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Modulation of Pediatric Brain Tumor Autophagy and Chemosensitivity

Jean M Mulcahy Levy¹ and Andrew Thorburn²

¹Department of Pediatrics, University of Colorado 12801 E 17th Ave RC-1 South, Rm 6400D Mail Stop 8303 Aurora, CO, USA, 80045

²Department of Pharmacology, University of Colorado 12801 E 17th Ave RC-1 South, Rm 6400D Mail Stop 8303 Aurora, CO, USA, 80045

Abstract

Brain and spinal tumors are the second most common malignancies in childhood after leukemia, and they remain the leading cause of death from childhood cancer. The role of autophagy, a catabolic cellular process used to provide energy during times of stress, in pediatric tumors is unknown. Here we present studies done in pediatric medulloblastoma cell lines (DAOY, ONS76) and atypical teratoid/rhabdoid tumor cell lines (BT-16, BT-12) to test this role. Autophagy was inhibited using siRNA against autophagy related genes ATG12 and ATG7 or pharmacologically induced using rapamycin or inhibited using chloroquine to test the effect of autophagy on chemosensitivity. We found that when pediatric brain tumor cells are under starvation stress or exposed to known autophagy inducers, they show increased levels of autophagy. We also found that current chemotherapeutics (CCNU, cisplatin) stimulate autophagy in pediatric brain cancer cells. Silencing of ATG12 and ATG7 prevents this increase and autophagy can be pharmacologically stimulated and inhibited. Although autophagy can be induced and inhibited in these cell lines, the effect of autophagy on tumor cell killing is small. This may have significant clinical relevance in the future planning of therapeutic regimens for pediatric brain tumors.

Introduction

Brain and spinal tumors, the second most common malignancies in childhood after leukemia, account for 22% of all childhood cancers in children up to 14 and 10% of tumors in children 15-19. Despite advances in therapy, they remain the leading cause of death from childhood cancer. Understanding mechanisms of tumor cell death and survival are vital to developing new, targeted therapies and improving the effectiveness of currently utilized therapies. One of these mechanisms is autophagy, a catabolic process that turns over long-lived proteins and organelles and contributes to cell and organism survival during nutrient deprivation and other stresses. One form of autophagy, macroautophagy (hereafter referred to as autophagy), is a ubiquitous process in eukaryotic cells whereby double membrane vesicles called autophagosomes engulf proteins, organelles and other cytoplasmic components into a structure that fuses with a lysosome to form an autophagolysosome allowing engulfed material to be degraded {Mizushima, 2007 #4138}. Autophagy is thought to be a tumor suppression mechanism because a genetic deficiency in various autophagy regulators (e.g. beclin 1 {Qu, 2003 #3324; Yue, 2003 #3323}, Atg4 {Marino, 2007 #4264}, Bif1 {Takahashi, 2007 #4127}, UVRAG {Liang, 2006 #3783}) leads to increased cancer while many oncogenes inhibit autophagy and tumor suppressors increase autophagy {Maiuri, 2009 #4232}. However, autophagy may also promote tumor progression and

metastasis, by, for example, helping tumor cells survive in a stressful microenvironment {Degenhardt, 2006 #3785; Kenific, 2010 #4395}.

There is great interest in manipulating autophagy to improve cancer treatment but considerable disagreement about how to use the effects of autophagy appropriately {Hippert, 2006 #3776; Kondo, 2005 #3688; Levine, 2008 #4166; Maiuri, 2009 #4232}. There remains a basic question of whether we should increase or decrease autophagy in cancer patients. Many publications report autophagy as a tumor cell killing mechanism by diverse anti-cancer agents {García-Escudero, 2008 #4242; Lin, 2008 #4243; Turcotte, 2008 #4244}. However, autophagy during tumor cell treatment often inhibits tumor cell killing as shown in our lab {Thorburn, 2009 #4231} and by others {Amaravadi, 2007 #4019; Carew, 2007 #4090; Park, 2008 #4246; Wu, 2006 #3755}. Thus there is evidence that autophagy can prevent or promote cancer and kill or protect cancer cells. This creates an important problem—should we try to inhibit autophagy or stimulate autophagy in people with cancer?

The urgency of answering these questions is underscored by the fact that clinical trials that increase or inhibit autophagy are already active. For example, a current trial (ClinicalTrials.gov NCT00728845) uses hydroxychloroquine to inhibit autophagy in combination with carboplatin, paclitaxel and bevacizumab in lung cancer and trials combining other agents with hydroxychloroquine are recruiting patients with glioblastoma, breast cancer, multiple myeloma, prostate cancer and other advanced tumors. Conversely, several trials in patients use mTOR inhibitors and other drugs that induce autophagy including a phase I pharmacokinetic and pharmacodynamic study of ridaforolimus in pediatrics and adolescents with refractory malignancies including tumors of the central nervous system (ClinicalTrials.gov NCT00704054) and a phase 2 study of everolimus in pediatric patients with refractory low-grade gliomas (ClinicalTrials.gov NCT00789828).

While autophagy's importance in adult tumors has been studied quite extensively and there is a developing consensus that autophagy may often lead to chemoresistance {Levine, 2008 #4166; Qu, 2003 #3}, pediatric cancers are different in both their genetics and their response to therapy and it is not known what the role of autophagy is in pediatric tumors. We therefore worked to define autophagy's role in the treatment of pediatric brain tumors. We hypothesized that autophagy could be induced in pediatric brain tumor cell lines by starvation and treatment with current chemotherapeutics and FDA approved drugs, and that by altering the levels of autophagy within the cells, we could affect chemosensitivity. Here we show that when pediatric medulloblastoma and atypical teratoid/rhabdoid tumor (AT/RT) cell lines cells are under starvation stress or exposed to known autophagy inducers, they show increased levels of autophagy as seen by LC3 and p62 flux by immunoblot analysis. We also showed that current chemotherapeutics used to treat these tumors stimulates autophagy. Silencing of autophagy genes (e.g. ATG12, ATG7) and the use of chloroquine prevents this increase of autophagy and the use of rapamycin induces autophagy in these cells. However, MTS assays and clonogenic studies, looking at both short and long-term endpoints, found no significant difference in survival in cells when autophagy was manipulated. These findings show the effect of manipulation of autophagy on tumor cell kill may be small at best and does not appear to have an effect on long-term tumor cell survival. This may have significant clinical relevance in the future planning of therapeutic regimens for pediatric brain tumors.

Materials and Methods

Cells and Reagents

Medulloblastoma and glioma cells were obtained from ATCC: Daoy, number HTB-186; ONS76, number ***; U87 MG, number HTB-14. Peter Houghton, St. Jude Children's

Hospital, provided the AT/RT cells (BT-16, BT-12). Lomustine (CCNU) and cisplatin were obtained from Sigma (St. Louis, MO). siRNAs and transfection reagents were obtained from Dharmacon (Lafayette, CO), and rapamycin and chloroquine from Sigma (St. Louis, MO)

siRNA Transfection

Medulloblastoma and AT/RT cells were transfected with either a control scrambled siRNA or an siRNA targeting ATG12 and ATG7 (Dharmacon, Lafayette, CO) using DharmaFECT 2 transfection reagents according to the manufacturer's recommendation (Dharmacon, Lafayette, CO). Cells were grown for 72 hours and then replated for experiments and cell lysate samples were collected for immunoblot verification of protein knockdown.

Immunoblotting

Autophagy flux assays were performed evaluating microtubule-associated light-chain 3 (LC-3) II formation and p62 in the presence or absence of lysosomal protease inhibitors as recommended by Mizushima and Yoshimori. {Mizushima, 2007 #59} Cell lysates were collected in RIPA buffer and quantified by Bio-Rad protein assay. For siRNA transfections, cell lysates were similarly collected 72 hours after transfection to demonstrate protein levels. For each lysate sample, 5µg of protein were resolved on SDS-PAGE 15% denaturing gels and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% nonfat dry milk in TBST buffer for 1 hour and were incubated overnight with antibodies that recognize LC3 (Novus Biologicals, Littleton, CO), p62 (Abnova, Walnut, CA) or Actin (Sigma, St. Louis, MO) and then washed in TBST and incubated with the appropriate peroxidase-conjugated secondary antibody. Bands were visualized with Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA) on X-ray film and densitometry measurements were performed. All experiments were performed a minimum of three times.

Viability Assays

For short-term viability assays, cells were plated in 96-well plates with 2×10^3 cells/well and incubated overnight in media. Cells were then treated with CCNU, Cisplatin, or a combination of CCNU and Cisplatin in decreasing doses for 24 hours. Cell viability was evaluated by MTS assay according to the manufacturer's recommendation (Promega, Madison, WI). All experiments were performed three times in triplicate and the proportion of living cells was normalized to control wells of untreated cells.

For long-term viability assays, cells were plated in 12-well plates with 1×10^4 cells/well and incubated overnight in media. Cells were then treated with CCNU or cisplatin in decreasing doses for 24 hours. Drug was then removed, the cells were gently washed and fresh media was provided every 4 days until control untreated wells had grown to approximately 70% confluence. Cells were fixed and stained using 0.4% crystal violet. Because the medulloblastoma and AT/RT cell lines do not form tight colonies, stained cells were solubilized in 33% acetic acid and absorbance were read at 540nm. All experiments were performed three times in triplicate and the proportion of cells was normalized to control wells of untreated cells.

Results

To test if medulloblastoma and AT/RT cell lines have functional autophagy programs, Daoy and BT-16 cells were treated with known autophagy inducers in the presence or absence of lysosomal protease inhibitors, Pepstatin A (PepA) and E64D, or chloroquine which prevents the fusion of lysosomes with autophagosomes (Figure 1). When autophagy is induced there is conversion of LC3-I to LC3-II and accumulation of LC3-II in the presence of PepA/E64D

or chloroquine indicates autophagic flux. p62, a scaffolding protein that localizes in the cell with LC-3 II inside the autophagolysosome also accumulates in the presence of PepA/E64D or chloroquine. Starvation stress is the gold standard for autophagy induction. When our cells were stressed with serum starvation using Earl's Balanced Salt Solution (EBSS), autophagic flux was demonstrated by LC3-II and p62 accumulation in the presence of PepA/E64D or chloroquine (Figure 1A). The average accumulation of LC3-II and p62 levels in the presence of chloroquine over three experiments is shown in Figure 1B. Accumulation of LC3-II and p62 was also seen when the cells are treated with rapamycin, which induces autophagy through its ability to inhibit the mTOR pathway (Figure 1). ONS76 and BT-12 cells undergoing starvation stress with EBSS in the presence of protease inhibitors also caused accumulation of LC3-II, indicating autophagic flux (Figure 2A). These findings demonstrate that all four of these medulloblastoma and AT/RT cell lines have functional autophagy systems.

Medulloblastoma and AT/RT are treated clinically with combination chemotherapy agents. Two currently used chemotherapeutics, CCNU and cisplatin, were used to treat all four cell lines and cell lysates were evaluated for accumulation of LC3-II in the presence of PepA and E64D. Representative immunoblots are shown alongside cells starved with EBSS in Figure 2A. Accumulation of LC3-II was seen in the presence of PepA/E64D for all cell lines in the EBSS treated groups verifying functional autophagy. When these cells were treated with CCNU and cisplatin, there are varying levels of autophagy induced by the different drugs and in the different cell lines. The average accumulation of LC3-II over three experiments is shown in Figure 2B. In all cell lines treated with CCNU, there was an increase in LC3-II levels both before and after addition of protease inhibitors. All cell lines demonstrated autophagic flux with further accumulation of LC3-II following the addition of PepA/E64D. Of note, the ONS76 cells did not have as large an increase of LC3-II before the addition of protease inhibitors. When the cells were treated with cisplatin there was an increase in LC3-II in the BT-16, BT-12, and Daoy cells both before and after addition of PepA/E64D. The ONS76 cells demonstrated an initial drop in LC3-II levels, but had accumulation of LC3-II with PepA/E64D. These findings demonstrate that CCNU and cisplatin induce autophagic flux in all cell lines tested albeit to varying degrees.

Autophagy is a complicated, multi-step process that requires multiple proteins to complete the formation of the double membrane autophagosome and conjugation of LC-3 I to LC-3 II; siRNA knockdown of required autophagy related proteins can be used to block this process. ATG12 is a protein that is conjugated to ATG5, which is then targeted to the autophagosomes during elongation of the double membrane and is required for autophagosome formation. ATG7 is an E1 enzyme essential for this conjugation. Using siRNA targeted to ATG12 and ATG7, we were able to achieve a significant decrease of ATG12 protein 72 hours after transfection as shown by western blotting (Figure 3A). When these cells were treated 72 hours after transfection with either CCNU or cisplatin, there was a dramatic decrease in the accumulation of LC3-II in all cell lines compared to cells transfected with a control scrambled siRNA (Figure 3B). This indicates a significant decrease in the ability of these cells to initiate autophagy during treatment with CCNU or cisplatin.

To determine if the genetic manipulation of autophagy had an effect on tumor cell killing in pediatric brain tumor cells, autophagy was inhibited using siRNA to ATG12 and ATG7 and cells were treated with increasing doses of CCNU, cisplatin, or a combination of both drugs. Cells were treated 72 hours after transfection and knockdown of ATG12 levels were confirmed by immunoblot analysis. All survival studies were conducted on cells achieving at least a 70% decrease in ATG12, which as noted above is sufficient to inhibit autophagy in these experiments (representative immunoblots, Figure 3A). Cell survival was compared

between cells treated with siRNA to ATG7 and ATG12 and cells treated with a control scrambled siRNA (Figure 4). There was no significant difference seen in tumor cell survival in a short-term MTS assay between any of the treatment groups.

Clinically, patients can be treated with chloroquine to inhibit autophagy or rapamycin to stimulate autophagy. Published reports have shown that inhibition of autophagy in adult brain tumor cell lines can improve responses to chemotherapy and radiation {Ito, 2005 #232; Wu, 2009 #62}. We used an adult glioma cell line (U87) to show that chloroquine inhibition and rapamycin induction of autophagy can influence cell survival in a setting where survival is influenced by autophagy. U87 cells were exposed to both chloroquine and rapamycin and levels of LC3-II were evaluated by Western immunoblot analysis (Figure 5A). The increased level of LC3-II seen with chloroquine is due to inhibition of lysosomal fusion with the autophagosomes leading to a building up of LC3-II produced during basal cellular autophagy. Rapamycin is also able to increase LC3-II levels through inhibition of the mTOR pathway, therefore increasing autophagy. U87 cells were treated with either chloroquine or rapamycin and placed in starvation conditions for 24 hours. Cells were then grown in regular media in a clonogenic assay and the percent survival was evaluated (Figure 5B). There is a significant decrease in cell survival in the chloroquine treated cells and increase in survival in the rapamycin treated cells. This shows these drugs, which inhibit or induce autophagy alter survival in adult brain tumor cells after starvation– i.e. that autophagy manipulation can indeed determine tumor cell survival.

We then used these pharmacologic manipulations of autophagy in pediatric brain tumor cells where we previously showed that autophagy is induced by chemotherapy (Figure 2). Cells were treated with increasing doses of CCNU or cisplatin in the presence of rapamycin or chloroquine and cell viability was assessed by MTS assay. There was a significant shift in the survival curve in a short-term assay when autophagy in BT-16 cells was inhibited by pre-treatment with chloroquine, with these cells showing improved survival compared to cells with baseline or up-regulated autophagy (Figure 6). There was no significant difference seen in tumor cell survival between any of the other treatment groups in a short-term MTS assay.

Cells were then treated with increasing doses of CCNU or cisplatin in the presence of rapamycin or chloroquine and allowed to recover to show long-term clonogenic survival (Figure 7). The small effect seen in the BT-16 cells treated with chloroquine evaluated by MTS assay was lost during clonogenic growth. Since the long-term clonogenic assay more rigorously tests the effect on chemosensitivity and resistance we conclude that the effects seen in the short-term assays did not reflect altered tumor cell killing but instead may be merely due to altered kinetics of cell death. There was no significant difference on long-term clonogenic growth in any of the cell lines treated with cisplatin (Figure 7B). In contrast, there was a small increase in tumor cell death with a complimentary decrease in cell death in ONS76 cells treated with the 12.5 ug/mL dose of CCNU (Figure 7A). The modest protective effect can also be seen in the rapamycin treated cells at the 25 ug/mL dose of CCNU. The remainder of the cell lines did not show a significant difference in long-term survival in CCNU treated cells.

Discussion

Autophagy is currently being targeted clinically to improve the treatment of a number of different cancers REFER to your recent review. While the manipulation of autophagy has been demonstrated to be important in the treatment of adult brain tumors and other cancer types {Levine, 2008 #13}, its importance in the treatment of pediatric tumors is still unclear. This question may be particularly important in pediatric patients due to an increased risk for

long-term side effects of autophagy manipulation. Autophagy is required in normal brain development; impaired autophagy in the brain causes behavioral defects, loss of brain cells, and early death {Komatsu, 2006 #60} and mice that lack a component of a multi-protein complex that is required for formation of autophagosomes called AMBRA display aberrant brain development {Fimia, 2007 #61}. Therefore treatments that alter autophagy may affect normal brain development, and altered autophagy resulting from brain tumor treatment may contribute to subsequent neurologic problems for pediatric brain tumor survivors. Thus, if as is suspected in adult tumors, autophagy inhibition might prove a useful way to increase chemosensitivity (an idea that provides the basis for over two dozen active clinical trials that combine hydroxychloroquine or chloroquine with other agents), the beneficial effect on chemosensitivity would need to be substantial in order to justify the risk of causing subsequent developmental problems in the brain.

We hypothesized that autophagy could be induced in pediatric brain tumor cell lines by starvation and treatment with current chemotherapeutics and FDA approved drugs, and that by altering the levels of autophagy within the cells, we could affect chemosensitivity. Our results are in contrast to findings in the study of adult brain tumors that often show an increase in tumor cell kill when autophagy is inhibited {Wu, 2009 #62; Shingu, 2009 #63}. Although there was a small effect seen in the BT-16 cells treated with chloroquine to inhibit autophagy and then treated with cisplatin, the cells showed improved survival with the loss of autophagy. Moreover, this effect was lost in long-term clonogenic assays, indicating any effect of autophagy manipulation on tumor cell kill may be transient. There was a small but significant change in long-term survival in one of the medulloblastoma cell lines treated with chloroquine and a complimentary response in cells treated with rapamycin with one of the CCNU treatment doses, but not in any of the cisplatin treatment doses. This suggests that altering autophagy may have an effect on chemosensitivity, but that it is dependent on the cell type being studied and the stimulus used to induce autophagy. Thus rather than autophagy being a general mechanism of chemoresistance in pediatric brain tumors, we propose that its effects may be highly context dependent. If this idea is correct, it will be necessary to identify the tumors and the treatments where autophagy manipulation is worthwhile rather than treating all brain cancer patients the same way.

Our study has some limitations. It is possible that there is a true effect of autophagy on pediatric brain tumor response to therapy, but that we used the wrong drugs, blocked the process at the wrong step, or used the wrong cell lines to be able to detect it. Also, autophagy is a dynamic process with multiple steps in the process of producing autophagosomes to fuse with lysosomes and complete the degradation of intra-vesicular contents and it is feasible that blocking autophagosome formation has different effects on tumor cell survival than blocking autophagic flux. There are over 115 currently known genes targeting autophagy at various steps in the process and this study was limited to affecting only two of those genes; thus a more comprehensive analysis may be worthwhile. Additionally, both chloroquine and rapamycin have other cellular effects that are not limited to altering autophagy that may influence these results; of course these autophagy-independent effects may also influence the results of ongoing clinical studies as well.

Conclusions

In this study, we have shown that AT/RT and medulloblastoma cell lines have functional autophagy systems and that current chemotherapeutics used to treat these tumors induce autophagy. We have also shown that levels of autophagy can be manipulated using siRNA and chloroquine to inhibit autophagy and rapamycin to induce autophagy. We are also able to show that chloroquine and rapamycin can influence cell survival in adult glioma cells under starvation stress thus showing that the manipulations we were making would have had a

detectable effect should there have been a role for autophagy in determining tumor cell survival. Despite the induction of autophagy during treatment of AT/RT and medulloblastoma cells with CCNU and cisplatin, the effect of manipulation of autophagy on tumor cell kill in these pediatric brain tumors is small at best and does not appear to have a significant effect on long term tumor cell survival in most of the conditions tested. The change in chemosensitivity in the ONS76 medulloblastoma cell line in one of the treatment conditions suggests that the effect of autophagy manipulation may be dependent on cell type and the stimulus used to initiate autophagy. Further evaluation of the effect of autophagy in pediatric brain tumors using a higher throughput system to evaluate a larger number of genetic and pharmacologic modifiers of autophagy is required.

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A

B

Figure 1. Starvation stress and rapamycin induce autophagy and chloroquine inhibits autophagy in pediatric brain tumor cell lines

AT/RT (BT-16) and medulloblastoma (DAOY) cells were treated with EBSS or rapamycin for 8 hours in the presence or absence of either lysosomal protease inhibitors (PepA/E64D) or chloroquine added for the last 4 hours of treatment. Protein lysate was collected and analyzed for LC-3 II and p62 using Western immunoblot analysis to detect autophagy flux. Representative immunoblots are shown (A). Experiments were repeated three times, and the average levels of LC3 and p62 normalized to actin are shown with SEM (B). The asterisk denotes statistical significance from the control group (p < 0.05).

Figure 2. Current chemotherapeutics induce autophagy in pediatric brain tumor cell lines AT/RT (BT-12, BT-16) and medulloblastoma (DAOY, ONS76) cells were treated with starvation stress, CCNU or cisplatin for 8 hours at the estimated LD50 based on previous MTS assays (data not shown) in the presence or absence of lysosomal protease inhibitors (PepA/E64D) added for the last 4 hours of treatment. Protein lysate was collected and analyzed for LC3-II using Western immunoblot analysis. Representative immunoblots are shown (A). Experiments were repeated three times, and the average levels of LC3 normalized to actin are shown with SEM (B).

A **B**

Figure 3. siRNA ATG7 and ATG12 knockdown is effective at inhibiting autophagy after treatment with current chemotherapeutics
 AT/RT (BT-16, BT-12) and medulloblastoma (Daoy, ONS76) cells were treated with non-targeted or ATG7 and ATG12 specific siRNA for 72 hours and protein lysates were evaluated for the presence of ATG7 and ATG12 by Western immunoblot analysis. Representative immunoblots showing knockdown of baseline ATG12 and ATG7 protein levels are shown (A). Cells were treated with CCNU or cisplatin 72 hours after transfection as described in figure 2. Representative immunoblots of LC3-II levels are shown for each cell line.

Figure 4. Genetic manipulation of autophagy has minimal affect on tumor cell survival AT/RT (BT-16, BT-12) and medulloblastoma (Daoy, ONS76) cells with autophagy inhibited as in Figure 3 were treated with increasing doses of CCNU, cisplatin, or a combination of CCNU and cisplatin for 24 hours. Cell viability was evaluated by MTS assay. The average of three experiments performed in triplicate are shown with SEM.

A **B**

Figure 5. Chloroquine and rapamycin influence survival in conditions where autophagy is important

Adult glioma cells (U87) were treated with chloroquine or rapamycin for 4 hours and levels of LC3-II were evaluated as in Figure 1. A representative immunoblot is shown (A). U87 cells were then placed under starvation stress in EBSS for 24 hours with either chloroquine inhibition or rapamycin induction of autophagy. Fresh media was provided and cells were allowed to grow for 10-14 days and then evaluated for long-term viability by clonogenic growth. Experiments were repeated three times in triplicate and the average level of survival is shown with the SEM. The asterisk denotes statistical significance from the control group (p < 0.001).

Figure 6. Pharmacologic manipulation of autophagy has a minimal affect on tumor cell survival in a short-term growth assay

AT/RT (BT-16, BT-12) and medulloblastoma (Daoy, ONS76) cells were pre-treated with either chloroquine to inhibit autophagy or rapamycin to induce autophagy for 4 hours. They were then treated with increasing doses of CCNU (A) or cisplatin (B) in the presence of chloroquine or rapamycin for 24 hours and cell viability was evaluated by MTS assay. Experiments were repeated three times in triplicate and the average level of survival is shown with the SEM.

Figure 7. Pharmacologic manipulation of autophagy has minimal affect on tumor cell survival in a long-term growth assay

AT/RT (BT-16, BT-12) and medulloblastoma (Daoy, ONS76) cells were pre-treated with either chloroquine or rapamycin for 4 hours, then treated with increasing doses of CCNU (A) or cisplatin (B) in the presence of chloroquine or rapamycin for 24 hours. Fresh media was provided and cells were allowed to grow for 10-14 days and then evaluated for long-term cell viability. Experiments were repeated three times in triplicate and the average level of survival is shown with the SEM.