Original Article Increasing colorectal cancer cell sensitivity to oxaliplatin through hyperthermia and chloroquine treatment

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Abstract: Colorectal cancer is the third most lethal form of cancer worldwide, with most patients dying of liver metastases. Patients with metastatic livers can be treated with hyperthermia therapy, which often uses IHP (Isolated Hepatic Perfusion). Hyperthermia has become as a highly anticipated method. Using traditional hyperthermia alone is unable to inhibit tumor cell proliferation. Tumor can be inhibited effectively by combining oxaliplatin with a 42°C hot water bath. However, this combination has been shown to induce autophagy in colorectal cancer cell lines, which inhibits tumor cell death to some extent. In order to combat this problem, we used oxaliplatin in combination with autophagy inhibitor chloroquine (CQ) at 42°C. We found that oxaliplatin combined with CQ at 42°C induces caspase-dependent apoptosis in colorectal cancer cells. This treatment not only impacts BCL-2 family proteins, but also downregulates the HSP and IAP family proteins. Activated caspase and PRAP (poly ADP-ribose polymerase) proteins are all upregulated. The results are suggesting that oxaliplatin combined with CQ at 42°C is a novel anti-tumor approach against colorectal cancer.

Keywords: Colorectal cancer, hyperthermia, oxaliplatin, chloroquine, apoptosis, autophagy

Introduction

As the incidence of colon cancer around the world constantly increases, there is a desperate need to develop new effective treatments. The current most effective treatment for colon cancer is surgical resection combined with chemotherapy, such as oxaliplatin and 5-Fu (5-fluorouracil). Unfortunately, 5-year survival rate of this treatment is still low, and side effects of oxaliplatin, including severe neurotoxicity, are extremely painful. While combination therapy is an important method for circumventing drug resistance, it has a poor overall efficacy in treating advanced metastatic cancer patients. Therefore, exploring new treatment options is still very actual.

Two major combination therapies have been explored: oxaliplatin combined with hyperthermia [1] and oxaliplatin combined with autophagy inhibitors [2]. However, both oxaliplatin and hyperthermia trigger autophagy [3], which inhibits tumor cell death. In order to combat this issue, oxaliplatin has been used in combination with autophagy inhibitors, which resulted in enhanced pro-apoptotic activity. Inhibiting autophagy induced by oxaliplatin and hyperthermia to enhance treatment efficacy has not been explored.

There are many key proteins which play roles in marking and regulating autophagy. For example, Beclin-1, a key protein that appears upstream, plays an important role in the regulation of autophagy. Autophagy markers LC3 and SQSTM1/p62 indicate the level of autophagy. SOSTM1/p62 provides a scaffold for several signaling proteins and triggering the degradation of proteins [4]. SQSTM1/p62 also binds protein LC3/Atg8, resulting in the transport of p62-containing protein aggregates to the autophagosome [5]. Lysosome degradation leads to a decrease of SQSTM1/p62 levels, thus, autophagy inhibitors stabilize SQSTM1/ p62 levels. During autophagy, LC3-I is converted to LC3-II that allows for LC3 to become associated with autophagic vesicles [6]. LC3-I and LC3-II, have been used as indicators of autophagy [7]. PARP, a nuclear poly (ADP-ribose) poly-

merase, appears in DNA repair when environment change sharply [8]. This protein is one of the main cleavage targets of caspase-3 in vivo [9, 10]. Activated PARP serves as a marker of apoptosis [11]. Bcl-2, Bcl-xl and Mcl-1 localize at the mitochondria, inhibits apoptosis by interacting with and antagonizes pro-apoptotic [12]. They exerts a survival function through inhibit mitochondrial cytochrome c release [13]. Heterodimerization with an apoptotic protein, and formation of mitochondrial outer membrane pores, which helps to maintain a stable membrane state under stressful conditions [14, 15]. IAP proteins inhibit apoptosis through direct inhibit the activity of several caspase proteins, including caspase-3, caspase-7, and caspase-9 [16]. Smac protein binds to IAP family members blocking their interaction with caspase-9, and then activated caspase proteins [17]. When x-linked IAP was limited due to severe hypoxic conditions, c-IAP2, an IAP, levels were also down regulated [18].

Hyperthermia triggers the expression of heat shock proteins (HSP), which has been shown to inhibit autophagy, though not significantly, as well as apoptosis. HSP27 inhibits Smac/ Diablo's function [19], HSP70 inhibits Bax/Bak oligomerization on mitochondria [20], thereby inhibiting the formation of the mitochondrial channel. HSP90 inhibits Casepase-8-mediated Bid activation to tBid [20], which is necessary for the opening and closing of the Bax/Bak channel [21]. The formed Bax/Bak channel also showed decreased activity, since proapoptotic factors Cyt-C (Cytochrome-C), AIF, EndoG (Endonuclease G) are unable to be released, which inhibits apoptosis severely. Therefore, HSP activation induces greater negative effects towards tumor cell death.

We report here that the combination treatment of oxlaplatin with CQ at 42°C hyperthermia, inhibit autophagy, downregulates anti-apoptosis proteins of BCL-2 and IAP family, and decreases the levels of HSP family proteins. This then active caspase, PARP, and other proapoptosis proteins, induce apoptosis cell death.

Materials and methods

Cell culture

HCT116 and SW620 cell lines were maintained in McCoy's 5A and L-15 (Hyclone, Logan, UT) and supplemented with 10% fetal bovine serum (FBS, Gibco) at 5% dioxide and 37°C.

Antibodies and stable-transfected cell lines

Anti-MCL-1, Anti-BCL-xI, Anti-BAK, Anti-BAX, Anti-β-Actin, Anti-PARP-1 (Activated PARP), Anti-AIF, Anti-Beclin-1, Anti-p62, and Anti-Caspase-3 were purchased from Cell Signaling Technology (USA, CST). Anti-HSP70, Anti-HSP27, Anti-LC3-I/II, Anti-HSP90, and Anti-BCL-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The pCDH-GFP-LC3b were transfected in HCT116 and SW620 cell lines with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and then HCT116/SW620-GFP-LC3b cell lines were filtered by Puromycin (p9620, Sigma).

Hyperthermia treatment

HCT116 and SW620 cell lines were cultured in 6-well plate, 60-mm dish, or and 96-well plate for 24 h before treatment. The plates were sealed with Parafilm and were placed in a 42°C water bath (Noki instrument, Changzhou, China), then treated with or without drug for 1 hour and transferred to the cell incubator for another 24 or 72 h.

Cell proliferation assay

Cells were plated 24 h before treatments in 96-well plates at 3000 cells/well. All cell lines were treated for 72 h before assaying. Proliferation (CellTiter 96® Aqueous One; Promega) assays were quantified with a compatible plate reader.

Western blot analysis

Cell lysates were equally loaded into 12% SDSpolyacrylamide gels, electrophoresed, and transferred to a PVDF membrane (0.45 µm, Merck Millipore, USA). Membranes were blocked 1 hour in TBS-Tween-20 containing 5% nonfat milk, and then incubated with primary antibodies at 4°C overnight. After being washed 3 times (10 min/time), the blots were incubated with horseradish peroxidase (HRP)-linked secondary antibodies at room temperature for 1 hour. The blots were visualized by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, USA) according to the manufacturer's instructions. All experiments were repeated three times with similar results.



Figure 1. Hyperthermia and oxaliplatin stimulated autophagy in CRC. Using fluorescence microscopy, autophagy induced by oxaliplatin was detected in HCT116-GFP-LC3b (A)/SW620-GFP-LC3b (B) stable-transfected cell lines, at 37 °C and 42 °C (1 hour, then cultured at 37 °C), respectively. GraphPad Prism 5 was used to analyze the number of GFP-LC3b puncta. (C) HCT116 cells treated by oxaliplatin at 42 °C (1 hour, then cultured at 37 °C) condition. Electron microscopy showed autophagic vacuoles, lysosomes, and the fusion of lysosomal autophagy: (a) junior lysosomes, (b) secondary lysosomes fusion with an autophagosome, (c) fragmentation substances result from autophagic degradation.

Electron microscopy

The cells were fixed for 1 hour at 4°C in 2% glutaraldehyde, washed, fixed again in 1% aqueous osmium tetroxide, then dehydrated in ethanol (30-100%), and embedded in Epon. Ultrathin sections (65 nm) were stained with 2% uranyl acetate and lead citrate, and then processed for electron microscopy with a PHILIPS CM-120 transmission electron microscope at 80 kV.

Fluorescence microscopy

Cells were plated on coverslips and, after treatment (70% confluence), fixed with 4% PFA for 15 minutes, washed, and mounted on slides with 2 μ g/mL DAPI (Beyotime, C1002) at 25°C



Figure 2. Hyperthermia and oxaliplatin-induced colon cancer cell death is enhanced by CQ. HCT116 (A) and SW620 (B) cell lines were treated with 10 μ M CQ, 50Q, oxaliplatin, 50 μ M oxaliplatin combined with 10 mb CQ, respectively, at 37 °C and 42 °C (1 hour, then cultured at 37 °C) conditions for 72 h. An MTS assay (CellTiter 96® Aqueous One; Promega) was used to detect cell viability, and the relative survival was calculated using GraphPad Prism 5. HCT116 (C) and SW620 (D) Cell lines were treated with 50 μ M oxaliplatin (3, 4), 50 μ M oxaliplatin combined with 10 μ M CQ (5, 6), 50 μ M oxaliplatin joint 20 μ M z-VAD-FM (7, 8), and 10 μ M CQ, 50 μ M oxaliplatin combined with 20 μ M z-VAD-FM (9, 10) for 72 hours. The control group was treated by saline (1, 2). Cell viabilities at 37 °C and 42 °C (1 hour, then cultured at 37 °C for 24 hours), and changes in the mitochondria was detected using scanning electron microscopy. Compared with the control group (a), mitochondrial ridges appear damaged in the drug treatment groups (b), in addition to mitochondrial vacuoles (c).

for 2-3 min. Then the cells were then washed twice with PBS (phosphate-buffered saline). JC-1 (Beyotime, C2005) reagent was used to cover the cells for 20 min at 37°C, and then coverslips were washed twice with PBS. A drop of PBS was added to the slides, and then the coverslips were placed on the slides. The cells

were observed immediately with a fluorescence microscope (Olympus, Japan).

Statistical analysis

Statistical analysis of the differences between the groups was performed using the Student's



Figure 3. CQ inhibits autophagy when treated with both hyperthermic conditions and oxaliplatin. HCT116 cells (A) and SW620 cells (B) were treated with 50 μ M oxaliplatin with or without 10 μ M CQ at 37 °C and 42 °C. Western blot was used to detected LC3-I/II and p62. 25 μ M and 50 μ M oxaliplatin or combined with 10 μ M CQ at 37 °C and 42 °C (1 hour, then cultured at 37 °C for 24 hours), Beclin-1 was detected by western blot. (C) The result of western blot was analyzed by Quantity one and plotted for the HCT116 cells. (D) The result of western blot was analyzed by Quantity one and plotted for the SW620 cells. (E) Beclin-1 was detected by western blot. (F) The comparison of the Beclin-1. All western blot pictures were analyzed by Quantity one and plotted result of plotted states and plotted by Quantity one and plotted plotted states analyzed by Quantity one and plotted states analyzed by Quantity one and plotted for the SW620 cells. (E) Beclin-1 was detected by western blot. (F) The comparison of the Beclin-1. All western blot pictures were analyzed by Quantity one and plotted plotted states analyzed by Quantity one and plotted states and plotted states and plotted states analyzed by Quantity one and plotted states analyzed by Quantity one

t-test. P<0.05 was considered statistically significant.

Results

Hyperthermia and oxaliplatin stimulated autophagy in colon cancer cells

Fluorescence microscopy was used to detect autophagy in HCT116-GFP-LC3b stable-trans-

fected cell lines (**Figure 1A**) or SW620-GFP-LC3b stable-transfected cell lines (**Figure 1B**), that were either treated with or without oxaliplatin at 37°C and 42°C (1 hour, then cultured at 37°C), respectively. We found autophagy occurred in both stable-transfected cell lines, with or without oxaliplatin treatment at 37°C and 42°C. GraphPad Prism 5 was used to analyze the number of GFP-LC3b puncta, the 42°C,



Figure 4. CQ increases hyperthermia- and oxaliplatin-induced apoptosis in CRC cell lines. (A) HCT116 cells were treated with 25 μ M and 50 μ M oxaliplatin with or without 10 μ M CQ at 37 °C and 42 °C, and saline as a control, (B) the result of western blot was analyzed by Quantity one and plotted using GraphPad Prism 5. HCT116 (C) and SW620 (D) were treated by 50 μ M oxaliplatin or oxaliplatin combined with 10 μ M CQ at 37 °C and 42 °C, control group was treated by saline, then stained nuclear or mitochondria with DAPI and JC-1. JC-1 can be set with high and normal potentials of the mitochondrial membrane. JC-1 gathers into oligomers, resulting in red fluorescence. When mitochondrial membrane potential declined, JC-1 can't gather into oligomers, resulting in a green fluorescence.

 50μ M oxaliplatin-treated group >37°C, 50μ M oxaliplatin-treated group >42°C, treatment group >37°C control group. Furthermore, we used electron microscopy to observe HCT116 cells that were treated with oxaliplatin at 42°C.

We observed a large number of autophagic vacuoles, lysosomes, and the fusion of lysosomal autophagy, junior lysosomes (Figure 1Ca), and secondary lysosomes, which were fused with an autophagosome (Figure 1Cb).



Figure 5. Combining oxaliplatin with CQ under hyperthermic conditions decrease HSP proteins. HCT116 cells (A) and SW620 cells (B) were treated with 50 µM oxaliplatin with or without 10 µM CQ at 37 °C and 42 °C. Western blot was used to detected HSP27, HSP70, and HSP90 proteins. (C) The comparison of the HSP proteins in the HCT116 cells. (D) The comparison of the HSP proteins in the SW620 cells. All western blot pictures were analyzed by Quantity one and plotted by GraphPad Prism 5.

Figure 1Cc shows fragmented substances after autophagic degradation.

Hyperthermia- and oxaliplatin-induced colon cancer cell death is enhanced by CQ

We initially explored the inhibitory effect of CQ, oxaliplatin, and oxaliplatin combined with CQ in HCT116 (**Figure 2A**) and SW620 (**Figure 2B**) cell lines at 37°C and 42°C. We found the cell viability of the CQ treatment group at 37°C to not have been inhibited, and the cell activity of the group stimulated by oxaliplatin combined with CQ was significantly lower than cell lines treated with oxaliplatin alone; the same trend appeared at 42°C, and cell viability was suppressed to a greater degree. After treating with oxaliplatin and CQ at 42°C, the cell viability of HCT116 was decreased more significantly than in SW620.

To explore the mechanism occurring when treated with oxaliplatin combined with CQ under 42° C-hyperthermia induced colon cancer cell death, we treated cells with 50 μ M

oxaliplatin (Figure 2C-3,4, 2D-3,4); 50 µM oxaliplatin, combined with 10 µM CQ (Figure 2C-5,6, 2D-5.6): 50 µM oxaliplatin combined with 20 µM z-VAD-FMK (V116, Sigma, Figure 2C-7,8, 2D-7.8); and 10 µM CO, 50 µM oxaliplatin combined with 20 µM z-VAD-FMK (Figure 2C-9,10, 2D-9,10), respectively. HCT116 and SW620 cells were treated for 72 hours at 37°C and 42°C, the control group cells was treated with saline (Figure 2C-1,2, 2D-1,2). The MTS assay (CellTiter 96® Aqueous One; Promega) was used to detect cell viability. As seen in the figures, z-VAD-FMK can largely revert the killing effects of oxaliplatin or oxaliplatin combination with CO on both HCT116 (C) and SW620 (D) cell lines. This shows that, the treatment of oxaliplatin combined with CQ under hyperthermia induced caspase-dependent apoptosis in colon cancer cell lines [22].

Scanning electron microscopy confirmed the results (Figure 2E). Compared with the control group (Figure 2Ea), mitochondria show damage both along the ridges (Figure 2Eb) and mito-



Figure 6. Combining oxaliplatin with CQ under hyperthermic conditions results in the downregulation of anti-apoptosis proteins. HCT116 cells (A) were treated with 25 μ M, 50 μ M oxaliplatin with or without 10 μ M CQ at 37 °C and 42 °C. The control group was treated with saline, (B) the result of western blot was analyzed by Quantity one and plotted by GraphPad Prism 5.

chondrial vacuoles (**Figure 2Ec**) as a result of drug treatment. These results further validate that oxaliplatin acted synergistically with CQ at 42°C, induces endogenous caspase-dependent apoptosis.

CQ still played the role of inhibiting autophagy in the case of hyperthermia plus oxaliplatin treatment

We detected the influence of treatment by oxaliplatin or CQ alone, and combined oxaliplatin with CQ at 37°C and 42°C (1 hour, then cultured at 37°C), using saline-treated cells as control group. In HCT116 (Figure 3A, 3C) and SW620 (Figure 3B, 3D) cell lines, CQ causes the accumulation of autophagy marking protein LC3-I/II. Comparing the control group with CO-treated group, it was observed that the p62 protein was not reduced significantly, but the LC3-I/II protein had a significant accumulated. A similar trend was seen when comparing samples treated with 50 μ M oxaliplatin and 50 μ M oxaliplatin combined with 10 µM CQ. This shows that CQ can inhibit the process of autophagy by blocking it at the stage of autophagosome fusion with lysosomes at 37°C and 42°C.

In addition, we detected Beclin-1, which is a protein that appears upstream of the autophagy pathway (**Figure 3E**, **3F**), and we found that Beclin-1 was upregulated more significantly at 42°C than at 37°C, regardless of the presence of oxaliplatin. Surprisingly, Beclin-1 was down-

regulated to the level even less than the same process conditions of 37°C. This shows that, CQ may regulate more upstream proteins, resulting in upregulation Beclin-1 reduction. Further studies on the molecular mechanisms need to be performed.

CQ increases hyperthermia- and oxaliplatininduced apoptosis in CRC cell lines

We examined the changes of the downstream markers of apoptosis pathway. We found that apoptosis induced by the 25 μ M oxaliplatin-treated group at 42°C was more significant than at 37°C. However, there was no a significant increase in 50 μ M oxaliplatin-treated group compared with the 25 μ M group under 42°C. PARP was activated significantly when hyperthermia (42°C) was combined with CQ, while CQ alone did not activate PARP. Furthermore, the 50 μ M oxaliplatin combination group was significantly stronger than the 25 μ M combination group. After association with CQ at 42°C, PARP-1 increased nearly two times.

JC-1 can be set with high and normal potentials of the mitochondrial membrane. When the JC-1 is gathered into oligomers, red fluorescence excitation occurs. JC-1 cannot gather into oligomers when the mitochondrial membrane potential is low, it exists in monomeric form, which emits green fluorescence [23]. HCT116 (**Figure 4C**) and SW620 (**Figure 4D**) cell lines were treated with 50 μ M oxaliplatin, 50 μ M oxalipla-



Figure 7. Combining oxaliplatin with CQ under hyperthermic conditions opens the apoptosis pathway, but not by upregulating caspase proteins. HCT116 cells (A) and SW620 cells (B) were treated with 50 μ M oxaliplatin with or without 10 μ M CQ at 37 °C and 42 °C, with a saline-treated control. Western blot was used to detected caspase-3, cleaved caspase-3, BAK, Bax, and c-IAP2 proteins. (C) The comparison of the caspase-3, cleaved caspase-3 and c-IAP2 proteins in the HCT116 cells. (D) The comparison of the caspase-3, cleaved caspase-3 and c-IAP2 proteins in the SW620 cells. (E) The comparison of the BAK, Bax proteins in the HCT116 cells. (F) The comparison of the BAK, Bax proteins in the SW620 cells. All western blot pictures were analyzed by Quantity one and plotted by GraphPad Prism 5.

tin combined with 10 μ M CQ at 37°C and 42°C, saline treated group was used as a control. In both cells, the control groups' JC-1 was concentrated in the mitochondrial membrane, and the red fluorescence was excited. On the other hand, oxaliplatin-treated cells showed a green fluorescence, indicating the potential of mito-

chondrial membrane was reduced by oxaliplatin. An even further reduction of mitochondrial membrane potential was observed when oxaliplatin was combined with CQ; and the red fluorescence almost disappeared, thus indicating that combination therapy can induce the mitochondrial-dependent endogenous apoptosis.



Figure 8. Schematic of colon cancer cell death treated by oxaliplatin combined with CQ under hyperthermic conditions at 42 °C. Both oxalipatin and hyperthermia induce autophagy, but CQ can inhibit autophagy, resulting in enhanced apoptosis. Using this treatment, HSP, IAP, and anti-apoptosis protein of BCL-2 family were all downregulated to different degrees. Also, the caspase-dependent apoptosis pathway became smoother.

Combining oxaliplatin with CQ under hyperthermic conditions decrease HSP proteins

HCT116 (Figure 5A, 5C) and SW620 (Figure 5B, 5D) cell lines were treated with oxaliplatin or CQ at 37°C and 42°C. In HCT116 and SW620 cell lines, HSP27 sharply reduced at 42°C, compared to 37°C, HSP70 showed a significant decrease in HCT116, but not in SW620 cells. HSP90 was downregulated in both cell lines, and even more when combined with CQ at 42°C.

Combining oxaliplatin with CQ under hyperthermic conditions in order to downregulate anti-apoptosis proteins

HCT116 cell lines were treated with 25 μM, 50 μM oxaliplatin, and combined with CQ at 37°C and 42°C for 1 hour, respectively (**Figure 6A**, **6B**). Without any drug treatment, MCL-1 was downregulated at 42°C, however, oxaliplatin-treated group and combination group showed no decrease at 42°C. BCL-2 was reduced more obviously at 42°C than 37°C regardless of the

treatment. Interestingly, BCL-2 did not show a decreasing trend when cells were treated with increasing concentrations of oxaliplatin alone at 42°C. However, after combining with CQ, BCL-2 was reduced significantly with increasing drug concentrations. The BCL-xI volume increased when cells were treated with increasing concentrations of oxaliplatin alone at 37°C and 42°C, but after combining with CQ, this trend was reversed at 42°C.

Combining oxaliplatin with CQ under hyperthermia conditions opens apoptosis pathway up, but not by upregulating caspase proteins

We used 10 μ M CQ, 50 μ M oxaliplatin, and a combination of oxaliplatin with 10 μ M CQ to treat HCT116 (Figure 7A, 7C, 7E) and SW620 (Figure 7B, 7D, 7F) at 37°C and 42°C for 1 hour, and then cultured for 24 hours at 37°C. We found that, in both cell lines, CQ used alone hardly promotes caspase-3 activation, while the oxaliplatin combined treatment group shows no obvious caspase-3 activation. This appears to be contradictory to enhanced apop-

tosis. Next, we examined the changes in proapoptosis proteins, BAX and BAK. We found that BAK was upregulated significantly in both HCT116 and SW620 cell lines, while BAX remained nearly unchanged in HCT116 cell lines. We know that BAK and BAX are oligomers at the mitochondrial membrane at the previous stage of apoptosis. Due to the interactions of caspase-8, Bid, and other apoptosis proteins, the mitochondrial membrane permeability was altered, and apoptosis inducing factor (AIF), cytochrome c, EndoG, and Smac/Diablo released. We have previously described the results of JC-1 experiments, which demonstrate that the mitochondrial membrane potential was decreased in the two cell lines under the same conditions. Because BAK levels were raised, the contents of the mitochondrial membrane were released and the potential was decreased. Increasing the presence of BAK oligomers will release cytochrome c and other factors from the mitochondria, resulting in apoptosis, with the possibility of enhancement. In addition, we monitored c-IAP2 expression under the same treatment of two cell lines. We found that oxaliplatin combined with CQ decreased c-IAP2 at 37°C. At hyperthermia conditions, including a 42°C water bath for 1 hour, even more downregulation is seen.

Discussion

In the present study, we selected colon cancer cell lines HCT116 and SW620. We used oxaliplatin or CQ alone, or combined oxaliplatin with CQ at 37° C and 42° C in a 1 hour water bath, then cultured at 37° C for an additional 24 hour. Then, the cell viability and the proteins changes in the apoptosis pathway were detected.

We found that the cell viability of the CQ-treated group at 37°C was not inhibited, while the oxaliplatin combined with CO-treated group was significantly lower than the group treated by oxaliplatin alone. The same trend also appears at 42°C, and the cell viability was suppressed to a greater degree. The drop in cell viability of HCT116 cells treated by oxaliplatin combined with CQ at 42°C was more significant than of SW620. z-VAD-FMK can largely revert the killing effect of two kinds of cell lines, which treated by oxaliplatin or oxaliplatin combined with CO. This shows that the cells that are subjected to conditions at 42°C, combined with oxaliplatin and CO in colon cancer cells die due to caspase-dependent apoptosis [22]. Scanning electron microscopy confirmed these results.

Colon cell lines treated by oxaliplatin at 37° C, as well as with or without oxaliplatin at 42° C underwent autophagy. The level of autophagy of the 50 μ M oxaliplatin-treated group at 42° C was higher than that at 37° C as well as the control groups at 42° C and 37° C. In the CQ-treated HCT116 and SW620 cells, p62 was not reduced significantly, while LC3-I/II accumulation was significant. This shows that CQ can block autophagy at the stage of autophagosome fusion with lysosomes, no matter the temperature condition.

In addition, we found that, Beclin-1 had a greater increased at 42°C compared to 37°C. Surprisingly, Beclin-1 was downregulated less at 37°C when combined with CQ. This shows that CQ may regulate more upstream proteins of the autophagy pathway, leading to a decrease in autophagy element Beclin-1. However, the detailed molecular mechanisms are required to be studied.

We found that the apoptosis of the 25 µM oxaliplatin-treated group at 42°C was more obvious than at 37°C, while the apoptosis of the 50 µM oxaliplatin-treated group had no marked increase. Under hyperthermic conditions at 42°C, CQ can't activate PARP obviously, while oxaliplatin combined with 10 µM CQ activated PARP significantly. Furthermore, the expression in the 50 µM combination group was stronger than the 25 µM combination group. In fact, oxaliplatin combined with CQ at 42°C causes PARP-1 increased nearly two times as compared to the group without CQ. In both cell lines, oxaliplatin promoted the decrease in mitochondrial membrane potential. The combined treatment by oxaliplatin and CQ induces greater endogenous apoptosis reliance on mitochondria [23].

HSP27 inhibits Smac/Diablo functions [16]. HSP70 restrains Bax/Bak oligomerization in the mitochondria [17], thereby inhibiting the formation of mitochondrial channels. Caspase-8-mediated Bid shear to tBid [18] is inhibited by HSP90. tBid is necessary to initiate the oligomerization of the Bax/Bak channel. If HSP70 production is upregulated, BAX/BAK lose their functionalities and pro-apoptotic factors Cyt-c, AIF, EndoG, Smac/Diablo cannot be released, consequently severely inhibiting apoptosis. When hyperthermia was combined with oxaliplatin and CQ in HCT116 and SW620 cells resulted in varying degrees of HSP regulation, but the general trend was analogous. In both cell lines, HSP27 was downregulated slightly at 37°C, and more markedly at 42°C. HSP70 was reduced significantly in HCT116 cells, while HSP90 was downregulated in both cell lines at hyperthermic conditions combined with oxaliplatin and CQ. These results show that the treatment overcomes the inhibition of HSP in order to induce apoptosis.

CQ reversed the trend of anti-apoptotic protein and pro-apoptosis protein expression in the hyperthermia treatment combined with oxaliplatin. In HCT116 cells, MCL-1 was down regulated at 42°C, even without drug treatment. However, the down regulation level of MCL-1 showed no difference between the 37°C and 42°C treatment groups. BCL-2 was downregulated at 42°C more than at 37°C with or without the drug treatment. Interestingly, BCL-2 did not decrease with increasing concentrations of oxaliplatin at 42°C, while when combined with CQ, BCL-2 was downregulated with increasing drug concentration. BCL-xl was upregulated with increasing oxaliplatin concentration at 37°C and 42°C, while CQ reversed this trend. Oxaliplatin-treated groups, pro-apoptosis protein Bim, BAK, and BAX were downregulated slightly at 42°C, while CQ reversed this trend once again. Downregulated anti-apoptosis proteins and upregulated pro-apoptosis proteins result in an enhanced apoptosis.

We know that in the previous stage of apoptosis. BAK and BAX gather on mitochondrial membrane. Interactions with caspase-8, Bid and other proteins, mitochondrial membrane permeability was altered, triggering the release of apoptosis inducing factor (AIF), cytochrome c, EndoG, and Smac/Diablo. Treatment with CQ alone results only in slight caspase-3 activation in HCT116 and SW620 cell lines, while treatment with oxaliplatin or oxaliplatin plus CQ, resulted in not significant activation of Caspase-3, seemingly contradicting the effect of enhanced apoptotic activity. Pro-apoptosis proteins BAK was upregulated significantly, while BAX remained almost unchanged in both cell lines. Increased levels of BAK caused the release of the mitochondrial contents and decreased in membrane potential. This increase of BAK oligomers encourages the release of apoptosis initiation factors, providing the possibility of enhance apoptosis. In addition, we studied the IAP family protein c-IAP2 in the same treatment in two cell lines, was downregulated by oxaliplatin plus CQ at 37°C, and 42°C-hyperthermia strengthened this effect very obviously.

In summary, both oxalipatin and hyperthermia induce autophagy, but CQ can inhibit autophagy, resulting in enhanced apoptosis. Under this treatment, HSP, IAP, and anti-apoptosis protein of BCL-2 family were all downregulated to different degrees. The caspase-dependent apoptosis pathway became smoother. This treatment mechanism is briefly descripted at **Figure 8**. Hyperthermia combined with oxaliplain and oxaliplain combined with CQ has already been used in clinical colon cancer treatment, and our study shows a potential of the combination therapy to inhibit colon cancer more effectively.

Disclosure of conflict of interest

None.

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