Chloroquine-Mediated Cell Death in Metastatic Pancreatic Adenocarcinoma Through Inhibition of Autophagy

Hermann B Frieboes1,2, Justin S Huang3, Wenyuan C Yin3, Lacey R McNally2,3

1Department of Bioengineering, 2James Graham Brown Cancer Center, and 3Department of Medicine; University of Louisville, Louisville, KY, USA

ABSTRACT

Context Cells in the interior of pancreatic tumors are believed to undergo continual autophagy to maintain homeostasis during unregulated growth in hypoxia caused by impaired vascularity. We hypothesize that treating metastatic cells with chloroquine, an inhibitor of autophagy, in hypoxia will decrease cell viability and induce cell death.

Design MiaPaCa2 (non-metastatic) and S2VP10 (metastatic) cell lines were treated with 25 and 50 µM chloroquine for 24 and 48 hours in normoxia and hypoxia (5-10% O2). Viability was measured using ATPlite™. After treatment, the cell stress was analyzed, and protein was lysed and quantified using the Bradford assay. Autophagy-associated protein levels were determined by Western blot.

Results S2VP10 cells treated for 48 hours with 50 µM chloroquine in hypoxia had 24% viability compared to normoxia control, with loss of 10% viability caused by low O2 alone. MiaPaCa2 cells under these conditions had 60% viability compared to normoxia control, with loss of 25% viability caused by low O2 alone. Analysis of cell stress pathways and dosimetry of Western blot data suggest that chloroquine inhibits the autophagy pathway in the metastatic S2VP10 cells.

Conclusion Autophagy blockage with chloroquine or similar-acting drugs may serve as a viable therapy for highly metastatic pancreatic cancers.

INTRODUCTION

For the past decade the incidence of pancreatic cancer has increased by about 1.5% per year; it is currently the fourth leading cause of cancer death in the United States [1]. In 2013, about 45,000 people will be diagnosed with this cancer and over 38,000 people will die from it. The most common pancreatic cancers are those that occur in the exocrine cells of the pancreas, out of which adenocarcinomas comprise about 95% [2]. At diagnosis, patients have a median survival shorter than 6 months and a 5-year survival rate of about 5%. The main treatment options for exocrine pancreatic cancers include surgery, radiation therapy, and chemotherapy. Following resection of the pancreas, 5-year survival rates range from 10% to 19% [3, 4, 5]. Although surgery is the most curative method, most patients (more than 80%) are unsuitable candidates due to systemic metastases. The outcome of chemotherapy in metastatic stages, where surgery is no longer a viable option, is a dismal 5-year survival ranging between 1% and 5% [6, 7].

Autophagy is a cellular response to stress in which organelles, cytoplasm, proteins, and metabolic byproducts are degraded. The process involves the packaging of unnecessary, worn out, or toxic products into autophagosomes. The autophagosomes then fuse with lysosomes to form autolysosomes in which target molecules are degraded into base components [8, 9]. In particular, autophagy is a critical process that allows cancer cells to manage the metabolic stress products created by local hypoxia and higher metabolic activity from increased cell turnover, as occurs within solid tumors [10, 11, 12]. This process is crucial to the survival and growth of apoptosis-deficient cancer cells [11]; autophagic processes protect the cellular genome and preserve limited resources in cancer cells in which dysregulated growth processes generate metabolic stress [13, 14]. Inhibition of autophagy disrupts this chain of events, resulting in the accumulation of metabolic stress products and thus inducing cell death [10, 11, 14], e.g., autophagy inhibition has been shown to induce tumor cell apoptosis [15, 16, 17] and defective autophagy has been linked to increased deoxyribonucleic acid (DNA) damage and genomic instability in breast cancer [14]. Hsp90 is another regulatory protein
involved in the cellular response to metabolic stress and the disruption of its associated mechanisms may also offer a target to induce death in cancer cells [18]. Pancreatic cancer is well established to survive in a hypoxic environment and to have high levels of autophagy [19, 20].

The anti-malarial drug chloroquine disrupts autophagy by inhibiting the acidification of the lysosomes that fuse with the autophagosomes, thus preventing the degradation of metabolic stress products and thereby inducing cellular apoptosis [21, 22, 23]. Chloroquine-mediated inhibition of autophagy has been demonstrated in melanoma cell lines in in vitro and in vivo subcutaneous tumor models [25, 26, 27]. The role of autophagy in pancreatic adenocarcinoma and the benefits of blocking it have recently been shown in a study in which animal survival was dramatically extended with chloroquine treatment against non-metastatic pancreatic adenocarcinoma [20]. Recent studies have shown that chloroquine-mediated chemosensitization to therapy may be an autophagy-independent event in some cancer cells, e.g., breast cancer [28]. However, there is evidence that the role of this chemosensitization in pancreatic cancer is autophagy-dependent [20].

Here, we seek to further elucidate the efficacy of chloroquine therapy in highly metastatic cancer, which is critical since most pancreatic cancer patients present with metastases at the time of diagnosis. Aggressive metastatic pancreatic cancer cells, such as S2VP10, are likely to be especially dependent on autophagic processes to maintain cellular homeostasis in the setting of unregulated cell growth. We have previously documented the propensity of S2VP10 cells to spontaneously result in metastasis from the orthotopic site [29]. Further, we investigate chloroquine treatment against these aggressive cells under hypoxic conditions, as would be characteristically found in the interior of primary, as well as secondary, pancreatic tumor lesions. We hypothesize that inhibition of autophagy in these aggressive cells causes accumulation of toxic metabolic byproducts and thus induces cell death. We compare the results against a less aggressive cell line representing non-metastatic disease. This work represents an initial step to help elucidate the suitability of chloroquine therapy to treat metastatic pancreatic adenocarcinoma, for which few curative options currently exist.

MATERIALS AND METHODS

Cell Lines

The pancreatic cancer cell line MiaPaCa2 (American Type Culture Collection (ATCC), Manassas, VA, USA) was used in this study to represent non-metastatic disease. The S2VP10 cell line (a generous gift from Dr. Michael Hollighsworth, University of Nebraska) was used to represent highly aggressive, metastatic disease. Cells were grown in DMEM and with 10% FBS and 1% L-glutamine at 37°C in a humidified incubator. Cells were grown and treated under both normoxic (20% O₂) and hypoxic conditions (5-10% O₂). Cells were plated in normoxia for 24 hours prior to moving to hypoxic conditions. S2VP10 cells spontaneously result in metastasis from the orthotopic site [29]. In contrast, MiaPaCa2 cells are typically injected into the spleen and give rise to deposition in the liver via the splenic vein [30]. We have investigated direct injection of the pancreas with MiaPaCa2 cells but have not witnessed any spontaneous metastases from the orthotopic site within 70 days.

Hypoxia

We performed parallel experiments under hypoxic conditions in environments with 5-10% O₂. There are two sets of rationale for the hypoxia experiments. The tumor microenvironment is known to be relatively hypoxic, and hypoxia is associated with genetic instability, metastatic spread, inadequate response to radiotherapy, and poor prognosis [31]. The use of a hypoxic environment more closely mimics the tumor microenvironment in metastatic cancer, thereby improving the clinical relevance and translational potential of the experiments. The second rationale is that we hypothesize that hypoxia will exacerbate cellular metabolic stress. Stressed cells are expected to increase their autophagic processes to conserve resources and dispose of stress byproducts, thus hypoxic cells would be more sensitive to inhibition of autophagy.

Treatment

Cells were treated with 25 µM and 50 µM of chloroquine (Acros Organics, part of Thermo Fisher Scientific Inc., Bridgewater, NJ, USA) in a 96 well plate for 24 and 48 hours in both normoxic and hypoxic conditions prior to assay. Cell viability was measured using ATPlite™ according to the manufacturer’s instructions (Perkin Elmer, Waltham, MA, USA). Adenosine triphosphate (ATP) levels were measured using a plate reader (Packard TopCount NXT, Meriden, CT, USA) and normalized to phosphate-buffered saline (PBS) treated control. S2VP10 or MiaPaCa2 cells were plated in 6 well plates and treated with 25µM and 50µM of chloroquine in hypoxic and normoxic conditions for Western blot at 24 and 48 hours. Each experiment was performed no fewer than 3 times.

Protein Analysis

After 25 µM and 50 µM treatment of chloroquine for 24 hours, protein from S2VP10 and MiaPaCa2 cells...
was lysed in a buffer solution containing 1% Nonidet P-40 or nonylphenoxypolyethoxyethanol (Pierce Biotechnology, Rockford, IL, USA) (NP-40), 1% phosphatase inhibitor, and 1% protease inhibitor in nuclease free water. Lysates were centrifuged at 13.3 g for 10 minutes at 4°C. Total protein in the lysate was quantified using the Bradford assay. Cell stress was analyzed using a cell stress array according to manufacturer's instructions (R&D Systems, Minneapolis, MN, USA) with the exception of using infrared-labeled secondary antibodies Streptavidin (Li-Cor, Lincoln, NE, USA) at a concentration of 1:2,500. Measurements were obtained via dosimetry. The levels of common autophagy-associated proteins were determined by standard Western blot analysis. Fifty μg of protein was added to NuPage® Novex® 4-12% Bis-Tris (Life Technologies, Carlsbad, CA, USA) gel and then transferred onto nitrocellulose membrane using iBlot (Invitrogen, Grand Island, NY, USA). Membranes were blocked with Li-Cor (Lincoln, NE, USA) blocking buffer. Proteins were incubated with LC3 (Novus Biologicals, Littleton, CO, USA) at a concentration of 1:500, ATG5, ATG12, and ATG7. The antibody detects LC3-I and LC3-II simultaneously; thus, only one beta-actin loading control is required. The membranes were incubated overnight at 4°C then washed three times using TBS. Secondary anti-rabbit IgG (Li-Cor, Lincoln, NE, USA) was added at a concentration of 1:2,500 and incubated for one hour at room temperature. The membranes were washed again using TBS. Membranes were scanned and analyzed using Li-Cor Odyssey (Lincoln, NE, USA). Statistical analyses were not performed since the values obtained from protein analysis were relative to control.

**Imaging**

Cells were imaged after therapy using phase contrast microscopy to follow the morphology changes associated with chloroquine therapy. Electron microscopy was used to determine ultrastructural changes in the lysosomes and autophagosomes. Acidine orange staining was performed and imaged with fluorescent microscopy to follow the changes in lysosomal trafficking.

**STATISTICS**

Data are shown as mean±SD. ANOVA analysis was performed with SAS software (Version 9.3, SAS Institute Inc., Cary, NC, USA). Significance was set at a two-tailed P value level of 0.05.

**RESULTS**

Both non-metastatic MiaPaCa2 and metastatic S2VP10 cells in normoxic monolayer culture were treated with two dosages of chloroquine, namely, 25 and 50 μM. Morphologic changes were observed in the S2VP10 upon treatment but not in the MiaPaCa2 cells (Figure 1), specifically regarding the accumulation of membrane bound vesicles. The

![Figure 1. Photographs after 24 hours of treatment in normoxia. Both non-metastatic MiaPaCa2 and metastatic S2VP10 cells in monolayer culture were treated with either 25 μM or 50 μM chloroquine. a. S2VP10 control; b. S2VP10 treated with 25μM chloroquine; c. S2VP10 treated with 50μM chloroquine; d. MiaPaCa2 control; e. MiaPaCa2 treated with 25μM chloroquine; f. MiaPaCa2 treated with 50μM chloroquine. Morphologic changes were observed in the S2VP10 upon treatment but not in the MiaPaCa2 cells. Arrows highlight large autophagic vacuoles plainly visible inside the metastatic cells (bars: 100 μm). Inset in panel c. shows electron microscopy photograph of autophagic vacuole in a treated cell; inset in panel f. shows electron microscopy photograph of an untreated cell showing absence of vacuoles.](http://www.serena.unina.it/index.php/jop)
presence of large autophagic vacuoles was plainly visible inside the metastatic cells (Figure 1bc, arrows). Electron microscopy further confirmed the presence of these vacuoles in treated cells (Figure 1c, inset) and the absence of such vacuoles in untreated cells (Figure 1f, inset). Evaluation of acridine orange staining did not result in orange staining. Both pancreatic cell types were observed to turn the environment acidic; since acridine orange staining is based on pH, this staining was determined to be not reliable for our system. Similarly, because the cells used in this study create an acidic environment, the GFP-LC3 does not fluoresce in our system. However, we noted very large vesicles (greater than 200 μm) in cells treated with chloroquine under hypoxic conditions (similar to Figure 1).

Next we compared normoxic treatment response to the response of cells in hypoxic conditions as they would exist in the interior of secondary and primary pancreatic tumor lesions. Figure 2 shows cell viability as a fraction of control in both normoxic (20% O2) and hypoxic (5% O2) conditions. Treatment with 50 μM chloroquine for 48 hours killed 50% of the aggressive S2VP10 cells in normoxia (Figure 2b) and 76% of these cells in hypoxia (Figure 2d). Under these conditions, the non-metastatic MiaPaCa2 cells, in contrast experienced inhibitions of 25% in normoxia (Figure 2b) and 40% after 48 hours in hypoxia (Figure 2d), i.e., about half that of the S2VP10. ANOVA analysis showed that chloroquine at 50 μM caused significant reduction in cell viability in S2VP10 compared to MiaPaCa2 in both hypoxia and normoxia (P=0.044, panel b; P=0.034, panel c; P=0.012, panel d; P=0.039, panel e; P=0.031, panel f).
for the MiaPaCa2 and 10% for the S2VP10 cells after 48 hours (Figure 2f). Therefore, the effect of hypoxia alone on the aggressive metastatic cells was minimal.

In order to further analyze the effect of chloroquine, we evaluated key cell stress pathways in S2VP10 cells in both normoxic and hypoxic (5% O₂) conditions. Sample stress array membranes were imaged using infrared dyes (Figure 3). In the membranes, bright pairs on upper right and bottom corners are positive controls. The 18 data points (in duplicate) were normalized by the controls and quantified using dosimetry as shown in Figures 4 and 5.

Most of the stress-associated proteins were altered upon treatment with chloroquine, indicating a cell stress response associated with autophagy. Positive controls are shown as pairs of dots in upper right and lower corners. The 18 data points (in duplicate) were normalized by the controls and quantified using dosimetry as shown in Figures 4 and 5.

Expression of p53 at the higher chloroquine dosage (50 µM) was almost the same in hypoxia and normoxia. Further analyses of stress proteins showed that while Cyt C was detected at about 3x higher levels in untreated cells and those treated with 25 µM chloroquine in hypoxia, possibly indicating an upregulation of apoptotic processes, the expression of a number of other proteins remained the same or lower (Figure 5). The amount of the other proteins was generally decreased upon treatment with 25 µM chloroquine, with an additional decrease in hypoxic conditions. The higher chloroquine dosage (50 µM) did not evince a similar effect in hypoxia; in this case, the protein expression, including Cyt C, was for the most part comparable to the normoxic control.

Analysis of the LC3-II protein suggests that chloroquine therapy does inhibit autophagy in metastatic pancreatic adenocarcinoma cells (Figure 6). LC3-II is associated with the cellular autophagosomes; accumulation of LC3-II suggests that the final step of autophagosome fusion with lysosomes to form the autolysosome is interrupted resulting in accumulation of LC3-II. Through the chloroquine-induced deacidification of the lysosomes, the cells are unable to complete autophagy and thus cannot completely process the excess of metabolic and cellular stress byproducts. Although S2VP10 cells had similar levels of LC3-II in both hypoxia and normoxia controls, LC3-II levels increased with chloroquine concentration (Figure 6ab). LC3-II levels were highest for S2VP10 with a combination of 50 µM chloroquine and hypoxia.

Further protein analysis (Figure 7) suggests an increase in LC3-II for S2VP10 cells when treated with chloroquine in normoxia for 24, 48, or 72 hours compared to untreated cells. These levels also increased in hypoxia, although apparently less than the amounts measured for the normoxic cases. The S2VP10 cells tolerate hypoxia better than the MiaPaca2 (Figure 2f) possibly due to the natively higher level of autophagy in the S2VP10 cells. Figure 6 shows that the proportion of LC3-I to LC3-II generally decreases with hypoxia for these cells, suggesting an upper (maximum) bound to an already-ramped up autophagic activity. In contrast, the proportion of LC3-I to LC3-II (Figure 6) is observed to stay stable for the MiaPaca2 cells under increasing hypoxia, suggesting a capacity to ramp up autophagic activity from a natively lower level. In general, variability in expression of Hsp60,
Hsp90, and ATG5 and 12 under all conditions (Figure 7) may reflect variation in cellular stress associated with chloroquine exposure, treatment duration, and hypoxic conditions.

**DISCUSSION**

Cellular autophagy can be induced by hypoxia, nutrient deprivation, and genetic stress [15]. Autophagy mediates recycling of the cell’s own components, e.g., damaged organelles and non-essential proteins, through the lysosomal machinery, and, in this manner, it can provide nutrients to cells [21]. Autophagy correlates with poor patient outcomes, especially in pancreatic cancer, suggesting that autophagy is associated with tumor growth and metastasis. However, the role of autophagy in cancer is controversial. It is still unclear whether autophagy upon activation by tumor therapy contributes to cell death or rather represents a resistance mechanism [21]. Recently, it has been observed that a peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist augments the anti-cancer effects of IFN-β through the induction of cell cycle perturbations and autophagic cell death in a non-metastatic pancreatic cancer cell line (BxPC-3) [32].

Mechanisms by which autophagy may promote metastasis and drug resistance are not well understood. Because autophagy promotes survival of cancer cells under starvation or other stressful cellular conditions, it has been hypothesized that higher autophagy is associated with more aggressive cancers. A better understanding of

![Figure 5](image-url) Analysis of cell stress pathways. Analysis of S2VP10 cells after treatment with chloroquine in normoxia and hypoxia (5% O2) conditions shows Cyt C levels in hypoxia at about 3x higher levels in untreated cells and cells treated with 25 μM chloroquine, possibly indicating an upregulation of apoptotic processes. The amount of the other proteins was generally decreased upon treatment with 25 μM chloroquine, with an additional decrease in hypoxic conditions. Higher chloroquine dosage (50 μM) did not show a similar effect in hypoxia; in this case, the protein expression, including Cyt C, was for the most part comparable to the normoxic control. The value of each column represents the average dosimetry measurements of the cell stress array membrane data in Figure 3 (one experiment run in duplicate).

![Figure 6](image-url) Western blot analysis of S2VP10 cells. a. Treatment with chloroquine in normoxia and hypoxia (5% O2) conditions shows accumulation of LC3-II under hypoxic as well as treatment conditions. b. Dosimetry data from the Western blot analysis quantifies the autophagy-associated protein flux. LC3-II accumulation suggests that the final step of autophagosome fusion with lysosomes to form the autolysosome is interrupted resulting in accumulation of LC3-II. Although S2VP10 cells had similar levels of LC3-II in hypoxia and normoxia controls, LC3-II levels increased with chloroquine concentration. LC3-II levels were highest with a combination of 50 μM chloroquine and hypoxia. Level of LC3-II compared to LC3-I is statistically different for the following cases: S2VP10 in hypoxia for control (P=0.021), 25 μ chloroquine (P=0.002), and 50 μM chloroquine (P=0.0004); and S2VP10 in normoxia for 25 μM chloroquine (P=0.009), and 50 μM chloroquine (P<0.001).
Autophagy may provide novel insight into mechanisms of cancer metastasis and also further the development of therapeutic approaches that target this cellular survival mechanism [20]. Aggressive cancers, including those that have metastasized, would be more dependent on autophagic processes to manage their metabolic stress byproducts; hence, these cancers would be more susceptible to inhibition of autophagy. Chloroquine combined with alternative agents is feasible and has been demonstrated, e.g., chloroquine treatment has been combined with nutrient deprivation in cancers such as melanoma, glioma, and fibrosarcoma [26, 27]. Further, autophagy inhibition is believed to sensitize cancer cells to chemotherapeutic or immune modulators [33, 34, 35, 36].

The results presented here support the hypothesis that chloroquine therapy inhibits autophagy in aggressive metastatic pancreatic adenocarcinoma. Because increased hypoxia found in the pancreatic tumors is associated with increased levels of autophagy [19, 20], blocking of autophagy is expected to result in pancreatic cell death. This is evidenced by reduction in LC3 proportion shown in Figure 6. Protein analysis shows an increase in stress-associated proteins when cells are treated with chloroquine especially in hypoxic conditions (Figures 4, 5, and 7). An increase in levels of LC3-II

Figure 7. Analysis of Western blot data using dosimetry. Data suggest an increase in LC3-II for S2VP10 cells when treated with chloroquine in normoxia for 24, 48, or 72 hours compared to untreated cells. These levels also increased in hypoxia, although apparently less than the amounts measured for the normoxic cases. In general, variability in expression of Hsp60, Hsp90, and ATG5 and 12 under all conditions may reflect variation in cellular stress associated with chloroquine exposure, treatment duration, and hypoxic conditions. (The values are reported for one experiment. These data are included as additional supporting information for the results in Figure 6).
(Figure 6) suggests that the final step of autophagosome fusion with lysosomes to form the autolysosome is interrupted by the chloroquine treatment, creating an accumulation of LC3-II. By chloroquine-induced de-acidification of the lysosomes, the cells are unable to complete the autophagy process and thus cannot completely digest the metabolic and cellular stress byproducts, leading to an increased p53 expression (Figure 4) and Cyt C release (Figure 5), and ultimately cell death (Figure 2).

Aggressive pancreatic cancer cell lines, such as S2VP10, are less sensitive to hypoxic conditions than non-aggressive cell lines such as MiaPaCa2, possibly due to having perpetually higher levels of autophagy. This suggests that autophagy is essential to the survival of the S2VP10 cells, and that blocking autophagy would lead to cell death. Photographs taken 24 hours post-treatment show an accumulation of autophagic vesicles in treated cells (Figure 1), also supporting the notion that chloroquine acts through inhibition of autophagy. Although the increase in LC3-II level (Figures 6 and 7) may largely be a hypoxia event, it seems reasonable to hypothesize that by blocking the autophagy-associated protein flux with chloroquine will result in cell death due to the cells’ reliance on maintaining high levels of autophagy in order to survive. Since the results demonstrate high levels of autophagy in response to hypoxia, blocking of autophagy could be a feasible therapeutic target.

Chloroquine is a well-tolerated, safe drug already in clinical use for treatment of malaria and other types of parasitic infections. We are pursuing in vivo studies to further help establish the efficacy of chloroquine for highly aggressive metastatic pancreatic cancer. Chloroquine treatment may provide a substantial benefit for patients with metastatic pancreatic adenocarcinoma, for whom few clinically effective therapies currently exist.

**Funding** Department of Bioengineering and James Graham Brown Cancer Center, University of Louisville, NIH CA-139050, and Brown Cancer Center Summer Intern Program for High School Students

**Conflict of interests** None to disclose

**References**


