Autophagy Inhibition Sensitizes Colon Cancer Cells to Anti-angiogenic and Cytotoxic Therapy

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Abstract
Autophagy is a critical survival pathway for cancer cells under conditions of nutrient or oxygen limitation, or cell stress. As a consequence of anti-angiogenic therapy, solid tumors encounter hypoxia induction and imbalances in nutrient supply. We wished to determine the role of autophagy in protection of tumor cells from the effects of antiangiogenic therapy and chemotherapy. We examined the effect of inhibiting autophagy on hypoxic colon cancer cells in vitro, and on bevacizumab- and oxaliplatin-treated mouse xenografts in vivo.

Experimental Design: The autophagic response to hypoxia and DNA-damaging agents was assessed by fluorescent microscopic imaging, autophagy-related gene expression, and by electron microscopic ultrastructural analysis. Pharmacological and molecular approaches to autophagy inhibition were taken in a panel of colon cancer cell lines. Mouse xenograft models were treated with combinations of oxaliplatin, bevacizumab and chloroquine to assess effects on tumor growth reduction, and on pharmacodynamic markers of autophagy inhibition.

Results: Autophagy was induced in colon cancer models by both exposure to hypoxia and to oxaliplatin. Inhibition of autophagy, either with chloroquine or by downregulation of beclin1 or of ATG5, enhanced sensitivity to oxaliplatin under normal and hypoxic conditions in a synergistic manner. Both bevacizumab and oxaliplatin treatments activate autophagy in HT29 murine xenografts. The addition of chloroquine to bevacizumab-based treatment provided greater tumor control in concert with evidence of autophagy inhibition.

Conclusions: These findings implicate autophagy as a mechanism of resistance to anti-angiogenic therapies, and support investigation of inhibitory approaches in the management of this disease.
INTRODUCTION

Colorectal cancer is the third most common malignancy and the second most common cause of cancer-related death in the USA. The application of both chemotherapy and targeted therapies including anti-angiogenic therapy has increased the survival of colorectal cancer patients (1-4). However, despite the success of anti-angiogenic therapy regimens in advanced disease, the use of bevacizumab with chemotherapy did not confer a survival benefit in disease confined to lymph nodes (Stage III), and the optimal use of this modality remains to be defined. It is critical to unravel the mechanisms that underlie the resistance of colon cancer cells to anti-angiogenic therapy.

In solid tumors, intratumoral hypoxic regions are implicated in poor prognosis and chemotherapy resistance (5,6). Hypoxic regions in the tumors are also associated with metabolic alterations as an adaptive response to nutrient deprivation (7,8). By inhibiting the tumor vasculature, bevacizumab and other anti-angiogenic agents cause substantial alterations in the tumor microenvironment (9). We have previously demonstrated that bevacizumab treatment induces hypoxia in colon cancer xenografts, and that tumor shrinkage as a consequence of treatment is variable (10,11). The degree to which hypoxia is induced in different models is similar: the production of tumor shrinkage by bevacizumab however, depends on susceptibility to hypoxia-induced cell death (11). An emerging concern with anti-angiogenic therapy is that under this selection pressure, more aggressive tumor behavior may ensue (12,13). Further, cells in hypoxic environments have been shown to accumulate mutations and more aggressive growth characteristics (14,15). Therefore, targeting this hypoxic population may have implications beyond immediate tumor shrinkage.
Both hypoxia and nutrient deprivation are known to induce the process of autophagy, which has recently emerged as a critical cellular process in cancer cell survival (16,17). Autophagy is a highly conserved catabolic process whereby long-lived proteins and organelles are engulfed in double-membrane structures called autophagosomes and targeted to the lysosomes for degradation for energy production (17,18). Some 20 autophagy-related proteins (ATGs) have been identified, initially from yeast genetic studies, followed by characterization of their mammalian homologues (17,19,20). Autophagy appears to play a role at multiple levels of tumor development, and may have a protective role in carcinogenesis (21). Autophagy is also a consequence of cytotoxic drug treatment, and more recently, has been appreciated as a means by which cells might survive the stress of cellular insults, and so become resistant to treatment (22). Evidence supports a role for inhibiting autophagy to enhance the effectiveness of cancer treatment, and genetic manipulation of cells to impede autophagy sensitizes resistant cells to treatment (23).

Autophagy is often localized to hypoxic regions of the tumor, where its induction is thought to facilitate cell survival (24-26). Hypoxia-induced autophagy mechanisms have been shown to depend on the Bcl-2 family member BNIP3 in the induction of cell death in apoptosis-competent cells (27-29). The displacement of beclin1 from beclin1/Bcl-2 complexes permits the rapid activation of autophagy (28). Interestingly, silencing of BNIP3 by histone deacetylation and methylation has been shown in colon cancer cell lines, and so other mechanisms must be sought in this disease (30). Hypoxia-induced autophagy has also been shown to contribute to chemoresistance in hepatocellular carcinoma cells (31). Accordingly, a strategy directed to
autophagy inhibition has the potential to overcome a key means of survival of cancer cells (22,32).

In this study, we examined the effect of hypoxia and oxaliplatin on autophagy regulation in colon cancer cells, and explored the impact of autophagy inhibition by chloroquine (CQ) on oxaliplatin- and hypoxia-induced cytotoxicity. We found a synergistic interaction of oxaliplatin and CQ on several colon cancer cell lines under oxic and hypoxic environments, and confirmed the role of autophagy inhibition in this interaction through silencing of beclin1 and ATG5 expression by RNA interference. We validated the interaction by showing a marked enhancement of tumor control by the incorporation of CQ with bevacizumab and oxaliplatin therapy in a colon cancer mouse xenograft model. Based on these findings a clinical trial of autophagy reversal is in progress in colorectal cancer.
MATERIALS AND METHODS

Cell lines and treatments

Colon cancer cell lines HT29, HCT116, HCT15, SW620, KM12, BE, WiDr, and LoVo were obtained from ATCC. HCT116 p53\textsuperscript{-/-} cells were provided by Dr. Bert Vogelstein, John Hopkins University. The WiDr\textsuperscript{OXL27} cell line was generated by an incremental increase in exposure of oxaliplatin to WiDr cells and maintained in culture with a high concentration of oxaliplatin. For hypoxia treatments, the colon cancer cell lines were exposed to hypoxia (0.1% O\textsubscript{2} for 24h), then returned to a standard culture environment (5% CO\textsubscript{2}, 95% air) for a further 48h. For chemotherapy treatments, cells in exponential growth were treated in hypoxia or normoxia followed by 48h of normal culture conditions, as previously described (10,11). The cells were cultured in DMEM supplemented with 10% FBS. Oxaliplatin and CQ were obtained from Sigma.

In vitro cytotoxicity assay and Combination Index analysis

Cytotoxicity was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (10). Briefly, after plating and overnight incubation at a density of 3000 cells/well in 96-well plates, cells were subjected to hypoxia for 24h in an anaerobic chamber (Forma Scientific Inc, San Jose, CA) which was gassed using oxygen-poor (less than 1 part per 10 billion) 95% N\textsubscript{2} and 5% CO\textsubscript{2}, and then cultivated for two more days under normal conditions, followed by MTT assay. The absorbance at 570 nm was determined using a microplate reader (Elx800, BioTek Instruments, Inc, Winooski, VT). The data shown are the means ± S.D. of two independent experiments done in triplicate. The combination index analysis was performed using the method of median effect method by Chou and Talalay (33,34). Initially, the IC\textsubscript{50} values were calculated for CQ and oxaliplatin for colon cancer cell lines using standard algorithms. The ratio
for combination of drugs was determined on the basis of IC_{50} values. The CI values for synergy, additivity and antagonism were determined using commercially available CompuSyn software (34). The CI is a quantitative measure of the degree of interaction between two or more drugs. When it equals to 1, it denotes additivity, > 1 antagonism, < 1 synergism, and < 0.3 strong synergism. The CI was calculated under the assumption of a mutually nonexclusive drug interaction.

**RNA interference**

The cells were transfected with 50 nM of control (ON-TARGETplus Non-targeting Pool, Dharmacon) or Beclin1 siRNAs (ON-Target plus SMARTpool, Dharmacon) with Lipofectamine™ RNAiMAX (Invitrogen) reagent using reverse transfection methods as described by the manufacturer. The sequences of the sense strands of the RNAs used in this study were as follows: Beclin1 siRNA-1 GAU ACC GAC UUC CUU A, Beclin1 siRNA-2 GGA ACU CAC AGC UCC AUU A, Beclin1 siRNA-3 CUA AGG AGC UGC CGU UAU A, Beclin1 siRNA-4 GAG AGG AGC CAU U UA UUG A and control siRNAs of ON-TARGETplus Non-targeting Pool from Dharmacon. The sequences of the sense strands of the RNAs used in this study for ATG-5 knock-down were as follows: ATG5 siRNA-1 GGC AUU AUC CAA UUG GUU U, ATG5 siRNA-2 GCA GAA CCA UAC UAU UUG C, ATG5 siRNA-3 UGA CAG AUU UGA CCA GUU U, ATG5 siRNA-4 ACA AAG AUG UGC UUC GAG A. HT29, HCT116, KM12, BE, SW620, and HCT15 colon cancer cell lines were initially cultured in 12-well plates, and transfected with either 50 nM of control siRNA pool, or with Beclin1 or ATG5 siRNAs SMARTpool, for 48 hrs. We confirmed down-regulation of beclin1 and ATG5 proteins by western blot analysis. For oxaliplatin sensitivity, cells were seeded in 96 well plates and reverse transfected with 50 nM of either control siRNA pool or beclin1 or ATG5 siRNA.
SMARTpool. After 24 hrs of transfection, cells were treated with oxaliplatin under hypoxic (24h) or normal conditions. Oxaliplatin cytotoxicity was determined by MTT assay as above.

**GFP-LC3 Assay**

HT29 cells were stably transfected with GFP-LC3 plasmids and selected with G418. GFP-positive cells were further sorted by flow cytometry. HT29-LC3 cells were seeded in 6-well plates with cover slip and treated with hypoxia. The cells were fixed in 4% paraformaldehyde and LC3 puncta were measured by light fluorescence and confocal microscopic counting of cells with GFP-LC3 puncta. A minimum of 50-100 cells per sample will be counted in triplicate samples per condition per experiment. To visualize autolysosomes in live cells, HT-29 cells expressing GFP-LC3 protein delivered by retroviral construct (Addgene, Cambridge MA) were seeded on glass slides 24 hours prior to hypoxic treatment (24 hours) with or without oxaliplatin (1 μM). At the end of treatment, LysoTracker Red DND-99 (Invitrogen) was added for 1 hour at 1 μM. Cells were removed from the hypoxia chamber, immediately washed twice with PBS and observed live, using EVOSfi fluorescent microscope (AMG, Seattle, WA) equipped with GFP and RFP filters. Images taken consequently with each filter were digitally overlapped to assess formation of autolysosomes.

**Western Blotting**

Proteins from a total cell extract (20 μg/lane) were separated by electrophoresis in a 12% SDS-polyacrylamide gel, and transferred to a Hybond-P membrane (Amersham, Arlington Heights, IL). Western blotting was carried out using LC3-IIIB (Novus Biologicals), beclin1, Atg7, (Santa Cruz), and ATG5 (Cell signaling technology) were used as primary antibodies (Calbiochem, La Jolla, CA) and horseradish peroxidase-conjugated second antibody (Santa Cruz Biotechnology
Inc, Santa Cruz, CA) was used to detect signals. The actin antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) was used as protein loading control. The ECL-plus detection system (Amersham, Arlington Heights, IL) was used to develop the signal.

Transmission electron microscopy (TEM)

For TEM quantitation of autophagosomes, tumors were fixed with 2.5% glutaraldehyde/2% formaldehyde with 0.1 M sodium cacodylate and stored at 4°C until embedding. Tumors were embedded in LX-112 medium (Ladd), and sections were cut ultra thin (90 nm), and placed on uncoated copper grids. The sections were stained with 0.2% lead citrate and 1% uranyl acetate. Images were examined with a JEOL-1010 electron microscope (JOEL) at 80 kV. For quantitation of cells using electron microscopy of tumor tissues, high-powered micrographs (x12, 000-20,000) of 25 single cells from multiple distinct low-powered fields in each tumor were obtained. For quantification of autophagic vesicle, cells with more than three to four double-membrane vesicles were scored as positive for autophagosomes.

Tumor Growth and Anti-angiogenic Therapy

To assess the efficacy of autophagy inhibition by chloroquine in anti-angiogenic and oxaliplatin combination therapy in mouse xenograft model, adult (8-10 weeks of age) female severe combined immunodeficient mice (C.B.17 SCID) were used. To generate tumors, HT29 cells (2 x 10^6 cells) were injected subcutaneously into the left flank of the mice. When the tumors reached approximately 400 mm³, mice were divided into eight groups (six mice per group) for treatment with bevacizumab (Genentech, Inc., San Francisco, CA), oxaliplatin, the combination of bevacizumab and oxaliplatin, chloroquine, the combination of bevacizumab and chloroquine, the combination of oxaliplatin and chloroquine, the combination of bevacizumab, oxaliplatin and chloroquine, and a vehicle control group. Mice in the bevacizumab treatment group received 5
mg/kg of bevacizumab by intraperitoneal injection every 3 days for 27 days. The oxaliplatin treatment group was injected intraperitoneally with 5 mg/kg oxaliplatin per week for 2 weeks. The chloroquine treatment group was injected intraperitoneally 3.5 mg/kg everyday for 27 days. The combination treatment groups received bevacizumab (every 3 days, 5 mg/kg for 27 days), oxaliplatin (weekly for 2 weeks, 5 mg/kg), and chloroquine (every day, 3.5 mg/kg). The control group received saline intraperitoneally every day. Tumor volume and body weight were measured every 3 days. Tumor volume was calculated using the formula $V = AB^2/2$, where $A$ is the largest diameter and $B$ is the smallest diameter. Tumor growth delay was calculated as the difference in the time for control and treated tumors to grow from 400 to 800 mm$^3$. Mice were sacrificed after treatments on day 27 for tumor processing for quantitation of autophagy by transmission electron microscopy. All animal experiments were performed according to an approved University of Pennsylvania IACUC (Institutional Animal Care and Use Committee).

**Tissue Immunohistochemistry**

Immunohistochemistry was conducted using the antigen retrieval protocol followed by primary antibody incubation previously described (11). Rabbit anti-human LC3 antibody (Cell Signaling Technology) was used to detect the expression of LC3 in HT29 xenografts. For blood vessels and Ki-67 markers detection, primary antibodies of CD31 (abcam) and Ki-67 (Dako) were used. Immunohistochemistry images were visualized using a Leica DMRBE upright microscope with QImaging MicroPublisher 5.0 RTV color camera with objective lens PL FluoTAR 20x/0.5 (Leica, Heerbrugg, Switzerland). Images were captured using iVision acquisition software, which were processed with Adobe Photoshop software (Adobe Systems, San Jose, CA). Tumor blood Vessels density were determined by counting per 40x high power field as described earlier (11). For quantitative analysis, ImageJ software, which measures the intensity of staining through
threshold analysis, coupled with the Color Deconvolution plug-in (http://www.dentistry.bham.ac.uk/landinig/software/cdeconv/cdeconv.html), was used to quantify LC3 and Ki-67 immunorecactivity in xenograft samples.

**Statistical analysis**

Tumor volume was represented as mean ± SD. Statistical analysis was performed using ANOVA. A P value < 0.05 was considered significant.
RESULTS

Hypoxia and oxaliplatin treatment induce autophagy in colon cancer cells.

To determine the importance of autophagy in colon cancer therapy, we began by examining the occurrence of autophagy in hypoxia- and oxaliplatin-treated colon cancer cells. We generated stable GFP-LC3-expressing HT29 cells by transfection and G418 selection. The LC3 proteins were measured by examining GFP-LC3 as punctate aggregations under fluorescence microscopy, and the conversion of LC3-I protein to modified form of LC3-II by western blot. The reason for the selection of HT29 cells for our study is that we have previously extensively characterized these cells for oxaliplatin and hypoxic cell signaling pathways and their ability to undergo antiangiogenic therapy in a mouse model (10,11). HT29 cells were monitored for GFP-LC3 dots after 24 hrs of hypoxia (0.1%) and oxaliplatin (IC50 dose) treatments. Clearly more cells with the punctate structures of autophagosomes are observed in hypoxia- and oxaliplatin-treated cells (Fig. 1A). Staining of cells for lysosomes, and overlapping of images further demonstrated induction of autophagy by both treatments, especially by hypoxia and the combination (Fig. 1A). Quantitation of cells positive for punctate structures revealed the induction of autophagy by oxaliplatin and hypoxia (Figure 1B). We confirmed autophagy induction in HT29 cells through electron microscopy ultrastructural analyses. As illustrated in Figs. 1C, 1D, and supplementary data Fig.S1, hypoxia and oxaliplatin significantly induce autophagosomal vesicles in HT29 cells (p< 0.05). To further confirm autophagy induction, protein extracts were made after hypoxia and oxaliplatin treatments for 24 and 48 hours. Western blots show increased LC3-II and LC3-I proteins following treatment. The induction of LC3 proteins is robust in hypoxic cells, and in cells treated with combined hypoxia and oxaliplatin, as seen in Figure 1E. The autophagy-related
genes beclin1 (BECN1) and ATG-7 were also induced with hypoxic and in combination treatments.

**Autophagy-inhibition sensitizes colon cancer cells to hypoxia and oxaliplatin cytotoxicity:**

To understand the role of autophagy induction in hypoxia and oxaliplatin treatments, we have used autophagy-inhibition strategy with pharmacological inhibitors 3-MA, bafilomycin A1 and chloroquine, and RNA interference technology for beclin1 and ATG5 knockdown. The autophagy inhibitor bafilomycin A1, which inhibits autophagosomal and lysosomal fusion, showed increased LC3-II accumulation in oxaliplatin- and hypoxia-treated cells, indicating enhanced autophagy flux under these conditions (Fig 2A, B &C). As expected, bafA1 has increased LC3 protein accumulation in western blotting analysis and inhibited autophagy as evidenced by reduction of p62 protein degradation in hypoxic treatments (Fig 2D). The use of bafA1 in MTT assays at IC$_{10}$ concentration, sensitized colon cancer cells to oxaliplatin in hypoxia (Fig 2E). Another autophagy-inhibitor 3-MA, which inhibits autophagy at initiation of double membrane encapsulation, also showed autophagy inhibition as evidenced by LC3 downregulation and p62 accumulation in hypoxia treatment (Fig 3A). 3-MA also sensitized hypoxia-induced oxaliplatin resistance at IC$_{10}$ concentration and showed synergy with oxaliplatin cytotoxicity in colon cancer cells (Fig 2B & C). To further understand the mechanistic role of hypoxia induced autophagy, we have used the inhibitor YC-1 and was used in other studies for pharmacological inhibition of hypoxia inducible factor-1 alpha (29). YC-1 treatment showed inhibition of HIF-1 alpha protein but not the autophagy inhibition or sensitization of oxaliplatin resistance as evidenced by LC3 and p62 protein levels and also in MTT based cell survival assays (Supplementary data Fig S2).
**Beclin1 knockdown sensitizes colon cell lines to oxaliplatin.** We also sought to downregulate autophagy by targeting the essential autophagy gene, beclin1. We used siRNA to decrease the levels of beclin1 in cells, and found that protein levels were considerably depleted by the intervention (Fig. 3D). As expected, knockdown of beclin1 in these cell lines caused marked inhibition of the accumulation of the autophagy markers LC3-II and ATG5, both upregulated by hypoxia in the parental line (Fig. 3D). We went on to transfect a series of colon cancer cell lines, and to determine the effect of beclin1 down regulation on sensitivity to oxaliplatin under oxic and hypoxic conditions (Table 1). As expected, five of the six lines show resistance to oxaliplatin under hypoxic conditions. In four of the six lines, beclin1 downregulation resulted in enhancement of sensitivity to oxaliplatin by 2-fold or more. In three of the lines, the sensitization was greater under hypoxic than under oxic conditions, while in the rest the degree of sensitization was equivalent. In no case was there a protective effect of downregulating beclin1. To confirm that this inhibitory activity was a consequence of autophagy downregulation, we also targeted the autophagy-related protein ATG-5 through RNA interference. We found comparable enhancement of sensitivity to oxaliplatin under both oxic and hypoxic conditions to that with beclin1 knock down (Supplementary data Fig.S3, and Table. S1). The results support targeting autophagy as a means to increase the effectiveness of colorectal cancer treatment.

**Effect of autophagy inhibition by Chloroquine in hypoxia and oxaliplatin induced cytotoxicity.** To determine the role of autophagy in colon cancer cell survival during hypoxia and oxaliplatin treatment, we used the known autophagy inhibitor chloroquine (CQ) in MTT assays. The addition of CQ at 1, 3, and 5 μM increased the sensitivity to oxaliplatin under oxic conditions in all of the cell lines (Figure 4). It will be seen that in three of the lines, CQ treatment has a further sensitizing effect under hypoxic conditions. We performed isobologram analysis
(Supplementary data Fig.S4) to characterize the interaction between these interventions. CQ demonstrated a synergistic interaction with oxaliplatin under both oxic (median CI = 0.75) and hypoxic conditions (median CI = 0.58) in six of eight colon cancer cell lines tested (Table 2). These data indicate a strong interaction between the treatments. The lower CI under hypoxic conditions suggests that sensitization of these tumors may occur both to DNA-damaging agents and to hypoxia.

**Autophagy inhibition by chloroquine sensitizes mouse colon cancer xenografts to bevacizumab.**

To determine the effect of chloroquine on bevacizumab, oxaliplatin, and in the combination therapy on colon cancer xenografts in vivo, mice bearing HT29 colon adenocarcinoma xenografts were randomly divided into eight groups for treatments as follows: PBS treated control, CQ alone, bevacizumab alone, bevacizumab and CQ in combination, oxaliplatin alone, oxaliplatin and CQ in combination, bevacizumab and oxaliplatin in combination, and bevacizumab, oxaliplatin and CQ in combination. CQ treatment alone decreased the tumor growth somewhat compared to PBS alone control tumor growth (Figure 5A). The tumor growth delay was for 4 days in CQ alone treatment (p<0.01). A much greater impact was seen with the combinations of CQ with both oxaliplatin and bevacizumab in combination. Tumor growth delay of 7.2 days was observed in the bevacizumab alone group (p<0.01), compared to 12 days with bevacizumab and CQ (p<0.01). Tumor growth delay was 6 days for oxaliplatin alone treatment (p<0.05), and 9 days for oxaliplatin and CQ (p<0.05). When all the treatments were combined, effects were even more dramatic: the growth delay was 15 days for bevacizumab and oxaliplatin (p<0.01), and 23 days for bevacizumab, oxaliplatin and CQ treatment (p<0.01). Therefore in all treatments, the
strategy of incorporating CQ increased the tumor growth delay significantly, suggesting that autophagy inhibition may have an impact on both cytotoxic and antiangiogenesis effectiveness (Table 3).

**Bevacizumab and oxaliplatin treatments induce autophagy in mouse HT29 xenografts**

Mouse tumors treated with bevacizumab and oxaliplatin were also processed for electron microscopy analyses. As depicted in Figure 5B, bevacizumab- and oxaliplatin-treated tumors examined by electron microscopy had more cells with vacuolar structures typical of autophagosomes than untreated or chloroquine-treated tumors. The quantitation of autophagosomal cells revealed significantly increased autophagy in bevacizumab- and oxaliplatin-treated tumors when compared to PBS alone controls (Figure 5C). In tumors treated in addition with CQ, we observed a reduced number of autophagosomal cells (Figure 5C). Incorporating CQ with bevacizumab and oxaliplatin treatments inhibited autophagosomal induction when compared to treatments without CQ (Figure 5B, Supplementary data Fig.S5). In the case of the triple (bevacizumab/oxaliplatin/CQ) combination, we did not observe a decrease compared with bevacizumab/oxaliplatin alone, and a number of practical issues may be adduced to explain this slight discrepancy, including sampling times for tumor harvesting (day 27) that in this group were close to the tumor growth delay, and the very much reduced tumor volumes in these groups.

Tumors from mice treated with bevacizumab, oxaliplatin, and both, were processed for immunostaining of LC3 protein to demonstrate autophagy induction by these therapies, and the effect of CQ on its inhibition. Immunohistochemical analysis confirms autophagy induction as evidenced by increased LC3 staining in bevacizumab and oxaliplatin treated tumors, and its
inhibition in CQ treated tumors (Figure 6A & B). Tumors were also stained for blood vessels and cell viability using CD31 and Ki-67 antibodies. As evidenced in Figure 6C & D, bevacizumab inhibited blood vessels density in the treated samples and the CQ and oxaliplatin treatments did not alter the antiangiogenic properties of bevacizumab. The cell proliferation marker Ki-67 staining depicted most reduced viable cells in the combination treatments of bevacizumab, oxaliplatin and chloroquine (Fig 6 E & F).
DISCUSSION

Autophagy is increasingly recognized as a contributor to the malignant phenotype, and as a possible mechanism for treatment failure in cancer (21,22,24-26). We hypothesized, based on our previous findings (10,11), that the hypoxic effects induced by bevacizumab in tumors in vivo were critical to its therapeutic action, and that autophagy might attenuate that response. We thought further that since one response to DNA-damaging drugs is the induction of autophagy, its inhibition could sensitize colon tumors to oxaliplatin. We confirm that induction of autophagy is a consequence of exposure both to oxaliplatin as well as to hypoxia in colon cancer cell lines. We note that the exposure to both hypoxia and oxaliplatin produces additive effects in autophagy protein expression (Fig. 1E), while autophagosome production appears to be maximal with either stress alone (Fig. 1D). Similar findings are obtained with Bafilomycin A inhibition (Fig. 2). The quantitative pharmacodynamic implications of this finding remain to be resolved, but it likely reflects a maximum effect level under these conditions. It is also worth noting that a basal level of autophagy is present in all of the cell lines as evidenced by activation of LC3-II and beclin1 – in this we find colon cancer cells to be similar to pancreatic cancer models as described recently by Yang et al (35). In that study, progressive activation of autophagy accompanied the progression of lesions through stages of increasing malignancy. In colon cancer also, activation of the autophagy pathway is a feature of the neoplastic phenotype, both in polyps and cancers (36).

Accumulating evidence also implicates autophagy in angiogenesis (37). Hypoxic endothelial cells derived from mice with heterozygous beclin1 disruption demonstrated greater proliferative and functional responses than those from wild-type animals, a finding associated with a switch in
hypoxia-response gene expression in these cells from HIF-1α to HIF-2α, the implications of which remain to be defined (38). In contrast, studies in bovine aortic endothelial cells with disrupted ATG5 demonstrated reduced angiogenesis under conditions of nutrient deprivation (39). Plausibly, autophagic responses from nutrient deprivation may differ from those under hypoxia, and the microenvironment of tumors, in which autophagy is reproducibly engaged in many models, may differ from that of isolated endothelial cells, but additional analysis of these apparent contradictions may provide the opportunity to refine approaches to autophagy inhibition. A recent publication from Hu et al (29) showed that glioblastoma tumors resistant to bevacizumab exhibited greater expression of autophagy markers than sensitive tumors, and that autophagy inhibition in glioblastoma cell lines sensitized them to bevacizumab in vivo. Further, the hypoxic regions of resistant tumors surrounding a necrotic core in post-treatment samples stained for autophagy markers, thus linking the microenvironmental conditions to bevacizumab responsiveness. These findings in human tumors support our contention that induction of hypoxia and the tumors response to it plays a key role in determining outcome of bevacizumab therapy.

We report here that the autophagy inhibitor chloroquine sensitizes colon cancer models to oxaliplatin under hypoxic conditions in vitro, and reduces the tumor growth of colon cancer xenografts in bevacizumab- and oxaliplatin-treated mice. In vitro, where such interactions can be explored in some detail, chloroquine enhanced the activity of both treatments individually, while the greatest synergy was observed with the three drug combination. These experiments cannot distinguish between the effects of inhibiting autophagy and some possible unrelated cytotoxic action of chloroquine. On the other hand, chloroquine has been widely used for many
years in malaria prophylaxis, without significant toxicity, a fact that may argue against a significant cytotoxic effect of the drug. To rule out such an effect, we undertook a molecular approach to autophagy inhibition through down-regulation of beclin1, a key mediator of the autophagy response. We found that the cells in which beclin1 was knocked down showed susceptibility to oxaliplatin similar to the degree of interaction with chloroquine, and that the potentiation of drug effect was greater under hypoxic conditions. We confirmed the autophagy-specificity of these findings by experiments in which ATG5 was knocked down with similar results. Consistent with the observations of others in different cancer models (23,40,41), it is reasonable to propose that the major therapeutic effect of chloroquine in this setting derives from autophagy inhibition. The effects of chloroquine are observed both with the DNA damaging agent and with hypoxia. The in vitro studies would suggest that there is synergy from the inhibition of both pathways – Table 1 shows clearly that, consistent with our previous findings (11,42,43), hypoxic cells are resistant to oxaliplatin compared to their oxic counterparts. The effect of chloroquine is to abrogate the resistance, and the synergistic nature of the interaction may suggest that the mechanisms by which autophagy is engaged by each cell stress may be different, and so possibly be amenable to more targeted intervention.

The mechanisms critical to cell death in this setting remain to be elucidated. Frezza (44) demonstrated induction of a catabolic metabolism profile in hypoxic (1% O₂) HCT116 colon cancer cells, and that this was sufficient to sustain ATP levels, albeit at reduced levels. These changes were accompanied by induction of autophagy, inhibition of which depleted ATP formation and led to cell death. While oxidative phosphorylation was impaired, preservation of
mitochondria suggested that mitophagy was not associated with the induction of hypoxic cell death. More profound hypoxia may be required for engagement of this process (45).

Previous studies of hypoxia-induced signaling events leading to autophagy identified BNIP3 as a key component of the cell’s detection and response to a hypoxic environment (28,46). However, BNIP3 protein levels in the colon cancer cell lines studied here (HT29, HCT116) were undetectable (data not shown), consistent with previous reports of it’s silencing by aberrant methylation and histone deacetylation in colon and gastric cancer (30, 47). In the glioblastoma work of Hu et al, BNIP3 was a key mediator of the autophagy response (29). When present, BNIP3 activates beclin1 by displacing its binding partners Bcl-2 and Bcl-XL, but in these colon cancer models, the basis for this signaling remains to be elucidated. Clearly, BNIP3 is not required for signaling to autophagy in this model: the additional participation of bcl-2 family proteins is being studied in human tumor samples. These findings may permit selection of patients for autophagy inhibition to sensitize tumors to therapy.

An issue of clinical importance is to define the molecular characteristics of colon cancers that might respond. The known link between disruption of growth and survival genes and metabolic responses renders this a potentially fruitful area of investigation in this context. We observe that in HCT116 and its derivative with biallelic deletion of p53 (Table 2), CQ synergizes with oxaliplatin in HCT116 under hypoxic conditions, but is only additive in its p53\(^{-}\) derivative. A number of investigators have questioned the strength of such findings to define p53-specificity of responses. We note that many of the models in which autophagy inhibition affords a therapeutic
benefit (SW620, KM12, HCT15) are p53-deficient, and one might be wary of over-interpreting the HCT116 data. Ultimately, this will be an important question to address in clinical trials.

The contribution of hypoxia to the effectiveness of anti-angiogenic therapy has not yet been definitively established. The work of Jain and colleagues demonstrated that the tumor vasculature is often disorganized, defective in maturation, and functionally impaired (48). They showed that an early effect of anti-angiogenic therapy is normalization of such vasculature, with restoration of normal permeability and that this may improve oxygenation in the short-term. These findings were recently corroborated in an elegant electron paramagnetic resonance study by Matsumoto et al (49) in a sarcoma model treated with sunitinib, and there is evidence for therapeutic benefit of vascular normalization (50). However in the longer term, with continued treatment both groups found vascular attenuation, and nutritional impairment of the treated tumors. Hence, these findings in colon cancer models, that induction of hypoxia may ultimately complement the effects of chemotherapy, and increase tumor cell kill, are not inconsistent with those that find early normalization of vasculature. Further, the therapeutic effects of CQ in vivo, with or without oxaliplatin, provide further support for a model in which the induction of hypoxia in colon tumors may confer therapeutic benefit, overriding any potential hypoxic cell resistance (10,11). Based on this notion, we have in progress a clinical trial of hydroxychloroquine with chemotherapy and bevacizumab in patients with advanced colorectal cancer.
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**Figure Legends: Figure 1:**

Induction of autophagy in response to hypoxia and oxaliplatin in colon cancer cells. A. HT29-LC3 cells were treated with hypoxia (0.1%), and/or oxaliplatin (IC\textsubscript{50} concentration) for 24 hrs. Lysotracker was used to visualize lysosomes. Cells were observed under fluorescence microscopy, and images obtained with GFP and RFP filters were overlapped to assess formation of autolysosomes (inclusion of autophagosomes’ LC3II into lysosomes). Green dots correspond to LC3II, accumulated in autophagosomes, red to lysosomes, and the overlapping of the two results in the orange/yellow color.

B. Cells with dot structures were quantified for autophagosomal formation. Mean and SD are shown from experiments that were performed thrice, and 100 to 200 cells counted in each.

C. Autophagosome accumulation after oxaliplatin and hypoxia. HT29 cells were processed for electron microscopy analysis after hypoxic and oxaliplatin treatments, and autophagosomes were quantitated.

D. Cells containing more than five vacuoles were counted as positive for autophagosomal structures and about 50 to 100 cells were counted in each well.

E. Induction of autophagy proteins in colon cancer cells after 24 and 48 hrs of hypoxia and oxaliplatin. The expression of LC3-I and II, beclin1, ATG-7 and actin proteins was monitored after hypoxia and oxaliplatin treatment.

**Figure 2:** The effect of bafilomycin A treatment on oxaliplatin- and hypoxia-induced autophagy in colon cancer cells.

(A & B) HT29-GFP-LC3 cells were treated with oxaliplatin (IC\textsubscript{50}) and hypoxia (0.1%) for 24 hrs, and bafilomycin A (1 μM) was added to the cultures for the last 3 hrs of incubation where
indicated. Cells were fixed for immunostaining with lysosomal protein LAMP-1 antibodies (Red). Representative images of oxaliplatin (A) and hypoxia (B), and combination (B) treatments are shown. (C) GFP-LC3 positive dots were quantified in approximately 30 cells from each experiment. Error bars represent SEM. (D) Western blotting analysis revealed inhibition of hypoxia-induced autophagy as evidenced by reduction in p62 degradation after 24 hrs of incubation with BafA1 (100 nM) in cultured HT29 cells. (E) BafA1 (20 nM) sensitized HT29 cells to hypoxia- induced oxaliplatin resistance in MTT assays (*= P<0.01).

**Figure 3:** The effect of autophagy inhibition by 3-MA and beclin1 knockdown on hypoxia-induced autophagy.

(A) Western blot analysis depicting reduction of LC3 protein and blocking of p62 degradation by 3-MA (1 mM) after 24 hours hypoxia treatment in HT29 cells.

(B) Incubation of 3-MA at IC_{10} concentration sensitized HT29 cells to hypoxia-induced oxaliplatin resistance in the MTT assays (*= P=0.01).

(C) Isobologram analysis showing synergistic interaction between oxaliplatin and 3-MA.

(D) Beclin1 knock-down by RNA interference abolished the induction of autophagy proteins after hypoxia exposure in colon cancer cells. Western blot analyses were performed for autophagy marker proteins after 6 and 24 hrs of hypoxic treatments in HT29 cells transfected with siRNA control pool, siRNA1-BECN1 and siRNA3-BECN1.
Table 1.

Beclin1 knockdown enhances oxaliplatin sensitivity in colon cancer cells under oxic and hypoxic conditions. Parentheses indicate the fold enhancement of oxaliplatin sensitivity.

Figure 4:

Autophagy Inhibition by CQ sensitizes colon cancer cells to hypoxia and oxaliplatin.

Inhibition of autophagy by chloroquine sensitizes colon cancer cells to oxaliplatin under oxic and hypoxic conditions. HT29 cells were tested for the effect of autophagy inhibition by CQ. The concentration of CQ at 1, 3 and 5 μM enhanced the cytotoxicity of oxaliplatin under normal and hypoxic conditions.

Table 2.

Synergistic interaction between CQ and oxaliplatin in colon cancer cells. IC$_{50}$ values for chloroquine and oxaliplatin were calculated using MTT assays. CI, combination index was calculated as described in Materials and Methods.

Figure 5:

(A) Autophagy inhibition sensitizes colon cancers to bevacizumab and oxaliplatin in vivo.

Efficacy of chloroquine in reducing the tumor size in oxaliplatin and bevacizumab
treatments in HT29-derived mouse xenografts. After formation of tumor, animals were treated with chloroquine (CQ, 3.5 mg/kg, every day for 27 days) alone or in combination with bevacizumab (Bev, 5mg/kg every 3 days for 27 days), oxaliplatin (L-OHP, 5 mg/kg weekly once for 2 weeks), or the combination of bevacizumab and oxaliplatin. Tumor size was measured every 3 days, and results are presented as average volume; bars represent standard deviation (n=6).

(B) Autophagy induction by bevacizumab treatments in colon cancer in vivo. Tumors were harvested from treated mice, and processed for EM studies after 21 days of treatment with bevacizumab alone, or oxaliplatin, or both in combination with CQ.

(C) The autophagosomal cells were quantified by EM analyses of about 100 to 200 cells from each tumor.

Table 3.

CQ enhances tumor growth delay to bevacizumab and oxaliplatin treatments.

Figure 6: (A) Immunohistochemical detection of LC3 in bevacizumab-treated HT29 tumors. Representative pictures of LC3-staining (Scale bars: 20 μm) from tumor samples harvested at 21 days after initiating treatment with bevacizumab alone, or oxaliplatin, or both, in combination with CQ. (B) Quantification LC3 staining by ImageJ analysis. (C) Immunohistochemical staining for blood vessels by CD31 antibodies. (D) Quantification of CD31 per high power field analysis. (E) Immunohistochemical staining for cell proliferation marker Ki-67. (F) Quantification of Ki-67 positive cells.
References:


46. Mazure NM, Pouysségur J. Atypical BH3-domains of BNIP3 and BNIP3L lead to autophagy in hypoxia. Autophagy 5:868-9, 2009


Translational Relevance:
The combination of cytotoxic chemotherapy and bevacizumab is the backbone of therapy for advanced colorectal cancer. Understanding the basis of resistance is the key to development of newer more effective therapies. Our previous work suggests that the induction of hypoxia by bevacizumab contributes to its interaction with chemotherapy, and may both increase cell kill, and at the same time promote the acquisition of resistance – much like cytotoxics. We show here that one of the ways in which colon cancer cells become resistant to bevacizumab and oxaliplatin is through induction of an autophagy program. In an animal model, inhibition of autophagy is associated with greater sensitivity to both oxaliplatin and bevacizumab individually, and especially in combination. These data may point the way to more effective colon cancer therapy, and clinical studies are in progress to that end.
**Figure 1**

- **A**
  - GFP-LC3B, Lysotracker, Overlay images for Ctrl, hypoxic, L-OHP, hypoxic conditions.

- **B**
  - Bar graph showing % LC3+ cells: Control, L-OHP, Hypoxia, Hypoxia + L-OHP.

- **C**
  - Images showing control vs. L-OHP conditions under hypoxia and hypoxia + L-OHP conditions.

- **D**
  - Graph showing % autophagosome+ cells: Control, L-OHP, Hypoxia, Hypoxia + L-OHP.

- **E**
  - Table showing time (hrs) 0, 24, 48 for Hypoxia and L-OHP conditions.
  - Western blots for LC3I, LC3II, BECN1, ATG7, Actin.
A. Figure 2

B. Figure 2

C. Figure 2

D. Figure 2

E. Figure 2
A. 

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>3-MA</td>
</tr>
<tr>
<td>LC3 I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC3 II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. Figure 3

Cell Survival (% of Control)

- 3-MA
- L-OHP
- L-OHP + 3-MA

C. 

OXIC (L-OHP + 3-MA)  
Hypoxic (L-OHP + 3-MA)

HT29
- L-OHP IC50 units
- 3-MA IC50 units

HCT-116
- L-OHP IC50 units
- 3-MA IC50 units

SW-620
- L-OHP IC50 units
- 3-MA IC50 units

D. 

<table>
<thead>
<tr>
<th>Hypoxia (hrs)</th>
<th>Si-Ctrl</th>
<th>Si1-BECN1</th>
<th>Si3-BECN1</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>6</td>
<td>24</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>6</td>
<td>24</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>6</td>
<td>24</td>
<td>-</td>
</tr>
</tbody>
</table>

BECN1

LC3B

ATG5

Actin
Table 1. Sensitization of oxaliplatin to colon cancer cells by beclin1 knock-down

<table>
<thead>
<tr>
<th></th>
<th>Oxaliplatin (IC₅₀ in μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia</td>
</tr>
<tr>
<td></td>
<td>si-Ctrl</td>
</tr>
<tr>
<td>1 HT29</td>
<td>0.71</td>
</tr>
<tr>
<td>2 HCT116</td>
<td>0.98</td>
</tr>
<tr>
<td>3 KM12</td>
<td>7.5</td>
</tr>
<tr>
<td>4 BE</td>
<td>1.56</td>
</tr>
<tr>
<td>5 SW620</td>
<td>0.38</td>
</tr>
<tr>
<td>6 HCT15</td>
<td>2.9</td>
</tr>
</tbody>
</table>
Table 2. Synergistic interaction of chloroquine and oxaliplatin cytotoxicity on colon cancer cells

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀ Concentration in μM</td>
<td>IC₅₀ Concentration in μM</td>
</tr>
<tr>
<td></td>
<td>Chloroquine</td>
<td>Oxaliplatin</td>
</tr>
<tr>
<td>1.WiDr</td>
<td>6.8</td>
<td>1.04</td>
</tr>
<tr>
<td>2.HT29</td>
<td>11.4</td>
<td>0.95</td>
</tr>
<tr>
<td>3.BE</td>
<td>12.74</td>
<td>1.02</td>
</tr>
<tr>
<td>4.SW620</td>
<td>13.56</td>
<td>0.4</td>
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<tr>
<td>5.HCT116</td>
<td>17.6</td>
<td>1.04</td>
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<tr>
<td>6.HCT116^{p53/−}</td>
<td>18.7</td>
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<tr>
<td>7.KM12</td>
<td>24.47</td>
<td>9.3</td>
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<tr>
<td>8.HCT15</td>
<td>27.56</td>
<td>2.65</td>
</tr>
</tbody>
</table>
A. Figure 5

![Graph showing tumor volume over days of treatment with different treatments: HT29, Control; HT29, CQ; HT29, Bev; HT29, Bev+CQ; HT29, L-OHP; HT29, L-OHP+CQ; HT29, Bev+L-OHP; HT29, Bev+L-OHP+CQ.](image)

B. ![Images of cellular structures under different treatments: Control, CQ, Bev, L-OHP, Bev + L-OHP, Bev + CQ, L-OHP + CQ, Bev+L-OHP+CQ.](image)

C. ![Bar graph showing percentage of autophagosomes in total cells: Control, CQ, Bev, L-OHP, Bev+L-OHP, Bev+CQ, L-OHP+CQ, Bev+L-OHP+CQ.](image)
Table 3. Effect of Chloroquine on Bevacizumab, Oxaliplatin, and the combination therapy of Bevacizumab and Oxaliplatin on the growth delay of HT29-derived tumors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor Growth Delay*</th>
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<tbody>
<tr>
<td></td>
<td>HT29</td>
</tr>
<tr>
<td>CQ</td>
<td>4</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>7.2</td>
</tr>
<tr>
<td>Bevacizumab + CQ</td>
<td>12</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>6</td>
</tr>
<tr>
<td>Oxaliplatin + CQ</td>
<td>9</td>
</tr>
<tr>
<td>Bevacizumab + Oxaliplatin</td>
<td>15</td>
</tr>
<tr>
<td>Bevacizumab + Oxaliplatin + CQ</td>
<td>23</td>
</tr>
</tbody>
</table>

*Tumor growth delay was calculated as time (days) needed for treated tumors to grow from 400 to 800 mm³ minus the time needed for control tumor to grow the same size control. P values are treated versus untreated tumors.
Clinical Cancer Research

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Muthu Selvakumaran, Ravi Amaravadi, Irina A Vasilevskaya, et al.

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