Cancer Therapy: Preclinical

Oncogenic B-RAF Signaling in Melanoma Impairs the Therapeutic Advantage of Autophagy Inhibition

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Abstract

Purpose: Metastatic melanoma is characterized by extremely poor survival rates and hence novel therapies are urgently required. The ability of many anticancer drugs to activate autophagy, a lysosomal-mediated catabolic process which usually promotes cell survival, suggests targeting the autophagy pathway may be a novel means to augment therapy.

Experimental Design: Autophagy and apoptosis were assessed in vitro in human melanoma cell lines in response to clinically achievable concentrations of the endoplasmic reticulum (ER) stress-inducing drugs fenretinide or bortezomib, and in vivo using a s.c. xenograft model.

Results: Autophagy was activated in response to fenretinide or bortezomib in B-RAF wild-type cells, shown by increased conversion of LC3 to the autophagic vesicle-associated form (LC3-II) and redistribution to autophagosomes and autolysosomes, increased acidic vesicular organelle formation and autophagic vacuolization. In contrast, autophagy was significantly reduced in B-RAF–mutated melanoma cells, an effect attributed partly to oncogenic B-RAF. Rapamycin treatment was unable to stimulate LC3-II accumulation or redistribution in the presence of mutated B-RAF, indicative of de-regulated mTORC1-dependent autophagy. Knockdown of Beclin-1 or ATG7 sensitized B-RAF wild-type cells to fenretinide- or bortezomib-induced cell death, demonstrating a pro-survival function of autophagy. In addition, autophagy was partially reactivated in B-RAF–mutated cells treated with the BH3 mimetic ABT737 in combination with fenretinide or bortezomib, suggesting autophagy resistance is partly mediated by abrogated Beclin-1 function.

Conclusions: Our findings suggest inhibition of autophagy in combination with ER stress-inducing agents may represent a means by which to harness autophagy for the therapeutic benefit of B-RAF wild-type melanoma.

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Introduction

The increasing incidence of cutaneous melanoma worldwide coupled with the limited response to current treatment modalities continues to reflect poor survival rates for patients with metastatic disease. Resistance to, or loss of, the apoptotic pathway is a key event in tumorigenesis; however, recent research has highlighted pro-autophagic therapy as a novel strategy to induce cell death in apoptotic-resistant tumors (1).

Autophagy (macroautophagy) represents the major lysosomal-mediated process for the degradation and recycling of intracellular components. Regulated by a complex signaling cascade involving mammalian target of rapamycin (mTOR) inhibition, the autophagy proteins (Atgs) and 2 ubiquitin-like conjugation systems, autophagy culminates in formation of a double-membraned vesicle (autophagosome) which ultimately fuses with the lysosomal compartment (autolysosome) to break down sequestered material (2, 3). Autophagy represents a homeostatic mechanism to regulate cellular metabolism and energy production, thus promoting cell survival under conditions of metabolic stress. Conversely, autophagy may also promote a tumor suppressor pathway; in an apoptosis-deficient background, autophagy-defective tumor cells accumulate damaged proteins and organelles, resulting in elevated genomic stress and tumorigenesis.
Translational Relevance

Survival rates for patients with metastatic melanoma remain extremely poor, emphasizing the acute need for novel therapies. Clinical endoplasmic reticulum stress-inducing agents, fenretinide and bortezomib, able to induce effective apoptosis in melanoma cells, are also able to activate autophagy, a lysosomal-mediated catabolic process used most frequently to promote cell survival. Targeting the autophagy pathway may therefore represent a novel means to augment therapy. Results from the present study show induction of autophagy in response to clinically achievable concentrations of fenretinide or bortezomib promotes melanoma cell survival, but that autophagy induction is abrogated in cells bearing oncogenic B-RAF. These findings therefore provide evidence for the development of novel therapeutic strategies based on autophagy inhibition in combination with fenretinide or bortezomib for B-RAF wild-type tumors, accounting for approximately 50% of all melanomas. Targeting autophagy according to B-RAF mutational status hence supports the development of personalized treatment for melanoma patients.

As well as their inhibitory effect on apoptosis, multiple oncogenes, such as Ras, Akt, mTOR, and Bcl-2, impede autophagy (3), thus exerting a dual role in the prevention of cell death. In the context of melanoma, oncogenic/activating mutations in B-RAF are present in 50% to 70% of melanomas and persist during melanoma pathogenesis (16, 17). B-RAF belongs to the RAF-extracellular signal-regulated kinase (ERK) kinase/ERK pathway, activation of which promotes melanoma proliferation and resistance to apoptosis (18). However, the role of B-RAF in the regulation of autophagy is controversial; ERK signaling is associated with autophagosome–lysosome fusion (19), and autophagic cell death (20). Furthermore, overexpression of mutant B-RAF in melanoma cells results in autophagy induction (21), suggesting activated B-RAF promotes autophagy. Nevertheless, the significance of this effect remains undefined in a therapeutic context.

The aim of this study was to understand the relationship between autophagy and cell death during the therapeutic induction of ER stress in melanoma, and the influence of oncogenic B-RAF in this context. Collectively, our data suggest that inhibition of autophagy in combination with fenretinide or bortezomib offers a more effective therapeutic strategy for B-RAF wild-type melanoma.

Materials and Methods

Cell culture, drug treatment, and analysis of apoptosis

Melanoma cell lines CHL-1, A375, and WM266–4 were cultured as described previously (22), verified as melanoma by melan A staining (23), and B-RAF mutational status confirmed using Custom TaqMan SNP genotyping assays (Applera Europe BV). Fenretinide (Janssen-Cilag, Ltd.) was added in ethanol, bortezomib (Millenium, Janssen-Cilag Ltd.), thapsigargin, acridine orange, bafilomycin A1, chloroquine, rapamycin, bacitracin (Sigma), ABT737, or its enantiomer A-793844 (Abbott Laboratories) were added in DMSO (24). For apoptosis assays, trypsinised cells were incubated with FITC-conjugated Annexin V and 7-Amino-Actinomycin (BD Biosciences). Ten thousand events were analyzed by flow cytometry with CellQuest Pro software. For cell death assays, flow cytometry of propidium iodide–stained methanol/acetone-fixed cells was used to estimate the percentage of cells in the sub-G₁ fraction (13).

Immunoprecipitation and Western blotting

Bcl-XL was immunoprecipitated from cell lysates using the Pierce Crosslink Immunoprecipitation Kit (Thermo Fisher Scientific). Preparation of whole-cell lysates and Western blotting for B-RAF, p62 (D-3), ATG7 (Santa Cruz Biotechnology), ATF4 (Calbiochem, Merck Chemicals, Ltd.), Beclin-1 (BD Biosciences) all diluted 1:1000, ERK1/2, phosphorylated (Thr202/Tyr204) ERK1/2, p70 S6 Kinase, phosphorylated (Thr389) p70 S6 Kinase, LC3B (Cell Signalling Technology) all diluted 1:2000, Ambra-1 (Covalab), UVRAG (Abgent), diluted at 1:500, and β-actin (Sigma) diluted 1:5000, were performed as described previously (12).

Transfection experiments

RNA interference-mediated gene knockdown was achieved using siRNA for ATF4 (SI03019345), B-RAF (SI0029-9488; Qiagen), Beclin-1 (HSS112741, HSS112742), and ATG7 (HSS116182, HSS173705; Invitrogen), and incorporated a validated negative control siRNA (Qiagen AllStars Negative Control siRNA/Invitrogen Negative Control siRNA). 2 × 10³ cells per well were transfected with 40 nmol/L siRNA using lipofectamine 2000 (Invitrogen) as...
described previously (24, 25). Expression vectors for
B-RAF<sup>WT</sup> and B-RAF<sup>V600E</sup> (provided by R Marais, Institute of
Cancer Research, London, UK), or pcDNA4 (Invitrogen)
were transiently transfected using lipofectamine 2000.

**Retroviral expression of GFP-LC3 and mRFP-GFP-LC3**

For retroviral expression, GFP-LC3 or mRFP-GFP-LC3
(provided by T. Yoshimori, Research Institute for Microbiol
Diseases, Osaka University, Japan; refs. 26 and 27)
were subcloned into pCLPCX vector as previously
described (25). Fifteen micrograms of the retroviral vec
tors were cotransfected with 5 μg of an expression plasmid
for the vesicular stomatitis virus G protein into 293 gp/bsr
cells using the calcium phosphate method. After 48
hours, the supernatant containing the retroviral particles
was recovered and supplemented with polybren (4 μg/ ml).
CHL-1, A375, and WM266-4 cells were infected by
incubation with retroviral-containing supernatant for
6–8 hours.

**Autophagy analysis**

For fluorescence microscopy, cells were grown on glass
cover slips before treatment and fixation in 4% paraformaldehye, and visualization of GFP-LC3 or mRFP-GFP-LC3 under a Leica TCS SP II laser-scanning confocal micro
scope with LCS Lite 2.61 software (Leica Microsystems).
For transmission electron microscopy, CHL-1 or A375
xenograft tumors were fixed in 2.5% glutaraldehyde and
embedded in Epon as described previously (25). Ultrathin
sections were contrasted with uranyl acetate and photo
graphed using a Zeiss CM 900 electron microscope. To
detect the formation of acidic vesicular organelles, trypsi
nized cells were resuspended in PBS and stained with
acidine orange (1 μg/mL) for 15 minutes at room tem
perature. Ten thousand events were immediately acquired
by flow cytometry and analyzed using CellQuest Pro
software.

**Xenograft mouse model and immunohistochemical analysis**

Female CD1 nude mice, 6 to 8 weeks old (Charles River),
were inoculated s.c. into the right flank with 7.5 × 10<sup>6</sup>
CHL-1 or A375 cells in 100 μL DMEM containing 4.5 g/L L-glucose (Invitrogen). On establishment of tumors 125
mm<sup>3</sup> in volume, mice were randomized into 3 treatment
groups (3–6 mice per group) and treated subsequently by
daily i.v. injection (tail) for 10 days with 0.1 mg/kg bortezomib or 1.45 mg/kg fenretinide as previously described
(11). The control group was treated with 100 μL of vehicle
only. Caliper measurements of tumor length (L) and width
(W) were taken each day, and tumor volume determined
through the formula \( V = [(L \times W) / 2] \). Mice were
humanely killed on the final day of treatment, and tumors
extracted and snap frozen in liquid nitrogen before storage
at −80°C. Tumors were processed for Ki67 and TUNEL
positivity as previously described (11), and analyzed using
a Leica TCS SP II laser-scanning confocal microscope and
LCS Lite 2.61 software.

**Statistical analysis**

Data were analyzed by drug treatment (fenretinide, bortezomib, rapamycin) with the use of 2-way ANOVA (cells, vector) or 1-way ANOVA with Tukey’s post hoc test, using
SPSS Release 15 (SPSS Inc.); where Levene’s test was sig
ificant, data were log transformed for analysis.

**Results**

**Fenretinide and bortezomib activate autophagy in melanoma cells**

Characteristic features of early and late stages of autop
hagy were used to determine the ability of fenretinide and
bortezomib to induce autophagy in 3 human melanoma
cell lines. Conversion of unconjugated LC3 (LC3-I) to the
lipidated form (LC3-II) is associated with autophagosome formation and localization to autophagosomes, whereas
acidine orange is used to stain acidic vesicular organelles
(AVOs) including autolysosomes (28). LC3-II induction
was seen in all 3 cell lines in response to clinically achiev
able concentrations (29, 30) of fenretinide or bortezomib (Fig. 1A), but the magnitude of response differed signifi
antly between each cell line (\( F_{2,18} = 51.8, P < 0.001 \)), with
the highest level of induction observed in CHL-1 cells
(Tukey’s HSD, \( P < 0.001 \)). Similarly, significantly greater
AVO staining was observed in response to fenretinide or
bortezomib in CHL-1 cells compared with A375 or
WM266-4 cells (Fig. 1A; Tukey’s HSD, \( P < 0.001 \)). In addition,
in cells stably expressing GFP-LC3, the number of LC3-positive autophagosomes increased in response to
fenretinide and bortezomib in CHL-1 cells, but not in
A375 or WM266-4 cells (Supplementary Fig. S1). Autop
hagic flux was assessed by use of the autophagy inhibitor
chloroquine, as well as visualization of tandem mRFP-
GFP–tagged LC3 to simultaneously detect LC3-positive
immature autophagosomes (yellow) and mature autolysosomes (red), denoted by characteristic punctate fluores
cence (26). Fenretinide or bortezomib treatment further
stimulated LC3-II accumulation in the presence of chloro
quine in CHL-1 cells, but not in A375 or WM266-4 cells
(Fig. 1B). Furthermore, CHL-1 cells expressing mRFP-GFP
LC3 displayed increased yellow and red punctate staining
in response to fenretinide or bortezomib (Fig. 1C). How
ever, although higher basal levels of red puncta were
observed in A375 and WM266-4 cells, there was little
increase in response to drug treatment, although induction
of yellow puncta was evident after bortezomib treatment.
Ultrastructural evaluation of CHL-1 or A375 xenograft
tumors treated for 10 days with fenretinide or bortezomib
revealed multiple double-membraned autophagosomes
and autophagic vacuoles in fenretinide- and bortezomib-
treated CHL-1 tumors. Conversely, increased numbers of
autophagic vacuoles were present in control A375 tumors
compared with CHL-1 tumors, but there was little evidence
for autophagy induction in response to either ER stress
inducing agent (Supplementary Fig. S2).

Increased basal autophagy in A375 and WM266-4
cells was consistent with significantly increased LC3-II
expression in these cells compared with CHL-1 cells (A375 vs. CHL-1, 2-fold increase, \( P = 0.027 \); WM266-4 vs. CHL-1, 8.5-fold increase, \( P = 0.006 \)). Furthermore, blockade of lysosomal-mediated protein turnover by bafilomycin A1 (28) resulted in accumulation of p62 in A375 and WM266-4 cells, but not CHL-1 cells (Fig. 1D). These data suggest that A375 and WM266-4 cells display an increased rate of basal autophagy but are resistant to autophagy induction in response to fenretinide or bortezomib.

**ER stress mediates fenretinide- and bortezomib-induced autophagy**

To determine whether ER stress signaling was required for fenretinide- or bortezomib-induced autophagy, the ER stress-associated activating transcription factor 4 (ATF4; ref. 24) was down-regulated by siRNA in CHL-1 or A375 cells before treatment with fenretinide, bortezomib, or thapsigargin (included as a positive control for ER stress). Knockdown of ATF4 increased LC3-II levels, but significantly reduced further LC3-II accumulation in response to drug treatment in CHL-1 (Fig. 2A,B; \( F_{1,18} = 46.2, P < 0.001 \)) and A375 cells (data not shown), as well as inhibition of p70 S6 kinase phosphorylation, a target of mTOR, indicating the ER stress response is required for autophagy induction, and is mediated, at least in part, by ATF4 activation and mTOR inhibition.

We have previously shown that A375 and WM266-4 cells display a reduced sensitivity to ER stress activation compared with CHL-1 cells, as evidenced by reduced upregulation of GADD153, ATF4, and GADD34 (12), suggesting an abrogated ER stress response may limit autophagy induction in these cells. To test this hypothesis, the ER stress response was enhanced through use of bacitracin (12). Fenretinide- and bortezomib-induced LC3-II accumulation were enhanced in CHL-1 cells by combined treatment with bacitracin compared with treatment with fenretinide or bortezomib alone, although this was only significant for fenretinide (\( P = 0.021 \)). Conversely, bacitracin did not augment LC3-II accumulation in response to either ER stress-inducing agent in A375 (Fig. 2C) or WM266-4 cells (data not shown). These data suggest resistance to autophagy induction in B-RAF–mutated melanoma cells lies downstream of the ER stress response.

To determine whether melanoma cells resistant to ER stress-induced autophagy were also resistant to autophagy activated by direct inhibition of mTOR complex 1 (mTORC1), CHL-1, A375, and WM266-4 cells were treated with rapamycin. CHL-1 cells were sensitive to rapamycin treatment, as evidenced by decreased phosphorylation of p70 S6 kinase, increased LC3-II expression and AVO staining, p62 degradation, induction of yellow and red punctate fluorescence in mRFP-GFP-LC3-expressing cells, and green puncta in GFP-LC3-expressing cells, indicating activation of autophagy (Fig. 3, Supplementary Fig. S1). Compared to CHL-1 cells, p70 S6 kinase phosphorylation was reduced basally in autophagy induction-resistant A375 and WM266-4 cells, but was further inhibited in A375 cells after rapamycin treatment. However, there was little effect

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**Figure 1. Fenretinide and bortezomib activate autophagy.** A to C, non- and mRFP-GFP-LC3-expressing CHL-1, A375, or WM266-4 cells were treated with vehicle (C), fenretinide (F; 10 \( \mu \)mol/L), or bortezomib (B; 200 nmol/L) for 24 hours, or B, for 18 hours with the addition of chloroquine (CQ; 10 \( \mu \)mol/L) for the final 2 hours. A and B, LC3-II expression was determined by Western blot analysis and band intensity normalized to \( \beta \)-actin. AVO induction was determined by acridine orange staining and flow cytometry, and expressed as fold increase compared to vehicle-treated cells, for each cell line (mean \( \pm \) SD, \( n = 4 \)). C, Representative fluorescent micrographs from melanoma cells expressing mRFP-GFP-LC3 (bar, 20 \( \mu \)m). D, CHL-1, A375, and WM266-4 cells were treated with bafilomycin A1 (Baf; 10 nmol/L) for 24 hours, p62 and \( \beta \)-actin expression were determined by Western blotting.
of rapamycin on autophagy induction in either A375 or WM266-4 cells compared with CHL-1 cells. Similar results were also obtained with respect to rapamycin treatment on autophagy induction in additional B-RAF wild-type (SK-Mel-23) and mutated (SK-Mel-28) cells, as well as in the transgenic mouse B-RAFV600E cell line, 4599 (31; data not shown). These data suggest resistance to autophagy induction is related to aberrant regulation of

Figure 2. ER stress mediates fenretinide- and bortezomib-induced autophagy. A and B, CHL-1 cells were transfected with siRNAs for ATF4 (siATF4), or with a nonsilencing control siRNA (siCtrl) before treatment with fenretinide (FenR, F; 10 μmol/L), bortezomib (Bort, B; 30 nmol/L), or thapsigargin (Thap, T; 7.5 μmol/L) for 18 hours. A, ATF4, p70 S6K, phospho-p70 S6K (p-p70 S6K), LC3, and β-actin expression were determined by Western blotting. B, LC3-II expression was quantified and band intensity normalized to β-actin. Data are expressed as fold increase compared to siCtrl untreated cells (mean ± SD, n = 3). C, CHL-1 and A375 cells were treated with vehicle (ctrl), fenretinide (F; 10 μmol/L), or bortezomib (B; 30 nmol/L) in combination with bacitracin (Baci; 500 μmol/L) for 24 hours. A, LC3-II expression was determined by Western blot analysis and band intensity normalized to β-actin. Data are expressed as fold increase compared to control cells, for each cell line (mean ± SD, n ≥ 3).

Figure 3. B-RAF–mutated melanoma cells are resistant to rapamycin-induced autophagy. Non- and mRFP-GFP-LC3-expressing CHL-1, A375, or WM266-4 cells were treated with vehicle (ctrl) or Rapamycin (Rap, R; 1 μmol/L) for 24 hours. A, p70 S6K, phospho-p70 S6K (p-p70 S6K), p62, LC3, and β-actin expression were determined by Western blotting. B, representative fluorescent micrographs from mRFP-GFP-LC3-expressing cells (bar, 20 μm). C, AVO induction was determined by acridine orange staining and flow cytometry, and expressed as fold increase compared to vehicle-treated cells, for each cell line (mean ± SD, n = 3).
mTORC1-dependent signaling and the resulting high basal levels of autophagy (21).

**Oncogenic B-RAF confers resistance to autophagy induction in melanoma cells**

Numerous oncogenes are known to inhibit autophagy (5); as autophagy-efficient CHL-1 cells are B-RAF wild type, whereas autophagy-defective A375 and WM266-4 cells harbor mutated B-RAF (B-RAFV600E and B-RAFV600D, respectively), this raises the possibility that oncogenic B-RAF confers resistance to the induction of autophagy. To test this hypothesis, B-RAFWT or B-RAFV600E were overexpressed in CHL-1 cells (Fig. 4). Rapamycin treatment of CHL-1 cells expressing B-RAFWT resulted in significant accumulation of LC3-II (n = 5, t18 = −3.325, P = 0.004) and increased yellow and red punctate fluorescence in mRFP-GFP-LC3–expressing cells, compared with untreated cells. Conversely, overexpression of B-RAFV600E alone significantly increased LC3-II levels (n = 5, t18 = −2.596, P = 0.018), compared with cells expressing B-RAFWT. However, rapamycin treatment had no significant effect on LC3-II accumulation (n = 5, t18 = −1.118, P = 0.278) or redistribution in CHL-1 cells expressing B-RAFV600E. These data are consistent with a role for oncogenic B-RAF in the promotion of basal autophagy but inhibition of mTORC1-dependent autophagy induction.

In the context of ER stress, however, fenretinide or bortezomib treatment resulted in a significant accumulation of LC3-II (F2,14 = 5.6, P = 0.016), but with no significant difference between CHL-1 cells expressing B-RAFWT or B-RAFV600E (n = 3, F1,14 = 0.007, P = 0.934) or the vector × treatment interaction (F2,14 = 0.822, P = 0.46; Fig. 4). Conversely, there was a decrease in fenretinide- or bortezomib-induced AVO formation (data not shown) and red punctate fluorescence in mRFP-GFP-LC3–expressing CHL-1-B-RAFWT compared to CHL-1-B-RAFV600E cells (Fig. 4B). Furthermore, siRNA-mediated B-RAF knockdown in A375 cells was associated

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**Figure 4.** B-RAFV600E confers resistance to autophagy induction. A and B, CHL-1 or mRFP-GFP-LC3-expressing CHL-1 cells were transfected with expression vectors for B-RAFWT or B-RAFV600E, or the empty vector (Vec) for 48 hours before treatment with vehicle (ctrl, C), fenretinide (FenR, F; 10 μmol/L), bortezomib (Bort, B; 200 nmol/L), or rapamycin (Rap, R; 1 μmol/L) for 24 hours. A, B-RAF, ERK1/2, phospho-ERK1/2 (p-ERK1/2), LC3, and β-actin were detected by Western blotting. B, representative fluorescent micrographs from CHL-1 cells expressing mRFP-GFP-LC3 (bar, 20 μm).
with increased red puncta in mRFP-GFP-LC3–expressing cells in response to fenretinide, but did not alter LC3-II accumulation in response to fenretinide or bortezomib (Supplementary Fig. S3). These data suggest autolysosome formation in response to fenretinide and, to a lesser extent, bortezomib is inhibited by oncogenic B-RAF.

**Relationship between fenretinide- and bortezomib-induced autophagy and apoptosis**

ER stress-inducing therapy can promote cell death through autophagy in apoptosis-defective cells (32, 33), whereas autophagy activation is commonly a prosurvival response in apoptosis-competent cells (7). CHL-1, A375, and WM266-4 cells activate apoptosis with differing sensitivity to fenretinide or bortezomib (11). This observation was supported by data demonstrating fenretinide and bortezomib activated apoptosis in CHL-1 cells to a greater extent than either A375 or WM266-4 cells (Supplementary Fig. S4: Post Hoc Tukey’s HSD CHL-1 vs. A375 or WM266-4, \( P < 0.001; \) A375 vs. WM266-4, \( P = 0.6 \)). Furthermore, evaluation of CHL-1 or A375 xenograft tumors treated with fenretinide or bortezomib revealed an increased inhibitory effect of fenretinide or bortezomib on CHL-1 tumor growth after 10 days treatment (Fig. 5A: 2-way ANOVA; \( F_{1,21} = 13.6, P = 0.001 \)). Furthermore, the percentage of apoptotic TUNEL-positive cells significantly increased in response to drug treatment, with a greater effect observed in CHL-1 compared with A375 tumors (Supplementary Fig. S4: \( F_{2,18} = 11.28, P = 0.001 \)). Collectively, these data suggest CHL-1 cells are able to activate both apoptosis and autophagy more efficiently than A375 or WM266-4 cells in response to ER stress.

To test the function of autophagy in relation to cell death in response to fenretinide or bortezomib, the expression of...
Beclin-1 or ATG7 was abrogated by siRNA-mediated knockdown in both CHL-1 and A375 cells (Fig. 5B,D). Beclin-1 or ATG7 knockdown significantly enhanced fenretinide-induced and, to a lesser extent, bortezomib-induced cell death of CHL-1 cells (1-way ANOVA: $F_{4,10} \geq 47.58$, $P \leq 0.001$), and ATG7 knockdown had a greater effect on fenretinide treatment compared with Beclin-1 knockdown (Fig. 5C). These data suggest that autophagy activation limits ER stress-induced cell death in CHL-1 cells. Conversely, individual siRNAs resulted in increased or decreased fenretinide- or bortezomib-induced cell death of A375 cells ($F_{4,10} \geq 17.5$, $P \leq 0.001$); however, taken together these data demonstrate that Beclin-1 or ATG7 knockdown does not affect the outcome of fenretinide or bortezomib treatment in A375 cells (Fig. 5E).

**ABT737 promotes fenretinide- and bortezomib-induced cell death in B-RAF–mutated melanoma**

Genetic ablation of Beclin-1 or ATG7 showed a pro-survival role for autophagy after therapeutic induction of ER stress in B-RAF wild-type CHL-1 cells. As oncogenic B-RAF acts to suppress both ER stress- and rapamycin-induced autophagy, it is possible that a common point of autophagy dysregulation is Beclin-1. Although Beclin-1 was expressed in melanoma cells, Beclin-1 function may be limited by increased expression of Bcl-XL in B-RAF–mutated melanoma cells (Supplementary Fig. S4; refs. 34 and 35). To reactivate autophagy and test the hypothesis that autophagy promotes cell survival in B-RAF–mutated melanoma cells, the BH3 mimetic ABT737 was employed to liberate Beclin-1 from its inhibition by Bcl-XL (36; Supplementary Fig. S4). Treatment of A375 cells with fenretinide or bortezomib alone increased the expression of p62, likely due to the generation of intracellular ROS or proteasome inhibition (13, 37, 38), however, combined treatment with ABT737 resulted in downregulation of both total p62 and ubiquitin-conjugated p62 (39), as well as enhanced accumulation of p62 in the presence of bafilomycin A1 (data not shown), and increased red puncta in mRFP-GFP-LC3–expressing cells, compared to treatment with the enantiomer, suggesting increased autophagy-mediated p62 degradation (Fig. 6A,B). Nevertheless, fenretinide- and bortezomib-induced cell death was significantly increased in the presence of ABT737, compared with treatment with either the enantiomer or vehicle ($P < 0.001$; Fig. 6C). Abrogation of autophagy by ATG7 knockdown resulted in a small but significant increase in A375 cell death in response to combined treatment with ABT737 and fenretinide or bortezomib (Fig. 6D; $F_{1,8} = 24.7$, $P = 0.001$). These data suggest the ability of ABT737 to promote autophagy, and hence cell survival, in B-RAF–mutated A375 cells has little effect on fenretinide- or bortezomib-induced cell death in comparison with its prevailing apoptosis-promoting action.
Discussion

It has become increasingly apparent that the therapeutic activation of ER stress may offer considerable benefit in cancer treatment (40), and we have recently shown that the ER stress-inducing drugs fenretinide and bortezomib promote melanoma cell death (11–13), suggesting activation of ER stress is a promising approach for melanoma therapy. Emerging evidence now implicates the unfolded protein response (UPR) in the activation of autophagy (40), which can either counteract the accumulation of unfolded proteins to promote cell survival, or participate in ER stress-induced cell death (8, 41). Targeting the autophagy pathway may therefore be a novel means to augment therapy. In this respect, autophagy activation has a prosurvival function in B-RAF wild-type cells, and autophagy inhibition potentiates ER stress-induced cell death in this setting. However, ER stress-induced autophagy is significantly less in B-RAF–mutated compared with wild-type melanoma cells.

The molecular mechanisms connecting ER stress and autophagy have been attributed to the inactivation of mTOR, UPR-dependent upregulation of ATG proteins, and disruption of the balance between Bcl-2 proteins (40). We have previously shown that fenretinide and bortezomib activate ATF4 (24), and here we demonstrate ATF4 is required for autophagy in response to ER stress in melanoma cells, suggesting the engagement of a common pathway upstream of both apoptosis and autophagy. Consistent with this observation, recent studies demonstrate ATF4 can mediate mTOR inhibition (42), and that LC3 is a direct transcriptional target of ATF4 (43). The inhibitory effect of oncogenic B-RAF on autophagy induction in this context is downstream of ER stress activation and is, at least partly, related to deregulation of mTORC1-dependent autophagy, as direct mTORC1 inhibition was unable to stimulate either LC3-II accumulation or redistribution in the presence of mutated B-RAF. Furthermore, ABT737 treatment partially restored fenretinide- and bortezomib-induced autophagy, indicating Bcl-XL overexpression prevents activation of the BH3-only protein Beclin-1 during ER stress in B-RAF–mutated cells. However, although fenretinide- or bortezomib-induced autolysosome formation was inhibited by oncogenic B-RAF, LC3-II accumulation was not affected by modulation of B-RAF expression. Furthermore, B-RAF knockdown did not enhance bortezomib-induced autophagy, suggesting additional mechanisms of autophagy regulation exist during ER stress that are not B-RAF dependent. Interestingly, accumulation of LC3-containing autophagosomes/aggregates during bortezomib treatment of B-RAF–mutated cells is potentially due to inhibition of proteasomal LC3 processing, signifying crosswalk between the autophagy and proteasome degradation pathways (44).

The simultaneous activation of autophagy and apoptosis in response to fenretinide or bortezomib suggests therefore that autophagy inhibition in combination with ER stress-inducing drugs is a promising strategy for B-RAF wild-type tumors. However, the lower sensitivity to autophagy induction conferred by oncogenic B-RAF limits the therapeutic advantage of autophagy inhibition in B-RAF–mutated melanoma cells. Autophagy activated in response to ABT737 had a small inhibitory effect on ER stress-induced cell death in comparison with its established apoptosis-promoting action in B-RAF–mutated melanoma cells, suggesting inhibition of autophagy in this setting would be of marginal benefit. Fenretinide or bortezomib in combination with ABT737 may therefore be an appropriate and effective therapeutic strategy negating the need for additional autophagy modulation in B-RAF–mutated melanoma.

The impact of deregulated autophagy in B-RAF–mutated melanoma cells can also be viewed within the broader context of tumor biology. The concept of B-RAF–mediated inhibition of autophagy induction is consistent with a role for oncogenes in autophagy repression (5) as well as a tumor suppressive role of autophagy, but contradictory to the reported promotion of autophagy by oncogenic B-RAF, attributed to decreased mTORC1 activity (21). The increased rate of basal autophagy in B-RAF–mutated compared with wild-type cells is therefore likely due to inhibition of mTORC1 signaling, although the relevance of this in vivo is unclear; mTOR is activated in the majority of malignant melanomas despite the presence of activating B-RAF mutations (45). Hyperactivation of mTOR (45) or increased expression of Bcl-XL (46) are 2 potential mechanisms by which autophagy inhibition could be achieved in vivo.

Collectively, these data establish a role for oncogenic B-RAF in the inhibition of fenretinide- and bortezomib-induced autophagy in melanoma. Although oncogenic B-RAF clearly plays a significant role in melanoma pathogenesis (16), up to 50% of tumors are nevertheless B-RAF wild type, with equally limited treatment options. This study therefore provides evidence to support the development of novel strategies based on autophagy inhibition in combination with ER stress-inducing therapy in B-RAF wild-type tumors. Differentially harnessing autophagy and selection of the most appropriate treatment for melanoma patients stratified according to B-RAF mutational status hence supports the current trend toward personalized therapy for melanoma patients to improve clinical outcome.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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