Autophagy Inhibition Improves Chemosensitivity in BRAF$^{V600E}$ Brain Tumors

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ABSTRACT

Autophagy inhibition is a potential therapeutic strategy in cancer, but it is unknown which tumors will benefit. The BRAF$^{V600E}$ mutation has been identified as important in pediatric central nervous system (CNS) tumors and is known to affect autophagy in other tumor types. We evaluated CNS tumor cells with BRAF$^{V600E}$ and found that mutant (but not wild-type) cells display high rates of induced autophagy, are sensitive to pharmacologic and genetic autophagy inhibition, and display synergy when the clinically used autophagy inhibitor chloroquine was combined with the RAF inhibitor vemurafenib or standard chemotherapeutics. Importantly, we also demonstrate that chloroquine can improve vemurafenib sensitivity in a resistant ex vivo primary culture and provide the first demonstration in a patient harboring the V600E mutation treated with vemurafenib that the addition of chloroquine can improve clinical outcomes. These findings suggest that CNS tumors with BRAF$^{V600E}$ are autophagy-dependent and should be targeted with autophagy inhibition in combination with other therapeutic strategies.

SIGNIFICANCE: Autophagy inhibition may improve cancer therapy, but it is unclear which tumors will benefit. We found that BRAF mutations cause brain tumor cells to depend on autophagy and display selective chemosensitization with autophagy inhibition. We present a pediatric case in which deliberate autophagy inhibition halted tumor growth and overcame acquired BRAF-inhibition resistance. Cancer Discov; 4(7); 773–80. ©2014 AACR.

INTRODUCTION

Despite treatment advances in childhood central nervous system (CNS) tumors and increased long-term survival over the past 50 years, CNS tumors remain a leading cause of childhood cancer death. New treatment strategies are urgently needed. Two potential strategies are (i) targeting specific mutations with small-molecule inhibitors and (ii) using a broader approach by inhibiting tumor cell survival pathways such as autophagy.

One of the most common mutations that can be targeted therapeutically is the V600E mutation in the v-RAF murine oncoprotein.
sarcoma viral oncoprotein homolog B1 (BRAF; ref. 1), which constitutively activates BRAF, resulting in increased tumorigenic growth. Recent studies have confirmed this mutation in pediatric brain tumors (2–6), highlighting its potential as a therapeutic target for these tumors using drugs such as vemurafenib, an ATP-competitive inhibitor, which has shown success in the treatment of late-stage melanoma (7). Case reports of patients with V600E mutations in other cancers, including lung adenocarcinomas (8), refractory hairy-cell leukemia (9), and multiple myeloma (10), have indicated potential therapeutic benefits of this drug. Nicolaides and colleagues reported the first pediatric patient treated with vemurafenib and demonstrated the potential for clinical improvement in pediatric patients using this targeted approach (11).

Recent studies have investigated the role of autophagy in cells with BRAF mutations. The ERK pathway induces autophagy in melanoma cells with oncogenic BRAF, and hyperactivation of ERK shows higher levels of autophagy (12). In BRAFV600E-driven lung tumors, autophagy was essential for mitochondrial metabolism and tumor growth, and mice where autophagy was blocked by knockout of an essential autophagy gene had a median survival nearly twice that of controls (13). Recent studies suggest that a high “autophagic index” in tumor biopsies of patients with melanoma is linked to poor therapeutic response and shorter survival. Furthermore, melanoma cells with high rates of autophagy had increased sensitivity and cell death with inhibition of autophagy (14).

Clinical trials evaluating the potential of autophagy inhibition in a variety of therapeutic protocols are ongoing in adult patients, including those with CNS tumors (15). We hypothesized that BRAFV600E cells would show higher levels of autophagy and, as a result, tumor cell death would increase when autophagy inhibition was combined with targeted BRAFV600E inhibition. We report here that high levels of autophagy are induced in BRAFV600E CNS tumor cells and these cells, but not BRAF wild-type (WT) cells, are highly sensitive to genetic and pharmacologic inhibition of autophagy. Moreover, inhibition with clinically available drugs is synergistic with both small-molecule inhibition of BRAFV600E and other chemotherapeutics in these tumors. In addition, we demonstrate that chloroquine can improve clinical efficacy of vemurafenib in a patient with a BRAFV600E tumor.

RESULTS

During autophagy, LC3 is cleaved, then conjugated to phosphatidylethanolamine to create LC3-II, and incorporated into the autophagosomal membrane. Autophagy can be measured by monitoring LC3-II on Western blots, by microscopy, or by flow cytometry using fluorescent protein–tagged LC3 (16). Using a tandem mCherry (mCh)–GFP–LC3 expression construct, changes in autophagy can be more quantitatively monitored in a cell population by measuring the ratio of GFP to mCh by flow cytometry. Using this method, we evaluated multiple BRAFV600E-mutant cell lines for autophagy induction in response to amino acid starvation, the current gold standard. Three astrocytoma cell lines, NMC-G1, DBTRG, and AM38, have previously characterized BRAFV600E mutations, with AM38 cells showing homozgyosity and the remainder heterozygosity (2). The 794 cell line was established and characterized in our laboratory from a ganglioglioma that developed a secondary atypical teratoid/rhabdoid tumor (ATRT) component. The 794 cells exhibit both INI-1 loss and the presence of BRAFV600E (5). This line provides an ideal in vitro model to test the effectiveness and specificity of vemurafenib in the context of a pediatric brain tumor, as it allows for stable, long-term growth that is otherwise difficult to achieve with low-grade tumors. V600E mutations in ATRTs evolved from a ganglioglioma or pleomorphic xanthoastrocytoma (PXA) have been previously noted (4).

Under starvation stress (Fig. 1A), all BRAFV600E cells induced autophagy to a greater degree than WT cells. This was confirmed by imaging of starved GFP–LC3 cells with and without chloroquine. Chloroquine prevents lysosomal fusion with autophagosomes, resulting in the buildup of membrane-bound LC3 that allows the quantification of GFP puncta before and after chloroquine. Increased autophagic flux was demonstrated by an increased number of GFP puncta in starved cells with chloroquine compared with starvation alone (Supplementary Fig. S1A). BRAFV600E cells showed a higher median number of puncta per cell compared with WT (Supplementary Fig. S1B).

To establish whether autophagy inhibition would be an effective therapeutic intervention in BRAFV600E cells, we measured cell survival after pharmacologic or genetic autophagy inhibition. BRAFV600E cells expressing shRNAs targeting ATG5 or ATG12 showed a 50% or greater reduction in the number of metabolically active cells compared with their nontarget controls by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay (Fig. 1B). This corresponded to an increase in propidium iodide–positive (PI⁺) 794 and AM38 cells (Supplementary Fig. S2A). In comparison, BT16 BRAFWT cells displayed only a minimal survival defect with ATG5 knockdown and no change with ATG12 knockdown (Fig. 1B) with a similar lack of PI⁺ cells (Supplementary Fig. S2A). Visualizing growth of BRAFV600E cells with continuous microscopic imaging demonstrated substantial decreased growth velocity of the knockdown cells compared with nontarget controls. The growth velocity of the BT16 BRAFWT cells was slightly affected, but the effect was much stronger in the BRAFV600E cells (Fig. 1C). Cells were verified to have effective RNAi of autophagy-related proteins (Fig. 1D) and a resultant high degree of autophagy inhibition (Supplementary Fig. S2B).

Because chloroquine is a potent autophagy inhibitor that is FDA-approved and available for rapid translation to pediatric clinical trials, we evaluated its effects on our CNS tumor cells. BRAFV600E-positive and WT BT16 cells were treated with increasing doses of chloroquine, and cell death/viability was assessed by lactate dehydrogenase (LDH) release and MTS assay (Fig. 1E). BRAFV600E cells showed significantly higher LDH release than WT cells and a much greater loss of cell viability by MTS assay. Importantly, these effects were not seen in WT RAF cells, suggesting that the BRAF mutation makes the survival of brain cancer cells autophagy-dependent even under nonstressed conditions. BRAFV600E–positive cells also demonstrated a higher percentage of PI⁺ cells.
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When autophagy was inhibited (17), in contrast, the pharmacologic inhibition of autophagy improved the effectiveness of vemurafenib in combination with chloroquine in BRAFV600E 794 and AM38 cells. When BRAFWT cells were treated with a similar range of doses of vemurafenib, there was a minimal effect on cell viability with the addition of chloroquine (Fig. 2A). Treating with a 100-times higher dose to produce a true dose curve also failed to show a significant improvement in the effectiveness of vemurafenib in combination with chloroquine in the BRAFWT cells (Supplementary Fig. S4). When treated with a broader range of doses of both drugs, the resultant Chou–Talalay combination indices (CI) were all less than 1 in the BRAFV600E cells, indicating synergy at all doses.

Figure 1. CNS tumor cells with BRAFV600E have high rates of induced autophagy and sensitivity to autophagy inhibition. A, cells with mCh–GFP–LC3 were exposed to either standard media or starvation Earle’s Balanced Salt Solution (EBSS) media for 4 hours and analyzed for the change in ratio of mCh to GFP signal as a measure of autophagic flux. *, P < 0.05. B, cells expressing control, ATG5, or ATG12 shRNAs were plated in standard media and allowed to grow for 72 hours before analysis by MTS assay. *, P < 0.05. C, cells were plated as in B and were monitored every 4 hours by light microscopy using real-time in vitro imaging. Quantitative analysis of confluence was performed using the IncuCyte system. Data, mean ± SEM of a representative experiment, D, representative immunoblot demonstrating knockdown of baseline ATG5 and ATG12 protein levels after 72 hours of RNAi for experiments shown in B and C. E, WT BT16 and BRAFV600E, mutant 794, AM38, and NMC-G1 cells were treated with increasing doses of chloroquine for 48 hours, and cell viability was evaluated by LDH release and MTS assay. F, cells were treated as in E and evaluated for the percentage of PI-positive cells at 48 hours.

Our previous studies in pediatric CNS cell lines with no RAF mutations found little difference in chemosensitivity when autophagy was inhibited (17). In contrast, the pharmacologic inhibition of autophagy improved the effectiveness of vemurafenib in combination with chloroquine in BRAFV600E 794 and AM38 cells. When BRAFWT cells were treated with a similar range of doses of vemurafenib, there was a minimal effect on cell viability with the addition of chloroquine (Fig. 2A). Treating with a 100-times higher dose to produce a true dose curve also failed to show a significant improvement in the effectiveness of vemurafenib in combination with chloroquine in the BRAFWT cells (Supplementary Fig. S4). When treated with a broader range of doses of both drugs, the resultant Chou–Talalay combination indices (CI) were all less than 1 in the BRAFV600E cells, indicating synergy at all doses.
Cells, at clinically relevant vemurafenib doses, the addition of clinically achievable ranges of chloroquine. Importantly, these doses are in the clinically achievable range for chloroquine (18). Conversely in the BRAFWT cells, at clinically relevant vemurafenib doses, the addition of chloroquine was predominantly antagonistic (Fig. 2B).

Vemurafenib treatment had little effect on autophagic flux in any of the cells (Supplementary Fig. S5). suggesting that synergy with autophagy inhibition is not a consequence of the drug itself inducing autophagy but rather a reflection of the combined effects of targeting two essential components in BRAF-mutant cells—i.e., autophagy and RAF signaling.
To investigate if synergy in BRAFV600E cells also applied to other drugs, we evaluated combination therapy with cisplatin and vinblastine (Fig. 2C and E). Chloroquine increased tumor cell death in 794 and AM38 cells with both drugs, but not in the WT BT16 cells. Evaluation of the synergistic effect of these combinations (Fig. 2D and F) found that in 794 cells, cisplatin CI values less than 1 were obtained for doses less than 12.5 μmol/L with chloroquine doses as low as 5 μmol/L and improving as the chloroquine dose increased in most cases. This was true for AM38 cells as well, though at 40 μmol/L chloroquine the majority of the evaluated cells were dead at all doses of cisplatin. Similar findings were seen for vinblastine, with synergy seen at low doses of cisplatin and chloroquine for doses starting at 5 μmol/L and generally improving with increasing concentration. WT BT16 cells again demonstrated an antagonistic effect of combination therapy with both cisplatin and vinblastine, with CI values predominantly above 1.

To ensure these findings were germane to true low-grade tumors with the mutation, we tested BRAFV600E-positive primary PXA samples. We evaluated two cell lines derived from BRAFV600E-positive PXA (Fig. 3A). UPN 858-2 and 794 lines had similar responses and IC50 to vemurafenib (111 and 273 nmol/L, respectively). In contrast, UPN678 cells displayed signs of resistance with an IC50 of 11 μmol/L (Fig. 3A). We therefore evaluated whether the relatively vemurafenib-resistant UPN678 cell line could be sensitized with chloroquine (Fig. 3B). Combination therapy produced additive effects at 5 to 10 μmol/L chloroquine starting at 300 nmol/L vemurafenib. With the addition of 10 μmol/L chloroquine, the IC50 for these cells decreased to 4.2 μmol/L, suggesting that chloroquine can overcome vemurafenib resistance.

Our group previously reported the case of a patient with a recurrent BRAFV600E-mutant brainstem ganglioglioma successfully treated with vemurafenib and vinblastine (11). This patient continued to do well with this regimen for approximately 1 year until she presented with reonset of progressive weakness (Fig. 4A). MRI found increased fluid-attenuated inversion recovery (FLAIR) hyperintensity at the pontomedullary junction, the site of her previous recurrence, and a slight increase in the overall size of her medullary mass (Fig. 4B), suggesting acquired vemurafenib resistance as is commonly seen in patients with melanoma (19). Vinblastine was stopped, and due to prior radiation injury she was not a candidate for reirradiation. After discussion with the family, she continued on vemurafenib with the addition of chloroquine in an attempt to improve vemurafenib’s therapeutic effect. Use of chloroquine in adult patients with CNS tumors had been previously published (20, 21), and this patient was treated similarly with 150 mg/d of chloroquine while continuing on standard-dose vemurafenib. She showed rapid clinical improvement and within 6 weeks had a 90% reduction in hiccup duration with significant improvement in her left facial nerve palsy. On imaging, there was significant improvement in the FLAIR signal abnormality in the pontomedullary junction and resolution of the abnormality by 4 months. Six months into therapy, there was continued clinical improvement, resolution of FLAIR signal abnormality, and stabilization of her medullary mass with no further tumor growth (Fig. 4C).

With continued monitoring, the patient had periods of time requiring discontinuation of vemurafenib, resulting in single-drug therapy with chloroquine alone. During these time periods, she showed clinical and MRI evidence of disease advancement that resolved with the reinitiation of vemurafenib treatment. Therefore, in this patient, neither the vemurafenib and vinblastine combination therapy nor the chloroquine alone was effective once resistance developed, but the vemurafenib and chloroquine in combination were able to stabilize her disease for, currently, more than 16 months. This suggests that the clinical improvement fits with the mechanism we found in cell culture—BRAFV600E-driven autophagy making cells sensitive to autophagy inhibition, which increases the efficacy of the RAF inhibitor—rather than being a response to the chloroquine alone. A comprehensive outline of her clinical course is shown in Supplementary Fig. S6.

**DISCUSSION**

There are over three dozen active clinical trials in adult patients with cancer exploring autophagy inhibition (15, 22), and similar trials in pediatric patients are likely to be forthcoming. These trials should target patients who will most likely benefit from this intervention by determining the tumor types and genetic lesions that increase sensitivity to autophagy inhibition. The link between autophagy and BRAFV600E is becoming clear, as shown by its importance in the growth of lung tumors harboring the mutation (13). Our data suggest a similar important role for autophagy in pediatric CNS tumors with BRAF mutation and, importantly, show that similar tumors without this mutation...
do not display a robust response to autophagy inhibition or synergy with other drugs when autophagy is inhibited. A recent article reported that targeting endoplasmic reticulum stress-induced autophagy can overcome BRAF V600E inhibitor resistance in melanoma, suggesting these effects may occur in other tumor types as well (23).

There is expanding interest in the use of targeted therapy against the V600E mutation in the pediatric brain tumor population. More patients will continue to be treated with RAF inhibitors, particularly as results become available from ongoing clinical trials, such as the first trial in patients with pediatric brain tumors, the Phase 0, and Pilot Efficacy Study in Children with Recurrent or Refractory BRAF V600E-mutant Gliomas (24). Our group reported on the first patient treated with vemurafenib (11), and since that time an additional 3 patients have been reported (25). One of these patients died of an intracranial hemorrhage following 2 weeks of therapy, but of the remaining 2 patients, one demonstrated a durable clinical response at 20 months, whereas the other had progressive disease following 2 months of therapy. With the additional experience of our patient showing progressive disease following 11 months of therapy with vemurafenib, it is clear that patients with CNS tumors acquire resistance to vemurafenib, and rational combination therapies are needed to improve the overall potential for RAF inhibitors.

The clinical response reported here is the first case in which a deliberate attempt at autophagy inhibition improved the response to another drug in a person with recurrent disease showing signs of acquired resistance and suggests that, as in the in vitro experiments, chloroquine treatment led to improved vemurafenib response. These data highlight an exciting possibility for identifying genetic markers, such as BRAF V600E, which sensitize tumors to autophagy inhibition combination therapy. Importantly, with this particular genetic marker, synergy was identified with both a specific, targeted therapy and other chemotherapies as well, suggesting that synergistic interactions are governed by the autophagy dependence of the tumor driven by its underlying mutations rather than being specific to the combined drugs. Therefore, in autophagy-dependent tumors, such as those

Figure 4. Combination therapy with vemurafenib and autophagy inhibition improved MRI appearance of BRAF V600E brainstem ganglioglioma. A, clinical timeline schematic. B, sagittal FLAIR MRI acquired at the time of relapse of clinical symptoms demonstrates increased signal at the pontomedullary junction (arrow 1) that had previously resolved with treatment. C, MRI following 6 months of combination therapy [vemurafenib plus chloroquine (CQ)] shows resolution of pontomedullary signal abnormality (arrow 1) and no further increase in size of the medullary tumor (arrow 2).
with BRAF<sup>V600E</sup>, there may be broad potential application of autophagy inhibition.

**METHODS**

**Study Approval**

Primary patient samples were obtained from Children’s Hospital Colorado and collected in accordance with local and federal human research protection guidelines and institutional review board regulations (COMIRB 95-500). Informed consent was obtained for all specimens collected.

**Statistical Analysis**

Statistical comparisons were completed using one-way ANOVA 9 (GraphPad Prism 5.0). A P value of less than 0.05 was considered statistically significant. Data shown are mean ± SD except where indicated.

**Reagents and Cell Lines**

Vemurafenib was obtained from LC Laboratories. BT16 ATRT cells were kindly provided as a gift from Dr. Peter Houghton (St. Jude Children’s Research Hospital, Memphis, TN). U87 and DBTRG-05 cell lines were purchased from ATCC. G1 cell lines were purchased from the Japan Health Sciences Foundation Health Science Research Resources Bank (Osaka, Japan). The 794 cells were established from a sample obtained during routine surgery at diagnosis. Cell lines were maintained in media supplemented with 10% FBS (Gibco) at 37°C and 5% CO<sub>2</sub>. The 794 line required Opti-MEM (Gibco) supplemented with 15% FBS. Cell line authentication was performed using short tandem repeat profiling and comparison with known cell line DNA profiles.

**LDH Assay**

Cells were seeded at 2,000 to 4,000 cells per well, dependent on optimal conditions per line, in 96-well plates (Corning) and incubated overnight. Cells were treated with increasing doses of chloroquine (Sigma) for 48 hours. LDH release was quantitated using the CytoScan-LDH Cytotoxicity Assay Kit (G-Biosciences) according to the manufacturer's instructions.

**MTS Assay**

Cells were seeded at 2,000 to 4,000 cells, dependent on optimal conditions per line, in 96-well plates (Corning). RNAi cells were plated 48 hours after knockdown. Cells were treated as indicated for 48 to 72 hours dependent on experimental conditions. Viable cells were measured using the MTS assay with CellTiter 96 AQueous One Solution Reagent (Promega) following the manufacturer’s protocol. The optical density of each well was measured with a Biotek Synergy 1.5 T Avanto scanner.

**Flow Cytometry**

Cells constitutively expressing mCherry–GFP–LC3 were seeded at 2 × 10<sup>5</sup> in 60-mm plates and allowed to equilibrate overnight. Cells were exposed to either standard media, Earle’s Balanced Salt Solution (EBSS) starvation media (Sigma), or vemurafenib as indicated for evaluation of induced autophagy. Flow data were acquired on a Gallios561 and analyzed using Summit v5.1 (Beckman Coulter). Autophagic flux was determined by the ratio of mCherry:GFP.

**MRI Images**

MRI images were obtained using standard protocols on a Siemens 3T Avanto scanner.

**Microscopy**

Cells constitutively expressing mCherry–GFP–LC3 were seeded onto poly-D-lysine-coated cell culture slides (BD Biosciences) and treated for 4 hours in media with or without 10 μmol/L chloroquine, and EBSS (Sigma) with or without 10 μmol/L chloroquine. Cells were fixed with 4% paraformaldehyde and imaged at 100× on a 3i Marianas

**CI Synergy Measurement**

The CI was calculated by the Chou–Talalay equation, which takes into account both the potency (IC<sub>50</sub>) and shape of the dose–effect curve (the M-value; 26). CI values less than 1, equal to 1, and more than 1 indicate synergism, additive effect, and antagonism, respectively.

**shRNA Transfection**

A pLKO system (Sigma-Aldrich) was used for transfection with shRNAs of autophagy-related proteins. The RNAi Consortium (TRC) numbers for shRNAs used are #151474 (ATG5), #7392 (ATG12), and SHC016 (nontarget). Cells were transduced with lentivirus using 8 μg/mL polybrene and selected with the puromycin dose determined appropriate for each cell line. Level of targeted knockdown was determined by Western blot.

**Western Immunoblots**

Cell lysates were harvested after treatments and timepoints indicated using RIPA buffer (Sigma) with phosphatase inhibitors (Roche). Membranes were blocked in PBS-Tween 5% milk and probed with primary antibodies at manufacturer-recommended concentrations. Anti–β-actin (Sigma) was used as the protein loading control. Primary antibodies used were ATG5, ATG12, and LC3 (Novus Biologicals).

**Tritiated Thymidine Uptake**

Cell proliferation was determined by <sup>3</sup>H-thymidine incorporation. Cells were seeded and treated with drug as indicated. Mitomycin C (25 mg/mL) was included as a negative control. Cells were pulsed with 0.5 mCi <sup>3</sup>H-thymidine and incubated at 37°C for 4 hours before cell harvest. After pulsing, short-term cultures were incubated overnight due to their slower rate of growth. Wells were washed with PBS, and 6% trichloroacetic acid was added to each well for 1 hour at 4°C. Wells were washed with 1 mL cold 6% trichloroacetic acid, and the acid precipitate was dissolved overnight in 50 μL 0.5 N NaOH. This solution was transferred to scintillation vials containing 3 mL of ScintiSafe-30%, and incorporated radioactivity was measured using a scintillation counter. DMSO showed no effect in control assays performed.
Spinning Disk Confocal Microscope. Imaging was performed in the Advanced Light Microscopy Core (University of Colorado Anschutz Medical Campus). Quantification of GFP-LC3 puncta was done using the Image-based Tool for Counting Nuclei (ITCN) on ImageJ 1.48a.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.M.M. Levy, J.C. Thompson, V. Amani, A.M. Donson, M.H. Handler, N.K. Foreman
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.M.M. Levy, J.C. Thompson, A.M. Griesinger, V. Amani, A.M. Donson, D.K. Birks
Writing, review, and/or revision of the manuscript: J.M.M. Levy, J.C. Thompson, A.M. Griesinger, A.M. Donson, M.J. Morgan, M.H. Handler, N.K. Foreman, A. Thorburn
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.M.M. Levy, J.C. Thompson
Study supervision: A. Thorburn
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