions
CXCL8	6.55	encodes a member of the CXC subfamily of chemokines; plays a role in inflammation
DDB2	4.16	encodes a protein that is necessary for the repair of DNA damaged by UV light
DDIT3	4.02	encodes a protein that prevents DNA binding activity and promotes apoptosis
EDN1	3.1	encodes a protein that is processed to secrete a peptide that is a vasoconstrictor
GADD45A	4.23	encodes a protein that mediates the p38/JNK pathway which may induce apoptosis
GADD45G	3.31	encodes a protein that mediates the p38/JNK pathway which may induce apoptosis
IL1A	3.05	encodes a member of the interleukin 1 cytokine family; induces apoptosis
IL1B	3.64	encodes a member of the interleukin 1 cytokine family; mediates apoptosis
MRE11A	3.43	encodes a nuclear protein that is involved in repairing double-stranded DNA breaks
SLC5A3	3.23	encodes solute carrier
SQSTM1	3.03	encodes a protein that acts as an adaptor protein and mediates nuclear factor kappa-B signaling
TNF	3.11	encodes a cytokine involved in regulating proliferation, differentiation, apoptosis, and more
TNFRSF10B	4.59	encodes a protein that contains an intracellular death domain and transduces apoptosis

Figure 2: Table of genes that are upregulated by at least 3 fold in AMQ-treated HCT116 cells. Fold change and function are described and those in bold are apoptotic or DNA damage repair genes. Gene descriptions obtained through the National Center for Biotechnology Information (NCBI).

Given our observation that so many pro-apoptotic genes were upregulated in response to amodiaquine, we chose to investigate the possibility that AMQ was inducing cellular death in HCT116 cells. For 48 hours these cells were either untreated as a control, treated with Etoposide, treated with AMQ (20μM), or treated with AMQ (80 μM). We then analyzed the expression of Poly (ADP-ribose) polymerase (PARP) and Actin via Western blotting. PARP was chosen because the gene Caspase 1 (CASP1) was found to be one of the upregulated pro-apoptotic genes

in AMQ-treated HCT116 cells (Figure 2), which is a gene that codes for a protein in the caspase family, and caspases cleave many proteins, including PARP, when apoptosis is occurring in a cell. Therefore, PARP cleavage is a hallmark of apoptosis (Erener et al., 2012). Actin was used as a control to ensure the presence of protein. Our western blot revealed that PARP is cleaved in HCT116 cells treated with Etoposide and HCT116 cells treated with AMQ 80μM for 48 hours (Figure 3). It is also shown in Figure 3 that PARP remained intact in both the control and HCT116 cells treated with AMQ 20μM for 48 hours, and that Actin was present at equal levels in all HCT116 cell treatment conditions. These results suggest that amodiaquine induces apoptosis in HCT116 colorectal cancer cells.

Western Blot at 48 Hours

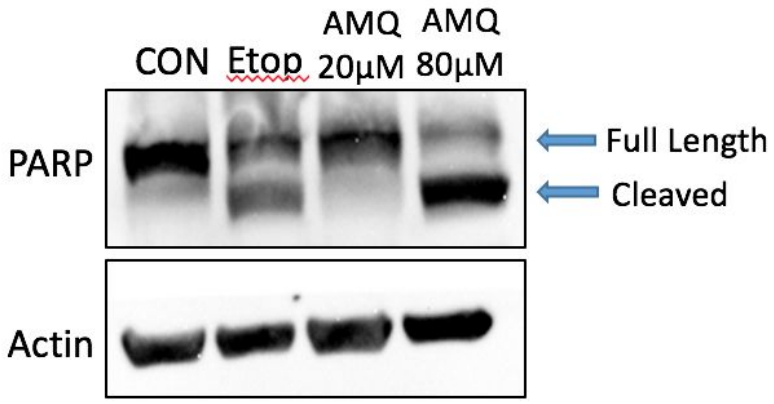

Figure 3: Amodiaquine induces apoptosis in HCT116 colorectal cancer cells. HCT116 cells were treated with control, etoposide, or amodiaquine, and PARP/Actin expression analyzed via Western blotting.

Discussion:

Colorectal cancer is of significant interest and is the third most commonly diagnosed cancer among men and women in the United States (Siegel et al., 2017). While preventative

measures and updated screening techniques have improved over time, conventional treatment options for those diagnosed with colorectal cancer still lack efficacy. This leaves colorectal cancer with relatively low survival rates compared to other cancers, and emphasizes the crucial need for new therapeutic options that will successfully treat colorectal cancer.

Amodiaquine, an analog of chloroquine, is an antimalarial drug that has been investigated for possible anticancer properties after it was shown to be toxic to healthy liver cells (Tafazoli & Brian, 2009). Previous research shows that amodiaquine successfully prevents the growth of melanoma cells and may make melanoma cells more susceptible to chemotherapeutics (Qiao et al., 2013). The effect of amodiaquine was also investigated on colorectal cancer cells in a previously unpublished study, and it was determined that the drug reduces viability of colorectal cancer cells, but a mechanism of action has not been determined for amodiaquine. Discovering the anticancer mechanism of this drug is essential to understanding how amodiaquine may be used to treat colorectal cancer in the future.

This study used gene expression analysis to explore the specific response to amodiaquine in HCT116 colorectal cancer cells. Real-time PCR revealed that 20 human stress and toxicity genes were upregulated by at least 3 fold in HCT116 colorectal cancer cells treated with AMQ. Of these 20 upregulated genes, 9 were found to be pro-apoptotic and 2 were DNA damage repair genes, which supports our original hypothesis that amodiaquine is inducing a stress response in these cells. These results are consistent with previous research done by Qiao et al. (2013), as DNA damage repair genes were among the genes upregulated in AMQ-treated melanoma cells. While our data show that the majority of upregulated genes were pro-apoptotic and only two

were DNA damage repair genes, DNA damage often triggers apoptosis, which suggests that apoptosis is of significant interest regarding the effect of AMQ exposure to HCT116 cells.

Gene expression analysis also revealed that CASP1 is one of the 20 genes upregulated in AMQ-treated HCT116 cells. CASP1 is a gene that encodes for a protein that is a member of the caspase family, and caspases are the main executors of apoptosis and inflammation (Kim et al., 2009). Due to the upregulation of CASP1 and many other pro-apoptotic genes, we decided to further explore apoptotic markers in AMQ-treated HCT116 cells. One clear and consistent marker of apoptosis is PARP cleavage, as PARP is one of many proteins cleaved by caspases when apoptosis is occuring in a cell (Pieper et al., 1999). A western blot revealed that PARP was cleaved in HCT116 cells that were treated with AMQ for 48 hours, but not in control-treated cells, which indicates that apoptosis was occuring in these cells. These results suggest that inducing apoptosis is the mechanism by which amodiaquine reduces viability of HCT116 colorectal cancer cells. As a result, we suggest that future research be conducted to further investigate the indicated apoptotic mechanism of AMQ, which could be done by using western blots to identify other markers of apoptosis, such as BCL-2. Apoptosis could also be detected through use of Annexin-V staining, as the Annexin-V stain reacts strongly with phosphatidylserine residues that are exposed when plasma membrane asymmetry is lost in the early stages of apoptosis (Van Engeland et al., 1998). In the late stages of apoptosis, DNA degradation takes place and could be detected by a TUNEL assay, which uses terminal deoxynucleotidyl transferase (TdT) to tag the blunt ends of double-stranded DNA breaks (Kyrylkova et al., 2012). Additionally, further gene expression analyses should be completed to

better understand the genes and pathways that are affected by AMQ exposure, as this could lead to the discovery of more mechanisms by which cells respond to AMQ.

In conclusion, our original hypothesis was supported, as 9 pro-apoptotic and 2 DNA damage repair genes were upregulated by at least 3 fold in AMQ-treated HCT116 cells, and our data suggest that apoptosis is the mechanism by which AMQ reduces HCT116 cell viability. Based on these results, we suggest that AMQ be further investigated for its anticancer properties and be further analyzed as a possible repurposed drug for treating colorectal cancer and other cancers with relatively low survival rates.

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