Dual Targeting of the Autophagic Regulatory Circuitry in Gliomas with Repurposed Drugs Elicits Cell-Lethal Autophagy and Therapeutic Benefit

Highlights

- Tricyclic antidepressants plus P2Y<sub>12</sub> inhibitors coordinately elicit death in glioma
- Dual therapy with autophagy enhancers impairs glioma progression
- By elevating cAMP levels via distinct mechanisms, IM and TIC increase autophagic flux

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In Brief
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Graphical Abstract

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Dual Targeting of the Autophagic Regulatory Circuitry in Gliomas with Repurposed Drugs Elicits Cell-Lethal Autophagy and Therapeutic Benefit

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http://dx.doi.org/10.1016/j.ccell.2015.08.012

SUMMARY
The associations of tricyclic antidepressants (TCAs) with reduced incidence of gliomas and elevated autophagy in glioma cells motivated investigation in mouse models of gliomagenesis. First, we established that imipramine, a TCA, increased autophagy and conveyed modest therapeutic benefit in tumor-bearing animals. Then we screened clinically approved agents suggested to affect autophagy for their ability to enhance imipramine-induced autophagy-associated cell death. The anticoagulant ticlopidine, which inhibits the purinergic receptor P2Y12, potentiated imipramine, elevating cAMP, a modulator of autophagy, reducing cell viability in culture, and increasing survival in glioma-bearing mice. Efficacy of the combination was obviated by knockdown of the autophagic regulatory gene ATG7, implicating cell-lethal autophagy. This seemingly innocuous combination of TCAs and P2Y12 inhibitors may have applicability for treating glioma.

INTRODUCTION
High-grade astrocytomas (anaplastic astrocytoma grade III [AA] and glioblastoma [GBM]) have the highest incidence and mortality rate among primary brain cancer patients (Friedman et al., 2000); survival benefits from current therapies are measured in months. The identification of new mechanism-based therapeutic targets and strategies that improve upon this bleak outlook is an important agenda.

In recent years, the roles of catabolic recycling of cellular components (autophagy) in malignant progression and in responses to therapy have become a focus in glioma, and other cancers. Although in some cases autophagy exhibits pro-tumorigenic effects (Hart et al., 2012), in gliomas autophagy has been suggested to inhibit tumor progression and to potentiate responses to conventional therapies (Aoki et al., 2008; Palumbo and Cominici, 2013). In the present study, we evaluated the mechanistic effects and therapeutic potential of manipulating autophagy in gliomas using repurposed USA Food and Drug Administration (FDA)-approved agents.

RESULTS
Imipramine Treatment Prolongs Survival of Glioma-Bearing Animals
Long-term use of tricyclic antidepressants (TCAs) has been associated with decreased incidence of gliomas (Walker et al., 2011). Intrigued by this observation, we asked whether TCA treatment might affect the progression of pre-existing low-grade lesions to secondary GBMs in genetically engineered mouse models of gliomagenesis. We and others have previously established that p53 deficiency promotes the formation of high-grade gliomas in animals expressing activated HRasV12 oncogene (Marumoto et al., 2009; Shchors et al., 2013). For this study, we generated two lines of mice, differing in their p53 status: GFAP-HRasV12;GFAP-CRE;GFAP-LUC; p53flox/wt (referred to here as GRLp53het mice) and GFAP-HRasV12;GFAP-CRE;GFAP-LUC;p53flox/flox (referred to here as GRLp53fko). The homozygous loss of p53 in this context significantly accelerated the progression of high-grade gliomas when compared with p53 heterozygous animals (Figure S1A) (Shchors et al., 2013). These mice

Significance
Glioma, a form of brain cancer, remains an intractable disease, for which standard-of-care treatments are largely inadequate. Discovering new therapeutic strategies to combat gliomas is therefore an important agenda. We describe a means to impair glioma growth and malignant progression by increasing autophagic flux upon combining two classes of clinically approved drugs: tricyclic antidepressants (TCAs) and certain anticoagulants. We show that combining a TCA and an inhibitor of the purinergic receptor P2Y12 promotes autophagy-associated cell death in glioma cells via the EPAC branch of the cAMP-signaling pathway. Pre-clinical trials in several models of glioma development and progression demonstrate therapeutic efficacy. Thus, combinations of TCAs and P2Y12 inhibitors could be considered for clinical evaluation as an adjuvant to conventional glioma therapy.
reproducibly develop high-grade astrocytomas (AA and GBM-like lesions) (Figure S1). The tumors developing in the GRLp53het and GRLp53fko animals varied in histopathological grade, with the GRLp53fko model having a higher incidence of the GBM-like tumors (80% versus 59% in the GRLp53het model) (Figure S1B). The molecular alterations detected in GBMs from the GFAP-HRasV12-driven model of gliomagenesis are similar to those observed in a range of high-grade human gliomas, including overexpression of EGF receptor and increased activity of the AKT/mTOR signaling pathway (Shannon et al., 2005) (Figure S1C). The incorporation of a luciferase reporter enables non-invasive monitoring of disease progression (Figures S1G–S1J). The scale bars represent 200 μm; insets are magnified 14×. *p < 0.001 by unpaired Student’s t-test.

We first assessed the impact on tumor-bearing GRLp53het animals of a TCA, imipramine (IM). Asymptomatic GRLp53het animals with similarly sized incipient gliomas were stratified into control and IM-treated cohorts. The IM cohort (5 days/week) was treated until a defined endpoint 13 weeks later or until the effects of tumor progression dictated euthanasia. The IM treatment prolonged the overall survival of treated animals, with a median survival of 31 days compared with 13 days in the control cohort (Figure 1A). The IM-treated tumors exhibited a lower histopathological score and had a reduced proliferative index, compared with the control group; only 36% of treated mice had progressed to high-grade GBM-like lesions, assessed by Ki67, compared with the control group. The size of each cohort is indicated. No statistically significant difference was detected by the Mantel-Cox test.

(A) Kaplan-Meier survival analysis of tumor-bearing GRLp53het animals in cohorts treated with vehicle control (CTRL) or with IM (40 mg/kg/day). Treatment was initiated 24 hr after tumor detection (see Experimental Procedures). *p < 0.02 by the Mantel-Cox test.

(B) The distribution of grade II, III, and IV tumors in end-stage GRLp53het mice, control (CTRL) and treated with IM. The histopathological score was determined as described in the Supplemental Information. Statistical analysis was performed using the chi-square test.

(C) Representative images of an IHC analysis of cell proliferation in GRLp53het tumors, control (CTRL) and treated with IM, assayed for Ki67 (see Supplemental Information). The scale bars represent 200 μm; insets are magnified 14×. *p < 0.001 by unpaired Student’s t-test.

(F) RT-qPCR analysis of mRNA expression of the p53 target genes: CDKN1a (p21cip1), puma, and gadd45a in tumor-derived primary cultures from GRLp53het and GRLp53fko that were followed in vivo. Data are presented as fold induction relative to control vehicle-treated samples. Ns, no statistical significance by two-way ANOVA. Three independently derived tumor cultures were analyzed, each in triplicate.

(G) Representative images from an IHC analysis of apoptosis assayed by activated caspase-3 (CC-3) staining in brains collected from GRLp53het and GRLp53fko mice, control or treated with IM for 72 hr (see Supplemental Information). The scale bars represent 50 μm. Quantification of CC3-positive cells in tumor fields is presented to the right. Statistical analysis was performed using a two-tailed Student’s t-test.
To understand the basis for the therapeutic efficacy of IM against low-grade gliomas, we examined the effects of IM action in tumors as well as cultured glioma cells of mouse and human origin. It has been suggested, depending on the context, that TCAs disrupt tumor cell homeostasis via several mechanisms, including accentuated autophagy, alterations in lysosomal turnover, and elevated apoptosis (Jachhan et al., 2013; Jeon et al., 2011; Petersen et al., 2013). Analysis of tumor samples collected from GRLp53het and GRLp53fko control and IM-treated animals did not reveal a heightened frequency of apoptosis in the gliomas treated with IM (Figure 1G). Therefore, we focused on the implication that IM could be modulating autophagy (Jeon et al., 2011).

The process of autophagy involves multiple stages. Following initiation, evidenced by autophagosome formation, the process can either become stalled at a midpoint (non-functional) or be productive (functional) at completing the degradation of enveloped cellular organelles, termed autophagic flux. A common metric for the initiation of autophagy involves the microtubule-associated protein light chain 3 (MAP1 LC3, hereafter referred to as LC3). The conversion of cytosolic LC3I into phosphatidyl-ethanolamine-conjugated LC3II closely correlates with the number of autophagosomes in the cell and is manifested as puncta by immunohistochemical (IHC) analysis or as a faster migrating LC3 isofrom in SDS-PAGE (Mizushima et al., 2010). Tumor samples were collected from symptomatic tumor-bearing GRLp53ko animals, subjected to IM or control-vehicle treatment for 72 hours, and analyzed for LC3 expression. We detected an increase in the abundance of LC3-II puncta per cell in tumors collected from the IM-treated animals compared with control tumors (mean ± SEM 3.174 ± 0.56 and 1.088 ± 0.35, respectively), as well as an increase in the LC3II isoform visualized by SDS-PAGE in tumor lysates and primary glioma cells derived from the GRLp53fko and GRLp53het mice and in human GBM cell lines (Figures 2A–2D). This effect was not associated with altered transcriptional regulation of LC3 (Figure 2E).

During the final stages of the autophagic process, the LC3-II protein is degraded in autolysosomes. Pharmacological inhibition of LC3-II degradation in lysosomes is used as a metric of productive autophagic flux (Mizushima et al., 2010). Indeed, an inhibitor of lysosomal acidification, Bafilomycin A1 (Baf1), was found to promote LC3 accumulation in IM-treated glioma cells (Figure 2F), indicating that it was otherwise being degraded in the end stage of autophagy.

**A Cell-Based Screen for Drugs that Enhances IM’s Effects on Autophagy and Cell Survival**

IM exposure interfered with the progression of low-grade incipient neoplasia in GRLp53het mice but was ineffective in GRLp53fko mice exhibiting more rapid disease progression (Figures 1A and 1E). Similarly, in a cohort of human glioma patients, in which high-grade glioma patients were overrepresented, post-diagnostic treatment with TCAs produced very limited survival benefits (Walker et al., 2012). We reasoned that IM’s modest activity in high-grade gliomas could be potentially enhanced in terms of therapeutic benefit by a combinatorial approach. Given that we (Figure 2) and others (Jeon et al., 2011) observed that IM modulates autophagic flux in this cell type, we screened for agents that could intensify IM-mediated regulation of autophagy and enhance its anti-tumoral activity in glial cells. Notably, other clinically approved drugs have recently been revealed to modulate autophagy by affecting different stages or regulators of this process (Hundeshagen et al., 2011; Williams et al., 2008). We evaluated a set of agents that were implicated to modulate autophagy at distinctive nodes (Figure 3A), focusing on drugs that were not associated with significant side effects in humans (Table S1).

We tested the effects of the selected drugs, alone and in combination with 20 or 40 μM IM, on cell survival in a panel of six human glioma cell lines differing in PTEN, p53, and INK4ARF status. Proliferation and survival were measured 72 hours following exposure (Figure S2A). The maximum tested dose of each drug was based upon its highest non-toxic concentration in plasma of human patients. Only one of the tested agents, the anti-platelet drug ticlopidine (TIC), synergized with IM in reducing cell survival in all tested lines (independent of the activity of the AKT/mTOR signaling pathway and of EGF receptor levels) (Figure S2B). The combination was also effective in primary GBM cultures derived from the GRLp53het and GRLp53fko mice and had minimal toxicity toward normal mouse astrocytes in vitro (Figures 3B, 3A, 3C, and 3A).

TIC inhibits the ADP receptor P2Y12, thereby abolishing the ADP-induced downregulation of adenylate cyclase. In normal tissue, expression of P2Y12 is limited to platelets and glial cells. In gliomas, P2Y12 is expressed at higher levels in cancer cells than in normal astrocytes (Barańska et al., 2004; Carrasquero et al., 2005) (Figure S3B). Concordantly, P2Y12 was elevated in GRLp53het gliomas compared with wild-type astrocytes and was detected in all tested human glioma cell lines (Figure S3C).

We hypothesized that inhibition of P2Y12 activity in conjunction with TCA treatment was increasing the cellular level of cAMP by upregulating adenylate cyclase (Hollopet et al., 2001; Toki et al., 1999), which in turn elevates the rate of autophagic flux (Ugland et al., 2011) (Figure 3C). If indeed IM and TIC act by targeting Gx and P2Y12, respectively, then other chemically distinct drugs targeting these proteins should have similar combinatorial effects in gliomas. Therefore, we analyzed two additional TCAs, desipramine (DMI) and trifluoperazine (TFP), and two additional P2Y12 inhibitors, prasugrel (PGL) and clopidogrel (CDL), alone and in combination, for their ability to reduce survival of glioma cells. All of the analogous combinations of a TCA and a P2Y12 inhibitor synergistically reduced the survival of glioma cells to varying degrees, strengthening the conclusion that IM+TIC treatment was elevating cAMP (Figure 3D). Among the agents tested, DMI was more potent in inducing cell death in vitro compared with IM, and the P2Y12 inhibitor CDL was similarly efficacious to TIC (Figure 3D). Beyond the immediate result of target validation, DMI and CDL warrant future evaluation as therapeutic agents.

Having established that drug combinations targeting the autophagic regulatory circuitry reduced cell survival (Figures 3B, 3D, and 3E), we next sought to characterize the mechanism of cell death. With regard to apoptosis, we found that the reduced survival of LN71 glioma cells treated with IM+TIC was not associated with the cleavage of the caspase-3 target protein poly ADP ribose polymerase (PARP), suggesting that IM+TIC treatment induces non-apoptotic cell death in LN71 cultured glioma cells (Figure 3F). Congruent with this observation, the
Figure 2. IM Treatment Induces Autophagy in Human and Mouse Glioma Cells

(A) Representative IHC analysis of LC3 expression and distribution in tumors collected from control (CTRL) and animals treated with IM for three days (see Supplemental Information). The scale bars represent 50 μm. Insets are magnified 8x.

(B) Quantification of LC3II puncta/cell in tumors described above. Statistical analysis was done by unpaired Student’s t test.

(C) Immunoblotting analysis of LC3 expression in GRLp53het and GRLp53fko tumors collected from control and IM-treated animals treated as described above. Quantification of LC3 levels in treated animals relative to the respective controls is presented below the immunoblot.

(D) Immunoblotting analysis of LC3 expression in mouse and human glioma cells, either control (sham-treated) (-) or IM treated (+) for 24 hr. Independently derived primary cell lines from the GRLp53het (H454, A923) and GRLp53fko (D798, 9312) animals and human GBM cell lines (LN229, LN71, LN 443) were treated.

(legend continued on next page)
pan-caspase inhibitor Z-VAD-(OMe)-fmk failed to protect glioma
cells from the IM+TIC-induced cell death (Figures 3G and 3H). In
some experimental systems, IM has been implicated as an inhibi-
tor of autophagy that acts by increasing lysosome membrane
permeability, thereby causing an increase in apoptosis (Ashoor
et al., 2013; Petersen et al., 2013). The lack of detectable
apoptosis and the elevated autophagic flux (further described
below) seen in the IM-treated glioma cells suggest that the re-
ported ability of IM to inhibit rather than accentuate autophagy
is not operative in this particular cancer cell type. In addition,
the inhibition of necroptosis (programmed cell death by necrosis)
by necrostatin-1 (25 μM), a small-molecular inhibitor of necro-
ptosis (Degterev et al., 2005), did not block IM+TIC-induced cell
death (Figures 3I and 3J).

Having excluded apoptosis and necroptosis as major determi-
nants of the induced cell death, we proceeded to evaluate the
role of heightened rates of autophagy in reducing survival of
cultured glioma cells. A number of studies suggest that in-
creases in cellular autophagy in vitro and in vivo can promote
non-apoptotic cell death (for reviews, see Jain et al., 2013; Tsu-
imoto, 2012). To determine whether IM+TIC was also modu-
lating the rate of cellular autophagy, as observed for IM

treatment (Figure 2A), we first analyzed expression of LC3-II
in LN71 glioma cells mock-treated or treated with IM, TIC, or
IM+TIC. Indeed, IM+TIC treatment further enhanced LC3-II
levels compared with IM or TIC (Figure 4A).

Autophagy is a dynamic, multi-stage process. The elevated
levels of LC3-II reflect the initiation of the autophagic process
but not necessarily its completion; as such, elevated LC3-II

can be indicative of an increase in autophagy or, alternatively,
stalling midcourse. To distinguish between these possibilities,
we performed immunoblotting analysis of the cellular levels of
p62/SQSTM1, which is known to recognize damaged proteins
and shepherd them in autolysosomes, where p62 is concomi-
tantly degraded. As such, cellular levels of p62/SQSTM1 reflect
the status of autophagic flux in the cell (Björkøy et al., 2009; Pan-
kiv et al., 2007). The levels of p62/SQSTM1 in LN71 glioma cells

treated with IM+TIC were found to be significantly reduced
compared with control, indicative of increased rates of functional
autophagy (Figure 4A). Both Baf1 and the lysosomotropically
agent chloroquine (CQ) inhibited degradation of LC3-II in
IM+TIC-treated samples (Figures 4B and 4C). The observed
changes in the MAP1LC3 and p62/SQSTM1 protein levels in
response to IM, TIC, and IM+TIC treatments were not a result
of transcriptional regulation (Figure S3D), consistent with a direct

effect on the autophagic machinery. Moreover, CQ treatment
partially impedes IM+TIC-induced cell death (Figure S3F), further
indicating that IM+TIC accentuates autophagy.

To further evaluate the outcome of IM+TIC treatment on gli-
oma cells, we used a mRFP-EGFP-LC3 tandem-tagged fluores-
cent protein (ptf-LC3) (Kimura et al., 2007). EGFP is sensitive
to lysosomal proteolysis, while red fluorescent protein (mRFP)
retains fluorescence in autolysosomes. Therefore, ptf-LC3 allows
one to measure both induction of autophagy (mRFP+EGFP pos-
tive puncta) and productive autophagic flux reflected in an in-
crease in the percentage of mRFP-only positive vehicles. A
48 hr IM+TIC treatment of LN71 glioma cells transfected with
ptfLC3 resulted in an increase in red-only fluorescent vesicles
compared with mRFP* EGFP+ puncta (Figure 4D), indicative of
EGFP proteolysis in lysosomes, consistent with the other data
indicating that the combination IM+TIC is promoting functional
autophagy in glioma cells.

To substantiate this conclusion, we interfered with the expres-
sion of two key autophagic regulatory genes, Beclin-1 and ATG-
7. Small hairpin RNA (shRNA)-mediated downregulation of
Beclin-1 resulted in statistically significant protection from
IM+TIC-mediated death in LN71 glioma cells (Figures 4E, S3G,
and S3H). Congruently, downregulation of ATG-7 in LN71 cells
also interfered with the IM+TIC-induced death (Figures 4E, 4G,
and S3G–S3I). Moreover, shRNA-mediated downregulation of
both Beclin-1 and ATG-7 impeded the IM+TIC-mediated induc-
tion of p62/SQSTM1 degradation (Figure 4F). These results
strengthen the conclusion that the cell death elicited by IM+TIC
is a consequence of elevated (and not reduced) rates of
autophagy.

To further characterize the status of autophagic flux in IM+TIC-
treated cells, we performed transmission electron microscopy
analysis (EM) of glioma cell lines LN71 and LN229 treated with
vehicle control or IM+TIC for 6 or 18 hours. We observed an in-
crease in cellular lysosomes as well as autophagic vacuoles
(AVs) following 6-hour exposure to IM+TIC. Prolonged exposure
to IM+TIC did not result in additional accumulation of lysosomes,
although LN71 exhibited a slight reduction in total lysosomal
number; there was, however, a statistically significant increase
in both early (or initial) AVs and late (degradative) AVs (AVd) (Fig-
ure 4H). Occasionally, IM+TIC treatment caused the formation of
“empty” autophagosomes, presumably with fully digested con-
ten (Figure 4H, red arrows). Taken together, our data suggest
that IM+TIC treatment causes non-apoptotic cell death and ac-
centuates autophagic flux in human glioma cell lines in vitro.

Co-treatment with IM and TIC Induces Autophagy In Vivo
Having established that combinatorial treatment of LN71 human
glioma cells in vitro results in induction of autophagy and non-
apoptotic cell death, we proceeded to investigate the outcome
of IM+TIC treatment on cell autophagy in vivo. IM+TIC treatment
of mice bearing incipient gliomas produced a significant increase
in LC3-positive puncta in tumor cells compared with control-
treated tumors (Figure 5A); this increase was not associated
with altered expression of the MAP1LC3 gene (data not shown).
We further visualized lysosomes with anti-LAMP1 (lysosomal
associated membrane protein 1) antibodies and observed an
evident co-localization with LC3 dots (Figure 5B). Therefore,
autolysosome formation is increased in IM+TIC-treated tumors
compared with control tumors, indicative of productive autopha-
gic flux.
Figure 3. TIC Synergizes with IM in Promoting Death in Cultured Mouse and Human Glioma Cells

(A) Schematic representation of the proposed regulation of cell autophagy by different clinically approved drugs. The agents used were either identified using cell-based screens (Hundeshagen et al., 2011; Williams et al., 2008) or were previously established to be modulators of autophagy (for references, see Table S1). Six drugs (highlighted in pink font) targeting different nodes in this circuit were tested, alone and in combination with IM (see Figure S2).
To further investigate the effects of increased autophagic flux in the IM+TIC-treated tumors, we performed a morphological analysis by EM. We found that 9.5 ± 4.6% of the IM+TIC-treated glioma cells in vivo exhibited features of autophagy-associated cell death (AACD) (as defined by Tasdemir et al., 2009; Petrini et al., 2012; Tinari et al., 2008) (Figure 5C). Namely, the dying cancer cells contained intact nuclei and had increased vacuolization, reduced numbers of cellular organelles, and increases in the number and size of AVs (Figure 5D) and in the size of AVd (Figure S4A).

We next sought to functionally connect autophagy with the effects of IM+TIC treatment on gliomas in vivo. LN229 glioma cells were engineered with an ATG7 shRNA lentiviral vector to have reduced expression of ATG7 (described in Supplemental Information). As presented in Figures 5E and 5F and Figure S4B, the ATG7 knockdown obviated the tumor growth inhibition and survival benefit elicited by IM+TIC, indicating that the autophagic regulatory circuit was important for the therapeutic effects of these agents.

The Combination of IM and TIC Has Therapeutic Benefit in Multiple Models of Glioma In Vivo

To further assess the therapeutic potential of IM+TIC in gliomas, we evaluated the efficacy of the combination in mice bearing de novo tumors. GRLp53het and GRLp53fko mice with asymptomatic incipient neoplasias were identified as described earlier, stratified into cohorts, and subjected to IM, TIC, or IM+TIC treatment (5 days/week) until a defined endpoint 13 weeks later, or until tumor progression dictated cessation. The IM+TIC treatment resulted in a significant survival advantage over either mono-therapy for both GRLp53het (59% GBM-like and 29% AA at end stage) and GRLp53fko (80% GBM-like at end stage) animals, without appreciable toxicity (Figures 5G and 5H). Greater than 50% of the GRLp53het animals survived more than 90 days following the identification of lesions, compared with 13 days in the control group (Figure 5G). The end-stage tumors in the IM+TIC-treated cohort had lower grade malignancy compared with both control cohorts and animals subjected to IM and TIC mono-therapy. The treatment also extended the median survival of GRLp53fko late-stage tumor-bearing animals, from 14–36 days in the IM+TIC-treated group (Figure 5H). The IM+TIC-treated GRLp53fko mice also had a higher percentage of lower grade (II and III) tumors compared with both the control group and animals treated with IM or TIC mono-therapy (Figure S4H). We further substantiated the therapeutic potential of IM+TIC treatment in vivo in an alternative model of gliomagenesis, GNLp53fko, elicited by the targeted deletion of the NF1 tumor suppressor gene that is implicated in a subclass of human glioma (Supplemental Information and Figures S4C and S4D). In this model, the survival of late-stage tumor-bearing mice treated with IM+TIC was 23.8 ± 3.7 days, compared with 5 ± 1.1 days in the control group (Figure S4E).

IM+TIC treatment was also beneficial when applied during the terminal stage of disease progression, when animals were exhibiting signs of neurological distress due to tumor burden. The mean survival of the IM+TIC treated animals was improved compared with the control group from 2.4–5.7 days while either IM or TIC mono-therapy was inefficacious (Figure 5I). The IM+TIC-treated tumors exhibited the reduction of cancer cell proliferation (Figure 5J). Thus, autophagy-promoting IM+TIC therapy is capable of slowing the rapid progression of late-stage disease. In notable contrast, inhibition of autophagy by CQ did not impart any survival advantage during analogous end-stage therapeutic trials (Figure S4F), further supporting the consensus of the data that IM+TIC is elevating autophagy to a cell-lethal level.

We further generated a syngeneic transplantable mouse model of glioma (Supplemental Information and Figures S4G and S4H), involving orthotopic inoculation of 5 × 10⁵ primary glioma cells derived from an end-stage GRLp53het mouse. Three days after transplantation, recipient animals were stratified into groups and mock-treated or treated with IM, TIC, or IM+TIC 5 days a week for 10 weeks or until the tumor burden required euthanasia. The animals treated with IM+TIC exhibited...
prolonged overall survival compared with the control cohort (Figure S4I).

As described above, the IM+TIC combination also reduced the growth of subcutaneous (s.c.) transplanted LN229 glial tumors (Figures S5E and SF). To substantiate this result in the context of the CNS microenvironment, we established an orthotopic transplant model of human GBM (described in Experimental Procedures). Four days after transplantation with LN229 cells, the animals were stratified into cohorts and treated as described above. IM+TIC treatment extended survival of the tumor-bearing animals from 51 days’ median survival in the control group to 61 days in the treated cohort (Figure S4J). In contrast, IM and TIC as single agents had no demonstrable effect in prolonging the survival of animals bearing aggressive transplanted tumors of mouse or human origin (Figures S4K and S4L). IM+TIC also elicited changes in tumor histopathology. The treated tumors contained large areas of tissue necrosis and displayed reduced cellularity compared with control tumors (Figure S4M).

**IM and TIC Coordinately Upregulate cAMP Levels in Gliomas**

A number of studies implicate cAMP in the induction of autophagy (Mestre and Colombo, 2012; Ugland et al., 2011) (Figure 3A). TCAs and P2Y12 inhibitors are suggested to upregulate cAMP levels in the cell by distinct mechanisms (Defreyn et al., 1991; Donati and Rasenick, 2005). To determine whether the observed synergistic effects of the IM+TIC treatment on survival and histopathology are mediated by modulation of cAMP levels in gliomas, we analyzed cAMP concentrations in GRLp53fko tumors untreated or treated with each agent alone or in combination. The 3-day treatment of animals with IM and TIC as monotherapy did not affect cAMP levels in tumors, whereas IM+TIC increased cAMP levels in the tumor tissue (Figure 6A). Similarly, although neither agent alone affected cAMP levels in glioma cell lines, IM+TIC treatment increased cAMP levels when adjusted to total protein concentration (Figure 6B).

The cAMP signaling circuit involves a number of downstream effectors, including PKA and CREB, and the recently described EPAC-1/2 (for review, see Gloerich and Bos, 2010) (Figure 6C). Both branches of the cAMP circuit are known to regulate the ERK-signaling pathway (Ster et al., 2007; Vossier et al., 1997), activation of which can in turn lead to an increase in autophagic flux (Ugland et al., 2011). A number of cAMP analogs with different specificities for these effectors have been characterized (Christensen et al., 2003) (Figure 6C). To determine if the activated cAMP signaling circuitry is involved in the reduced survival of IM- and IM+TIC-treated glioma cells, we first tested a cAMP analog, Dibutyryl-cAMP (dbcAMP), which has high specificity for PKA (Christensen et al., 2003), alone and in combination with IM or TIC in LN71 cells. The co-treatment of cells with IM or TIC in the context of ectopically increased levels of dbcAMP had no impact on glioma cell survival (Figure 6D). Congruent with this observation, the PKA-specific inhibitor KT5720 had little effect on IM+TIC-mediated death in vitro. We also did not observe increased phosphorylation levels of PKA targets in response to IM+TIC (Figure S5).

In contrast, perturbation of the EPAC branch had a discernible effect. A non-hydrolyzable cAMP analog, 8-CPT-cAMP, which selectively activates EPAC, and a highly specific EPAC agonist, 8-CPT-Me-cAMP-AC (Vliem et al., 2008), both intensified the IM- and TIC-mediated cell death in glioma cells (Figures 6E and 6F). Conversely, shRNA-mediated downregulation of EPAC1 levels using two different shRNAs partially blocked the IM+TIC-induced cell death in gliomas (Figures 6G–6J). In light of these results, we conclude that elevating cAMP levels by combining TCAs and inhibitors of purinergic receptor P2RY12 (Figure 7) is retarding gliomagenesis. Consequently, we establish the importance of this pathway in malignant brain tumor progression as suggested by epidemiological analysis in the case of TCAs.
and **p (J) IHC analysis of cell proliferation assayed by Ki67 expression (shown) and BrdU incorporation (not shown) in the tumor samples from the cohorts analyzed in (I) n = cohort size. ***p < 0.001. ns, no statistical significance by a two-tailed Student’s t test.

In this study, we assessed the hypothesis that perturbation of autophagy could have a deleterious effect on glioma progression, motivated both by an epidemiological study suggesting that chronically depressed patients being treated long term with TCAs had a reduced incidence of glioma (Walker et al., 2011) and by experimental evidence that one such agent, IM, modulated autophagy of glioma cells in culture (Jeon et al., 2011). Because IM mono-therapy only modestly prolongs the survival of tumor-bearing animals, we hypothesized that combining IM with other agents known to potentiate autophagy at distinct nodes in the autophagic regulatory circuit might increase its therapeutic benefit. Among six such drugs tested in a screen of human GBM cell lines, only TIC, an antiplatelet agent targeting the purinergic receptor P2Y12 (P2RY12), exhibited a synergistic effect with IM in impairing cell survival.

Although P2RY12 is not reported to be highly expressed in normal tissues other than platelets and glia (Hollopeter et al., 2001), it is upregulated in different types of human tumors and appears to be associated with poor prognosis for patients with glioma, colon, and non-small-cell lung cancer (NSCLC) (Figure S6). Unconventional roles for purinergic receptors in cancer progression are beginning to be explored (Di Virgilio, 2012). Here we present evidence that P2RY12 inhibitors, although ineffective as mono-therapy in glioma, can synergize with three different TCAs, markedly elevating cell death. Although treatment with a prototypical TCA and a P2Y12 inhibitor (IM and TIC, respectively) exhibits therapeutic benefits in several distinctive mouse models of glioma, the question of whether all human GBMs will be similarly responsive remains open, recognizing their heterogeneity and the diversity of driver mutations, including rearrangements, and amplification of the EGFR and PDGFR genes. Therefore, it will be important in future studies to assess IM+TIC therapy alone and in combination with therapies targeting distinct driver mutations in the broader genetic landscape of glioma.

The experimental evidence confluenceantly supports the interpretation that IM+TIC accentuates autophagy by coordinately elevating the level of cAMP. The importance of cAMP signaling in gliomas has been highlighted in several studies. Thus, CXCL12- or phosphodiesterase 4A1-mediated reduction in cAMP levels in the brain promotes gliomagenesis following the loss of NF1 (Warrington et al., 2007, 2010). Conversely, reactivation of cAMP signaling, or exposure of glioma cells to cAMP analogs, inhibits growth of xenografted brain tumors or decreases proliferation and survival of glioma cells in vitro (Goldhoff et al., 2008; Hill et al., 2009; Sugimoto et al., 2013; Yang et al., 2007). We now describe an alternative mechanism of increasing cAMP levels in gliomas with a combination of FDA-approved agents that elicit elevated rates of autophagy, with therapeutic benefit. Our data suggest that although the PKA branch of the cAMP signaling circuit is dispensable for this process, the EPAC-branch mediates the cellular response to IM+TIC. Collectively, our data indicate that IM+TIC increases cAMP levels in the cell, which, via the EPAC branch of the cAMP signaling cascade, induces AACD in glioma cells (Figure 7). Notably, we exclude

Figure 5. Combined Treatment with IM+TIC Elevates Autophagy and Prolongs Survival of Mice with Brain Tumors

(A) Representative IHC analysis of LC-3 expression in tumors collected from tumor-bearing GRLp53fko animals treated with vehicle (CTRL) or IM (40 mg/kg/day) + TIC (1 mg/kg/day) daily for 72 hr (see Supplemental Information). The scale bars represent 10 μm. Graph shows the quantification of LC3 in tumors (puncta per cell). Data are presented as mean ± SEM. Statistical analysis was performed using the unpaired Student’s t test.

(B) Intracellular localization of LC-3 and the lysosomal marker LAMP1 in tumors, either vehicle control (CTRL) or treated with IM+TIC for 24 and 72 hr, determined by confocal microscopy (see Supplemental Information). The graph presents LC3 and LAMP1 co-localization as a percentage of total LAMP1 dots. ***p < 0.0001, ns, no statistical significance by a two-tailed Student’s t test.

(C) Representative EM images of GRLp53fko tumors treated either with vehicle control (I, II) or with IM+TIC for 24 hr (III, IV) or 48 hr (V–VIII). For organelle definition and experimental details, see Supplemental Information. The scale bars represent 1 μm. IM+TIC-treated tumors show different stages of AACD (V–VIII), characterized by cell shrinkage, extensive vacuolization, and depletion of organelles. Graph shows the quantitative analysis of tumor cells exhibiting the features of AACD, presented as percentage of cells showing these features in ultrathin sections. At least 22 independent images per section were analyzed. nd, not detected.

(D) Exemplary images of organelles identified in GRLp53fko tumors. Avd (L), late degradative AV; ER, endoplasmic reticulum; Li, lipofuscin; Mi, mitochondrion. (Note the typical double membrane [red arrows] characterizing AVL.) To the right is a quantification of cellular organelles in GRLp53fko tumors, control and treated for 48 hr with IM+TIC, per 100 μm² cytoplasm. At least 20 cell bodies with clearly visible nuclei were considered for each treatment. ns, no statistical significance.

**p < 0.005 and ***p < 0.001 by a two-tailed Student’s t test.

(E) Effects of vehicle control or IM+TIC treatment on tumor volumes (left column) or survival (right column) of s.c. xenografts from LN229 cells infected with shRNA control (shCTRL) or shATG7. Tumor volumes were determined at a defined endpoint of 39 days, when control mice were at end stage (left). Survival was determined when the average tumor volume of each cohort reached end stage, approximately 870 mm³ (right). ***p < 0.0001. ns, no statistical significance by a two-tailed Student’s t test.

(F) Therapeutic potential of IM+TIC treatment presented as the reduction in the average tumor volume of s.c. xenografts from LN229 cells described above, calculated when tumors from vehicle-treated mice reached an average size of 870 mm³.

(G) Survival of tumor-bearing GRLp53fhet animals subjected to the indicated treatments initiated 24 hr after tumor detection (Supplemental Information). n = cohort size. ***p < 0.0001, **p < 0.005, and *p ≤ 0.02 by the Mantel-Cox test. The graph below shows the distribution of tumor grade (G) in end-stage GRLp53fhet animals; see the Supplemental Information for grading metrics.

(H) Survival of tumor-bearing GRLp53fko animals subjected to the indicated treatments initiated 24 hr after tumor detection (Supplemental Information). *p < 0.02 and **p ≤ 0.005 by the Mantel-Cox test. n = number of animals per cohort. The distribution of tumor grade (G) in end-stage GRLp53fko animals is shown below. (L) Scatterplot shows the survival of symptomatic (end-stage) tumor-bearing animals following enrollment into trials. Data are represented as mean ± SEM. (M) Scatter plot shows the survival of symptomatic (end-stage) tumor-bearing animals following enrollment into trials. Data are represented as mean ± SEM. (N) Scatter plot shows the survival of symptomatic (end-stage) tumor-bearing animals following enrollment into trials. Data are represented as mean ± SEM.

See also Figure S4.
Figure 6. Dual Treatment with IM+TIC Elevates cAMP Levels in Gliomas
(A) cAMP levels in tumor tissue adjusted to tumor weight. CTRL, vehicle-treated tumor-bearing animals; IM, TIC, and IM+TIC, tumor-bearing animals treated daily for 3 days (Supplemental Information). At least three animals per condition were analyzed. The analysis was performed in triplicate. Statistical analysis used two-way ANOVA.

(legend continued on next page)
apoptosis and necroptosis as mediators of cell death in response to these agents and functionally associate autophagy. In contrast, TCAs have been shown to enhance apoptosis and impair tumor growth in a mouse model of lung cancer (Jahchan et al., 2013). Although autophagy was not assessed in this study, the result suggests there may be context-dependent effects of TCAs in different tumor types, warranting further investigation.

The role of autophagy in cancer progression is complex. The outcome of autophagy activation in cancer cells depends on the stage of the disease progression, cell type, oncogenic drivers, and the intensity of the activating signal (White, 2012). For example, in some instances, pharmacological induction of autophagy can be cytoprotective, whereas subsequent inhibition of autophagy by lysosomotropic agents can result in apoptosis (Fan et al., 2010). On the other hand, increasing evidence suggests that some anticancer agents promote Aacd and that inhibition of autophagy under these conditions reduces their cytotoxicity (for review, see Eberhart, 2014). It is currently believed that Aacd is the result of excessive rates of autophagic flux this is distinct from death by apoptosis or necroptosis (Marino et al., 2014). Recently a panel of experts in the field of cell death suggested the following criteria to define Aacd: (1) increased autophagic flux, (2) cell death without the evident involvement of apoptosis, and (3) genetic inhibition of autophagy by at least two regulatory factors suppresses the cell killing (Galuzzi et al., 2012). The IM+TIC-mediated cell death in vitro meets all the proposed requirements.

The tumor-inhibiting role of autophagy in certain human cancers is increasingly being demonstrated (Aita et al., 1999). For example, in GBM patients, comparatively higher levels of autophagy are associated with better survival prognosis (Aoki et al., 2008). Increased autophagic flux was recently shown to impair tumor growth of NSCLC in mice (Xia et al., 2014), whereas inhibition of autophagy by hydroxychloroquine accelerated tumor formation in a model of pancreatic ductal adenocarcinoma (Rosenfeldt et al., 2013). These results are all consistent with the hypothesis that excessive autophagy can be tumor suppressive. Our data add glioma to this growing list.

Aside from surgical removal, which is often not possible because of tumor location and infiltration, the current conventional therapy for glioma patients consists of ionizing irradiation alone or in combination with the DNA alkalytng agent temozolomide (HeGi et al., 2005; Stupp et al., 2005). Each of these therapies elicits an increase in autophagic flux. A compelling body of data obtained in vitro suggests that the observed autophagy is enhancing therapeutic modalities by evoking Aacd in cells resistant to apoptosis (reviewed by Palumbo and CominCini, 2013). We now show that the use of agents that enhance Aacd reduces survival of glioma cells in vitro and restrains glioma progression. It is reasonable to suggest that incorporation of IM+TIC might enhance the therapeutic benefit of conventional treatments for gliomas, and we are currently investigating this possibility.

In summary, we describe the successful application of two FDA-approved pharmacological agents to target glioma cell
survival and malignant progression by eliciting AACD via induction of the cAMP-signaling pathway. Notably, other analyzed inhibitors of the P2RY12 receptor, such as CDL or PGL, may prove applicable to this mechanism-based therapeutic targeting, as might other TCAs, affording flexibility in identifying combinations with the best efficacy and toxicity profiles in humans.

The results presented herein offer a provocative strategy to target apoptosis-resistant brain tumors by hyperactivating levels of cellular autophagy; such dual targeting of autophagy with repurposed, pharmacologically tractable drugs may warrant consideration for clinical trials.

**EXPERIMENTAL PROCEDURES**

**Mouse Models and Bioluminescent Monitoring**

Mice were housed, fed, and treated in accordance with protocols approved by the committee for animal research of Canton Vaud, Switzerland. The de novo and orthotopic models and manipulations are described in detail in Supplemental Experimental Procedures.

Luminescent images were obtained 5 min after injections of firefly D-luciferin, potassium salt (L-8220; Biosynth) using an IVIS-100 Imaging System (PerkinElmer) at field view D with the following parameters: 1 min exposure, small binning, F/stop 1, emission filter open. The images were analyzed with the Living Image 3.2 Analysis software package (Caliper, PerkinElmer). The data were expressed as total photon flux (photons per second), Mice whose tumor burden produced 3.5 × 10^6 to 4.5 × 10^6 photons per second in square centimeters in regions of interest were enrolled into therapeutic trials (see Figures 1A, 1E, 5G, and 5H).

**Immunoblotting, Histology and Immunohistochemistry, Cell Culture, and Transmission EM**

These procedures were described as described by Schchers et al. (2013) and in the Supplemental Information.

**cAMP Measurement**

cAMP was measured by competitive immunoassay using the cAMP Direct Immunooassay Kit (K371-100; BioVision) according to the manufacturer’s instructions, as described in the Supplemental Information.

**Statistical Analysis and Quantification of Immune Staining**

Data are presented as mean ± SEM from at least three independent experiments, unless indicated otherwise. Kaplan-Meier survival curves were generated using GraphPad Prism 5. The analysis of the survival curves was done according to the Mantel-Cox test. The quantification of tumor proliferation and apoptosis was performed using the Fiji software package (http://rsb.info.nih.gov/ij).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.012.

**AUTHOR CONTRIBUTIONS**

D.H. and K.S. designed the experiments and analyzed the data. K.S. and A.M. performed the experiments. D.H. and K.S. wrote the manuscript. All authors agree with the conclusions presented in the manuscript.

**ACKNOWLEDGMENTS**

This work was supported by grants from Fondation S.A.N.T.É. and the School of Life Sciences at EPFL. We thank M. Hegi (Department of Neuro-oncology, Centre Hospitalier Universitaire Vaudois) for providing the LN series of human GBM cultures. We are grateful to P.Y. Dietrich (University Hospital Geneva), P.S. Mischel (University of California, San Diego, and Ludwig Institute for Cancer Research), and Jeffrey A. Kasten (École Polytechnique Fédérale de Lausanne [EPFL]) for valuable comments on the manuscript. Special thanks to members of the Hanahan laboratory for valuable discussions. We further acknowledge G. Knott, S. Rosset, and M. Crozier in the BioEM Facility, EPFL, for EM, as well as the Bioimaging & Optics Platform (PT-BIOP) and the Histology Core Facility at EPFL for their services. We thank G. Romain for help defining an algorithm for quantification of LC3 puncta.

Received: November 10, 2014 Revised: April 13, 2015 Accepted: August 31, 2015 Published: September 24, 2015

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Published: September 24, 2015

Cancer Cell 28, 456–471, October 12, 2015 ©2015 Elsevier Inc. 469


