Dean&Francis
ISSN 2959-409X

MS003842

Artemisinin treats SNU-C2A cell line for colorectal cancer by inhibiting cell growth through undergoing ferroptosis.

Yueh-Shan Chi

Shanghai Soong Ching Ling School, Shanghai 201703, China
sammichi071004@gmail.com

Abstract:

Colorectal cancer is one of the major types of cancer that humans are suffering from; however, drugs that can be used for treatment with no side effects have still not been discovered. By research, Chinese traditional medicine called artemisinin (ART) was known for undergoing ferroptosis in cells. Therefore, some experimental tests need to verify the effect of whether artemisinin allows ferroptosis to apply to colorectal cancer cells. The result of this experiment can allow a better understanding of the direction of colorectal cancer treatment and how Chinese traditional medicine can perform as treatments for CRC. Therefore, during this experiment, ART was used to test ferroptosis in the SNU-C2A colorectal cell line to see whether it's a suitable drug to use for CRC patients.

Keywords:- Artemisinin; Colorectal Cancer; Ferroptosis, SNU-C2A Cell Line

1. Introduction

Colorectal cancer, also called CRC, is a common cancer that exists in the human colon. In 2020, approximately 147,950 patients were diagnosed with CRC, and among them, 53,200 died [1]. There are some symbols for early CRC diagnosis, such as tumor bleeding and the appearance of blood in feces. However, there were no specific symptoms for every case of early diagnosis, so CRC diagnosis was mainly determined through colonoscopy[2]. Recommendations were to screen adults aged 50-75 to seek for the appearance of CRC [3]. Some current treatments for CRC are ablation therapy, which is used to destroy small tumors, and embolization therapy to threaten cancer infection in livers[4]. Within the ablation therapy, there is the Percutaneous ethanol ablation (PEI) or Alcohol ablation, which destroys cancer cells by injecting alcohol into the cells. In combination with other treatments, it can successfully kill cancer cells and tumors. Cryoablation was also a treatment used for CRC. It includes freezing the tumor with a thin metal probe, which allows liquid nitrogen and other cold substances to pass through and directly destroy the cancer cells. However, all ablation therapies have side effects, including abdominal pain, fever, and infection of the liver. When the livers were infected by CRC cancer cells, embolization therapy was used. It involves injecting substances into the artery of the liver to stop blood transfusion to the tumor. Albeit, side effects such as fever, abdominal pain, and Gallbladder inflammation still exist, which makes the founding of new therapies indispensable

ISSN 2959-409X

SNU-C2A is one of the 14 colorectal cancer cell lines, taken from a 43-year-old female diagnosed with CRC. It's a tumorigenic cell and can develop a tumor in mice within 21 days at 100% frequency[5]. Experiments done before such as Mechanisms related to [18F]fluorodeoxyglucose uptake of human colon cancers transplanted in nude mice [6] had succeeded using the same cell line. This emphasizes how the SNU-C2A CRC cell line is a suitable choice for testing the effect of medicines on the treatment of colorectal cancer

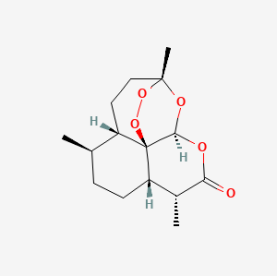

Figure 1. Chemical structure of artemisinin molecule (PubChem, n.d.)

Artemisinin (ART) is isolated from a plant called Artemisia annua, it's a Chinese medicine that is used as a treatment for malaria.[7] Its chemical formula is $C_{15}H_{22}O_5$ [8]. The ability of ART to reduce the amount of Plasmodium parasites in blood makes it an excellent drug for malarial treatment. It is shown to form covalent interaction with Human serum albumin (HSA), red cell membrane protein, and heme to decompose into free reactants. During ferroptosis, ART can degrade ferritin which is storage for iron in cells, causing the concentration of iron molecules in cells to increase which leads to ferroptosis. ART can also decrease the GPX4 function and increase lipid peroxidation leading to ferroptosis. ART's appearance also regulates the Nrf2-ARE pathway PERK-ATF4-HSPA5 pathway and ATF4-CHOP-CHAC1 to allow ferroptosis to occur in the cell. [9]

Ferroptosis is a new form of cell death due to iron accumulation and lipid peroxidation[10]. With the increase of intracellular ions, the amount of reactive oxygen species (ROS) also increases which overwhelms the antioxidant mechanism and causes membrane lipid peroxidation and cell death. During iron metabolism, a protein on the plasma membrane called the transferrin receptor binds to the iron resulting in endocytosis and bringing the iron inside the cell. Then the iron will turn from ferric form to ferrous form through the enzyme STEAP3, then later on transferred into the cytosol with the help of DMT1 protein[11]. After the protein-bonded redox-active ions of iron were in the cytosol, PCBP1 and 2 helped transfer these ferrous and stored them in the form of ferritin which can be utilized and degraded in the lysosome to release the ferrous iron and transported out the cell by ferroprotein[12]. However, under the condition of ferroptosis, the number of ferric entering the cell increases causing the concentration of iron in the cell to increase, which means that not all irons will be turned into the storage form of ferritin. During the antioxidant mechanism. There's an amino acid anti-transporter called system Xe with two subunits, SLC3A2 and SLC7A11 on the plasma membrane[12]. It transports one molecule of cystine inside the cell for an exchange of one molecule of glutamate. The cystine that enters the cell is then immediately turned into cysteine which gets turned into glutamate. This cysteine with the help of γ-glutamyl cysteine synthetase converted into Glu-Cys with the addition of glycine formed reduces glutathione (GSH) which tackles all the ROS within the cell. Another important enzyme glutathione peroxidase (GPX4) turns lipid peroxide into lipid alcohol which prevents lipid peroxide from causing membrane peroxidation [13]. Ferroptosis will occur when the lipid oxide is not converted into the alcohol form, PUFA on the membrane with the ferrous iron will then lead to lipid peroxidation and cell death. As patients intake ART, the number of GPX4 proteins inside their cells will be reduced causing lipid peroxidation to increase leading to ferroptosis of the cell [14].

2. Research Purpose

Since artemisinin being an antimalarial drug is capable of causing ferroptosis in cancer cells, itsr hypothesize that increasing the concentration and treatment duration of artemisinin decreases cell viability of the SNU-C2A CRC cell line and shrink in size of SNU-C2A mouse xenograft tumor size and increase lipid peroxidation in which increases its potential of treating colorectal cancer.

3. Material and Methods

3.1 MTT Assay

The MTT assay is a method of measuring cellular viability by looking at mitochondria activity. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) turning from purple to yellow indicates an active mitochondrion [15].

Seed the SNU-C2A CRC cancer cells in 96 different wells at appropriate concentrations that allow incubation to happen. After incubating the cells at 37°C for 4 hours, add ART at gradient concentrations of 0.01μM, 0.1μM, 1μM, 10μM, and 100μM to the wells for 1 hour, 5 hours, 12 hours, 24 hours, and 48 hours. Add the taxol as positive control and DMSO/ PBS as negative control for observation for comparison add the MTT solution to each well

and observe the color change.

3.2 BODIPY

BODIPY is a method used to measure the amount of lipid peroxidation in cells and indicates whether ferroptosis has happened to cells. This will be shown when the C11-BODIPY dye is added to wells with different cells [16].
Seed the SNU-C2A cells in different wells and add ART at the concentrations of 10 µM, 30 µM, 50 µM, and 100 µM for 1 hour, 5 hours, 12 hours, 24 hours, and 48 hours. Add the taxol as positive control and DMSO/ PBS as negative control for observation for comparison. Then add the C11-BODIPY dye to all wells and observe the amount of staining to indicate if lipid peroxidation occurred.

3.3 Xenograft in vivo

Inject the SNU- C2A CRC cells into the mouse through their tail into the vein for the cells to land on their colon and form tumors. Wait for 1 week, 2 weeks, 4 weeks, 6 weeks, and 8 weeks for the tumors to form. Then inject ART for the concentration of 30mg/kg, 100mg/kg, 200mg/kg, 500mg/kg, and 800mg/kg per mouse. Measurement of the size of the tumor will be done by cutting open and taking out the tumor in the colon [17].

3.4 Statistical analysis

Each experiment will be repeated 3 times to ensure accuracy of the results. Student T-test will be used to compare the significance of the result with the control groups for both the MTT assay and the BODIPY assay. The significance level of $p<0.05$ will be considered for statistical significance.

4. Result

Table 1: Possible results for the experiments.

Combination Result # (CR#)	Artemisinin decreases cellular viability by MTT assay?	Artemisinin increase lipid peroxidation by BODIPY FACS?	Artemisinin shrinks tumor size in mice by xenograft by caliper?	Supporting hypothesis?
CR1	+	+	+	Yes
CR2	+	+	-	Partially
CR3	+	-	+	Partially
CR4	-	+	+	Partially
CR5	+	-	-	Partially
CR6	-	+	-	Partially
CR7	-	-	+	Partially
CR8	-	-	-	No

Table legend: "+" indicates the condition described at the top of the column observed and results are statistically significant compared to the positive control taxol, "–" indicates the condition described at the top of the column not observed and results are statistically significant compared to the negative control DMSO/ PBS.
CR1: The ART-injected SNU-C2A cell line analyzed by MTT assay shows the cell viability decreased throughout treatment. The lipid peroxidation observed by BODIPY increased through the treatment period. When the SNU-C2A cell line is injected into a mouse vein creating the CRC tumor cell in vivo, the tumor size shrinks because of the ART given to the mouse.
CR2: The ART-injected SNU-C2A cell line analyzed by MTT assay shows the cell viability decreased throughout treatment. The lipid peroxidation observed by BODIPY increased through the treatment period. When the SNU-C2A cell line is injected into a mouse vein creating the CRC tumor cell in vivo, the tumor size did not shrink because of the ART given to the mouse.
CR3: The ART-injected SNU-C2A cell line analyzed by MTT assay shows the cell viability decreased throughout treatment. The lipid peroxidation observed by BODIPY did not increase through the treatment period. When the SNU-C2A cell line is injected into a mouse vein creating the CRC tumor cell in vivo, the tumor size shrinks because of the ART given to the mouse.
CR4: The ART-injected SNU-C2A cell line analyzed by MTT assay shows the cell viability did not decrease throughout treatment. The lipid peroxidation observed by BODIPY increased through the treatment period. When the SNU-C2A cell line is injected into a mouse vein creating the CRC tumor cell in vivo, the tumor size shrinks because of the ART given to the mouse.
CR5: The ART-injected SNU-C2A cell line analyzed by MTT assay shows the cell viability decreased throughout

ISSN 2959-409X

treatment. The lipid peroxidation observed by BODIPY did not increase through the treatment period. When the SNU-C2A cell line is injected into a mouse vein creating the CRC tumor cell in vivo, the tumor size did not shrink because of the ART given to the mouse.

CR6: The ART-injected SNU-C2A cell line analyzed by MTT assay shows the cell viability did not decrease throughout treatment. The lipid peroxidation observed by BODIPY increased through the treatment period. When the SNU-C2A cell line is injected into a mouse vein creating the CRC tumor cell in vivo, the tumor size did not shrink because of the ART given to the mouse.

CR7: The ART-injected SNU-C2A cell line analyzed by MTT assay shows the cell viability did not decrease throughout treatment. The lipid peroxidation observed by BODIPY did not increase through the treatment period. When the SNU-C2A cell line is injected into a mouse vein creating the CRC tumor cell in vivo, the tumor size shrinks because of the ART given to the mouse.

CR8: The ART-injected SNU-C2A cell line analyzed by MTT assay shows the cell viability did not decrease throughout treatment. The lipid peroxidation observed by BODIPY did not increase through the treatment period. When the SNU-C2A cell line is injected into a mouse vein creating the CRC tumor cell in vivo, the tumor size did not shrink because of the ART given to the mouse.

Variables of concentration and treatment duration: When treatment concentration and duration are dependent, as they increase, the effect of ART increases, less cellular viability, more lipid peroxidation, and more shrink are observed in mice. When Treatment concentration and duration are dependent but are inverse with the result, as they increase, the effect of ART decreases, more cellular viability, less lipid peroxidation, and tumors in mice don't shrink in size. When treatment concentration and duration are independent, as they increase or decrease, the outcome won't be affected. Therefore, the results will end up being the same under different concentrations and durations.

5. Discussion

CR1 shows a result that supports the hypothesis. ART successfully caused ferroptosis in the SNU-C2A cells in vitro, entered the body of the mice, and shrunk the size of the tumor in vivo. This result shows that ART is a suitable drug to use against CRC and is capable of relieving symptoms. This might be caused by using the correct amount of treatment concentration and duration in the right cell, and that there's no existence of anti-drug in the mouse body. After knowing this drug is capable of treating CRC, we will examine if there are any side effects on the mouse. While studying this, the concentration of ART will be increased to see whether this drug can cause any danger. Consider making this drug usable in every CRC patient, the next step might be testing this treatment on a human patient to see if the same thing that happened to the mouse happened to the human.

CR2 shows a result that partially supports the hypothesis. ART only worked in the SNU-C2A cells that are in vitro, not in vivo when it's injected into a mouse. This might be the result of inhibitors in the animal body, which caused the drug to not function well. If this is the case, future investigations need to focus on what caused the inhibitor or anti-drug in the animal's body and how to overcome it or find a new drug to research. Another possibility is that there was an error during the experiment. The error might be that the SNU-C2A cells didn't go to the colon but instead directly infected the liver or the tumor formed wasn't caused by cancer. Or that the drug was given the wrong way. To avoid the error, future experiments might change the way of creating the tumor, such as injecting the CRC cell line from the back of the mouse into the subcutaneous fat layer and forming the tumor on the back instead of in the colon. Or repeat the experiments more times to find the mistake.

CR3 shows a result that partially supports the hypothesis. In this result, cancer cells did die, and tumors did shrink in size, but the cells didn't die due to ferroptosis. Since the lipid peroxidation measured by BODIPY didn't increase, this indicates that the cell didn't die because of ferroptosis. One reason that might have caused this result is that ART met substances in the animal's body that activated the drug and made it function in vivo. If this is the case, then future experiments need to focus on researching the substance in the animal's body that activates the drug and makes it a part of the treatment grouping up with ART. Another cause of this result is that the CRC cancer cell didn't die because of ferroptosis. Maybe it died by apoptosis. In this case, the new experiment will be researching the ART effect on apoptosis or other death pathways.

CR4 partially supports the hypothesis. Tumor size did decrease, and cells did undergo ferroptosis, but the cells didn't die. This might be the result of shrinking a non-cancer tumor by undergoing ferroptosis, but lipid peroxidation was not enough to fully kill the SNU-C2A cancer cell by ferroptosis. If this is the case, future experiments will try different concentrations and treatment durations to see if higher concentrations and duration make a difference in the amount of lipid peroxidation formed that allows ferroptosis to happen to cancer cells.

CR5 partially supports the hypothesis. Cancer cells did die, but it wasn't caused by ferroptosis, and the tumor size did not shrink in its size. This might be the result of cells undergoing death pathways other than ferroptosis, such

as apoptosis, autophagy, necrosis, and ptosis. If this is the case, then future experiments need to focus on how ART affects proteins in other death pathways and leads to cell death. The result of the tumor not shrinking in size indicates that the drug might not get to the colon where the tumor was; it might have decomposed or deactivated on its way due to substances in the animal's body that might work as anti-drug or inhibitors. In this case, the next step of investigation will be focused on drugs used together in the animal body that allow other death pathways to take place in the animal's tumor.

CR6 partially supports the hypothesis. Cellular viability doesn't decrease, tumors in the animal's body don't shrink, but lipid peroxidation does increase. This result shows that even though lipid peroxidation increases in amount, it's still not enough for the cell to establish ferroptosis. For this result, future experiments need to research drugs that can increase the amount of ROS that can lead to an increase in lipid peroxidation, and cause the cell to die through ferroptosis. In addition to the cause of this result above, the animal might have resistance towards ART, causing it to not function in the animal body. Then, researchers will need to investigate another drug that can overcome resistance and anti-drugs in the animal.

CR7 partially supports the hypothesis. The tumor size in the mice did shrink, but cellular viability and lipid peroxidation tested in vitro didn't change. The reason that might have caused this situation is that ART only works under specific circumstances, and luckily, the body of the mouse has this substance that catalyzes ART to function. In this case, the next step of the experiment will be to find out the catalyst that exists in the mouse body and adopt it in a way that cooperates with ART and allows the treatment to be done in humans. Another reason might be that ART in the mouse body shrinks a tumor that's not caused by cancer, in this case, the drug needs to be adjusted or changed.

CR8 fully contradicts the hypothesis. ART doesn't decrease cellular viability, doesn't increase lipid peroxidation, and doesn't shrink the size of the tumor in mice. If this result shows up, it means that ART is the wrong drug to use for treating colorectal cancer. In this case, researchers need to conduct a new experiment on a new Chinese traditional medicine or try a new signaling pathway that ART might affect. Another reason might be that it's the wrong concentration used for ART, so the next step will be to widen the range of concentration and treatment duration for ART and try the experiment again.

Concentration and treatment duration are directly dependent, indicating that the more the amount and time, the more powerful the function of the drug. This might be because more ART used will cause more GPX4 protein to be influenced, causing more ferrous iron in cells that leads to more ferroptosis. With more time, ART has more time to interact with more GPX4, causing more cells to die. This means that for patients with more serious colorectal cancer, treatment concentration and duration can be added. For less serious patients, treatment concentration and duration can be lowered. If concentration and treatment duration are inversely dependent, the more drug used, the less powerful the drug is. This might be that the more ART enters, the higher the possibility it will be found and killed. The more time ART stays in the cell, the more time is given for the reparation of the cell. If concentration and treatment duration are independent, then the amount of time and concentration won't make any difference. This might be caused by the fixed concentration of GPX4 that can be affected. Therefore amount and time of ART make no difference in its effect.

6. Conclusion

In this experiment, artemisinin was being tested in the SNU-C2A colorectal cancer cell line to indicate whether it can cause ferroptosis in cancer cells and shrink tumors in the colon. Artemisinin successfully increases the lipid peroxidation in the SNU-C2A cell line by decreasing the function of the GXP4 protein which works for decreasing lipid peroxidation in cells. As a result, ferroptosis will happen to the cell. The results will provide a piece of better knowledge on treating CRC with Chinese medicine and allow further investigations on this field. Colorectal cancer, being a disease with a great impact on society, currently doesn't have the best therapy with fewer side effects. Therefore, new drugs need to be invented and more researches need to be done in order to consume a broader knowledge for CRC and SNU-C2A.

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