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RETRACTED ARTICLE: Endoplasmic reticulum stress-induced autophagy determines the susceptibility of melanoma cells to dabrafenib

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Abstract: Melanoma is one of the deadliest skin cancers and accounts for most skin-related deaths due to strong resistance to chemotherapy drugs. In the present study, we investigated the mechanisms of dabrafenib-induced drug resistance in human melanoma cell lines A375 and MEL624. Our studies support that both endoplasmic reticulum (ER) stress and autophagy were induced in the melanoma cells after the treatment with dabrafenib. In addition, ER stress-induced autophagy protects melanoma cells from the toxicity of dabrafenib. Moreover, inhibition of both ER stress and autophagy promote the sensitivity of melanoma cells to dabrafenib. Taken together, the data suggest that ER stress-induced autophagy determines the sensitivity of melanoma cells to dabrafenib. These results provide us with promising evidence that the inhibition of autophagy and ER stress could serve a therapeutic effect for the conventional dabrafenib chemotherapeutic.

Keywords: melanoma, dabrafenib, ER stress, autophagy, apoptosis

Introduction

Melanoma, one of the deadliest skin cancers, is derived from melanocytes and is poorly differentiated.[1–3] It accounts for most of the mortality rate in humans of all skin cancers, and the incidence of melanoma has been rising worldwide during the last 20 years, mostly in white populations.[1,4–6] Several gene alterations have been reported in this lethal cancer, among which B-Raf^{V600E} has been considered to be largely related to the aggressive metastatic characteristic and high mortality rate. Although at present this malignant tumor can have a good prognosis with early diagnosis and sufficient surgical treatment, the 5-year survival rate of patients in the advanced stages is less than 5%.[7–10] Current single-drug chemotherapies based on inhibition of B-Raf are not effective and eventually develop drug resistance. Dabrafenib, B-Raf inhibitor, is currently in use and effective in preventing the growth of late-stage melanoma. However, according to previous clinical studies, during B-Raf inhibitor treatment patients eventually develop drug resistance and fail to respond to chemotherapy. For example, vemurafenib is also taken and, although the clinical trials showed tumor shrinkage and improved rates of overall and progression-free survival, 40% of the cases still developed resistance to the treatment of vemurafenib.[11] Thus, we still face a chemotherapeutic challenge in treating advanced stage melanoma. It is crucial to understand the mechanism underlying drug resistance in therapies, such as with dabrafenib.

Efforts have been put into the drug resistance studies in melanoma and several different hypotheses on the mechanism have been reported such as enhanced DNA

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repair, resistance to apoptosis, drug-induced autophagy, etc. Autophagy is a catabolic process by which subcellular membranes undergo dynamic morphological changes that result in the removal of cellular proteins and organelles within the lysosome.[12–14] To date, autophagy has been widely considered to be critical in the chemotherapy of multiple cancer types. However, the exact roles of autophagy in cancer biology still remain debated as drug-induced autophagy can play dual roles in the cancer type and stage context.[15] This process can be induced by many physiological and pathophysiological conditions, such as infection, reactive oxygen species, endoplasmic reticulum stress (ER stress), etc.[16–18] A key factor contributing to autophagy is ER stress, which occurs in response to the accumulation of misfolded proteins within the ER. So far, whether drug-induced autophagy in chemotherapy resistance to dabrafenib plays a role in cell survival or cell death in melanoma is still unknown. In addition, the mechanism regulating autophagy and the sensitivity of melanoma cells to dabrafenib still needs to be clearly defined.

In this study, first, we want to investigate whether dabrafenib can cause autophagy in two melanoma cell lines; second, whether this autophagy induced by dabrafenib is regulated by ER stress; and third, whether blocking ER stress and autophagy can increase the efficacy of dabrafenib in treating melanoma cells. We provide evidence that ER stress-induced autophagy protects the melanoma cells from the toxicity of dabrafenib and that blocking both ER stress and autophagy can enhance the drug efficacy in treating melanoma. These results provide us with a novel and more effective avenue of dabrafenib-based chemotherapy for melanoma.

Materials and methods

Cell lines and culture

The melanoma cell lines A375 and MEL624 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (Biomeda Corp., Foster City, CA, USA) and 1% penicillin/streptomycin/glutamine (ThermoFisher Scientific, Waltham, MA, USA). Cells were incubated in a humidified incubator with 5% CO_2 and 95% air at 37°C.

Antibodies and reagents

Dabrafenib, 3-methyladenosine, and 4-phenylbutyrate were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). The antibodies to phosphoprotein kinase RNA-like endoplasmic reticulum kinase (PERK), CHOP, inositol-requiring enzyme 1α, and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA); anti-LC3 I/II was purchased from Novus (St Louis, MO, USA); and anti-p62 was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA).

RNA interference

Small interfering RNA against PERK (Sigma) and non-target control small interfering RNA were transfected into cells by the Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions.

Western blotting

The methods have been described in other studies.[19,20]

Cell viability assay

The methods have been described in another study.[21] Cells were plated at a density of 5×10[3] cells/well in 96-well plates in 100 mL medium. After each treatment, cell viability was assessed by the Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) test according to the manufacturer's instructions.[22]

Statistical analysis

All data represented in this study are the mean values ± standard deviation of at least three separate experiments. *P*-values were calculated with the appropriate statistical tests using the GraphPad Prism software 7.0 (GraphPad Software, Inc, San Diego, CA, USA). A significant difference was considered to be present at $P<0.05$.

Results

Dabrafenib induces both autophagy and ER stress in a dose-dependent pattern in melanoma cells

Since autophagy can be induced by other B-Raf inhibitors in different types of cancers such as vemurafenib in nonmelanoma and B-Raf mutant colorectal cancers,[23,24] we wanted to identify whether dabrafenib can increase the level of autophagy in two human melanoma cell lines, A375 and MEL624. These two human melanoma cell lines are categorized as either B-Raf inhibitor sensitive (A375) or B-Raf inhibitor resistant (MEL624). We tried to determine the effect of dabrafenib in these different feature cell lines. We first tested the level of autophagy in melanoma cells after treatment with different concentrations of dabrafenib. We found a dose-dependent activation of autophagy via Western blotting analysis of LC3 I/II and p62 levels (Figure 1A and B). LC3 I/II levels were

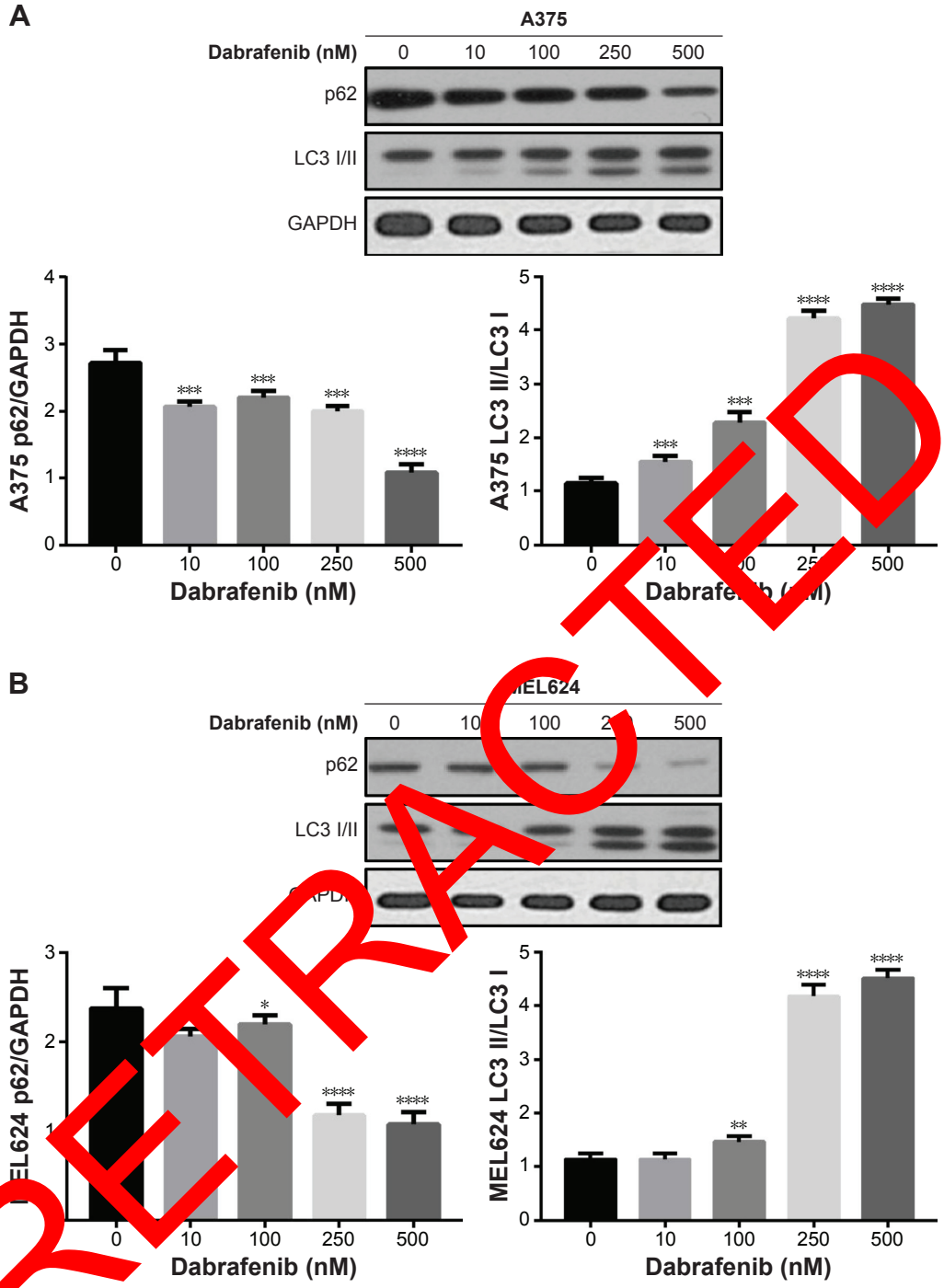

Figure 1 Dabrafenib induces autophagy in melanoma cell lines (A375 and MEL624).
Notes: Western blotting analysis of dabrafenib treatment on autophagy level of A375 (**A**) and MEL624 (**B**) cells. Both cell lines were treated with vehicle or the indicated concentrations of dabrafenib for 24 hours. At the end of the treatment, Western blotting analysis was done with antibodies specific for autophagy marker, LC3 I/II, p62, and GAPDH as indicated, respectively. GAPDH was used as a loading control. The data are presented as the mean ± standard deviation of at least three independent experiments (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$, Student's *t*-test).
Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

notably higher following exposure to dabrafenib for 24 hours. This was accompanied by a significant decrease in p62 level. We then assessed the dabrafenib-induced ER stress response in both A375 and MEL624 cells. We treated both melanoma cells with the indicated doses of dabrafenib, followed with a Western blotting assay (Figure 2A and B). As expected, the dabrafenib treatment of melanoma cells at different doses provided us with evidence that this drug can induce ER stress in a dose-dependent pattern as shown by our Western blotting analysis of ER stress markers (Figure 2A and B). Surprisingly,

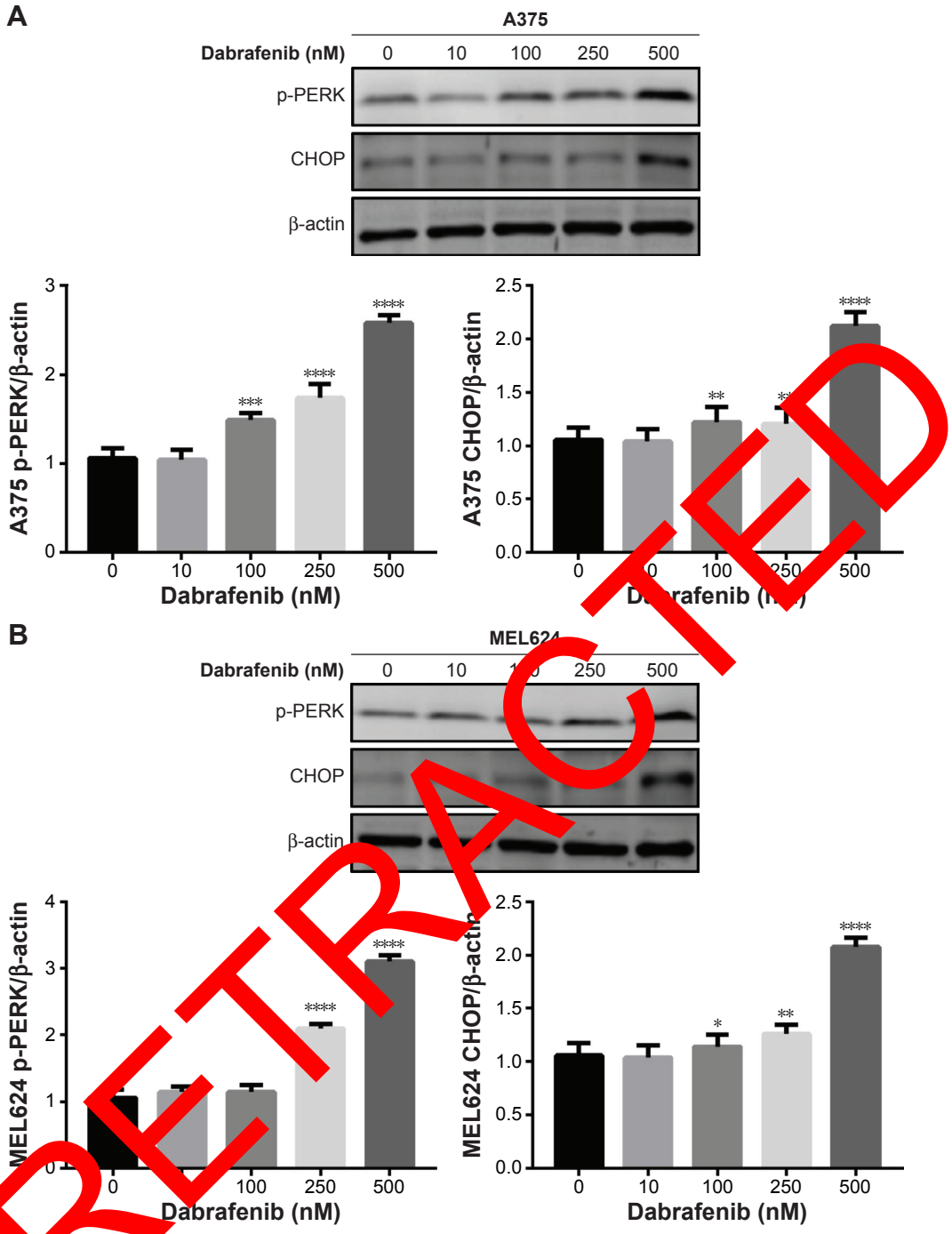

Figure 2 Dose-dependent effects of dabrafenib on ER stress of melanoma cells (A375 and MEL624).
Notes: Western blotting analysis of dabrafenib treatment on ER stress of A375 (**A**) and MEL624 (**B**) cells. Both cell lines were treated with vehicle or the indicated concentrations of dabrafenib for 24 hours. Western blotting analysis was done with antibodies specific for ER stress marker, phosphorylated (p-)PERK, CHOP, and β-actin as indicated, respectively. β-actin was used as a loading control. The data are presented as the mean ± standard deviation of at least three independent experiments with Student's *t*-test between two groups (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$, Student's *t*-test).
Abbreviations: PERK, phosphoprotein kinase RNA-like endoplasmic reticulum kinase; ER, endoplasmic reticulum.

similar results were observed in both cell lines except for the effective induction dose of autophagy and ER stress. Taken together, we verified that dabrafenib triggers both autophagy and ER stress in both B-Raf inhibitor-sensitive and B-Raf inhibitor-resistant melanoma cells.

The inhibition of ER stress regulates the autophagy-associated pathways in melanoma cells

To determine the effect of ER stress in the regulation of autophagy to dabrafenib treatment in melanoma cells, we

established procedures to reduce PERK protein levels by roughly 70% using small interfering RNA (Figure 3A). We then treated the transfected melanoma cells with dabrafenib (100 nM) for 24 hours. At the end of the treatment, cells were harvested and tested by Western blotting for ER stress and autophagy pathways. We have observed that the dabrafenib-stimulated increase in ER stress signaling is attenuated by PERK knockdown (Figure 3B and C). In addition, autophagy

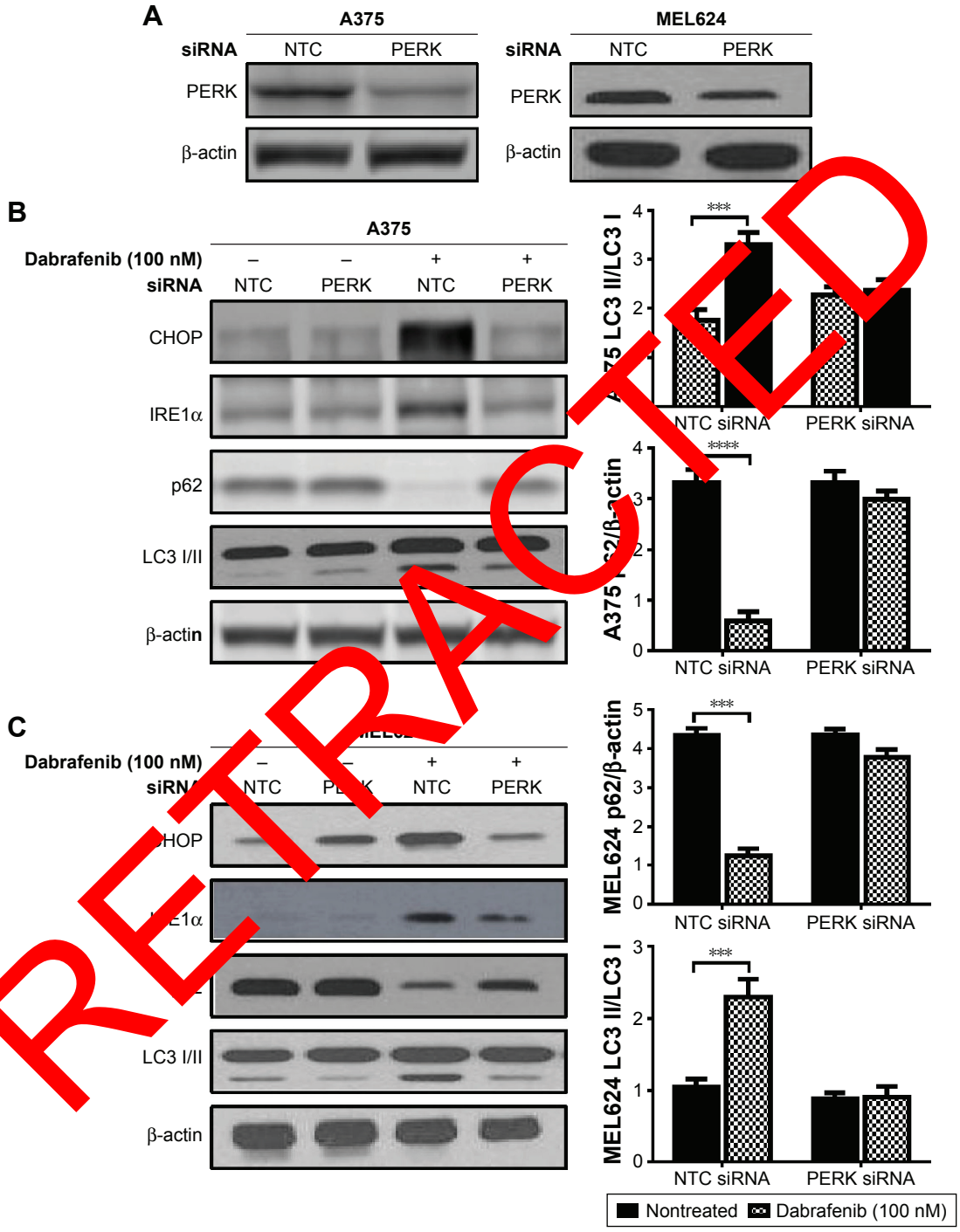

Figure 3 Silencing of PERK expression attenuates dabrafenib-induced autophagy.

Notes: (**A**) Treatment with PERK siRNA significantly reduces PERK protein levels in both A375 and MEL624 cells. A375 (**B**) and MEL624 (**C**) cells were transfected with a NTC siRNA or a siRNA targeting PERK for 48 hours, followed by the treatment of dabrafenib (100 nM) for 24 hours. At the end of treatment, whole cell lysates were prepared, resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and subjected to Western blotting analysis for CHOP, IRE1α, p62, LC3 I/II, and β-actin. β-actin was used as a loading control. The data are presented as the mean ± standard deviation of at least three independent experiments (***$P<0.001$, ****$P<0.0001$, two-way analysis of variance with Bonferroni correction).

Abbreviations: NTC, nontarget control; PERK, phosphoprotein kinase RNA-like endoplasmic reticulum kinase; siRNA, small interfering RNA.

signaling is also impaired by knocking down PERK, which supports our notion that dabrafenib-induced autophagy is regulated by ER stress (Figure 3B and C).

ER stress response plays a protective role and provides resistance to dabrafenib mediated cell death in melanoma

Based on the fact that dabrafenib induces ER stress response in melanoma cells, we then tried to determine whether ER stress induced by dabrafenib will protect the cancer cells from the cytotoxicity of the drug. We first treated the human melanoma cells A375 and MEL624 with vehicle or ER stress inhibitor, 4-phenylbutyrate (10 mM) for 1 hour, followed by the 24 hours treatment with or without dabrafenib (100 nM). We harvested and examined cell viability by the Cell Counting Kit-8 and found that the group treated with only dabrafenib was more resistant to the drug in comparison to the group that was cotreated with 4-phenylbutyrate. This is indicative that ER stress plays a protective role in melanoma cells exposed to the dabrafenib (Figure 4).

Autophagy induced by dabrafenib protects the melanoma cells from the cytotoxicity of dabrafenib

We treated the melanoma cell lines A375 and MEL624 with dabrafenib in the presence or absence of autophagy inhibitors, 3-methyladenosine, to determine if inhibition of autophagy induced by dabrafenib will affect the viability of melanoma cells to the drug. Both melanoma cells were treated with different concentrations of dabrafenib in the presence or absence of 3-methyladenosine (2 mM) for 24 hours. At the end of the treatment, we examined cell viability by the Cell Counting Kit-8 assay and verified that the dabrafenib group was more resistant to the treatment in comparison to the cotreatment group (Figure 5). This finding supports the protective role of autophagy in melanoma cells to dabrafenib treatment.

Discussion

In the last two decades a large number of chemotherapeutic drugs have been developed; however, melanoma in advanced stages still has poor prognosis and contributes to most of the mortality rate.[25,26] The high mortality rate is mostly due to ineffective chemotherapy and strong resistance of tumors. Dabrafenib, a B-Raf inhibitor, is a drug currently in use to treat melanoma associated with a B-Raf gene mutation that is critical in the regulation of cell growth. Clinical data have shown that single dabrafenib treatment for patients with an advanced stage B-Raf^{V600E} mutation melanoma is effective for 6–7 months after which resistance occurs.[27] Drug resistance is now a big issue in the treatment of melanoma and many pathways have already been associated with drug resistance. However, the specific mechanism of drug resistance to dabrafenib treatment is still in need of clarification.

Various anticancer reagents are known to induce autophagy and autophagy is widely known as an important process associated with the regulation of cancer development and progression. Whether autophagy in cancer therapy acts as a tumor promoter or suppressor is still controversial.

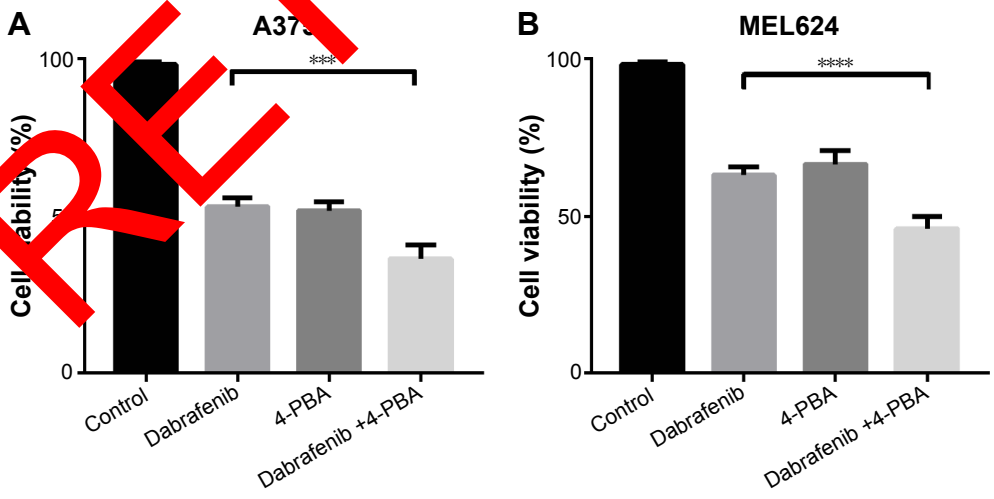

Figure 4 Inhibition of ER stress increases the sensitivity of melanoma cells to dabrafenib.
Notes: A375 (**A**) and MEL624 (**B**) cells were treated with 4-phenylbutyrate (10 mM) for 1 hour, followed by the treatment of vehicle or the indicated concentration of dabrafenib (100 nM) for 24 hours. At the end of treatment, cell viability was measured by the Cell Counting Kit-8. The data are presented as the mean ± standard deviation of at least three different independent experiments (***$P<0.001$, ****$P<0.0001$, Student's *t*-test).
Abbreviations: ER, endoplasmic reticulum; 4-PBA, 4-phenylbutyrate.

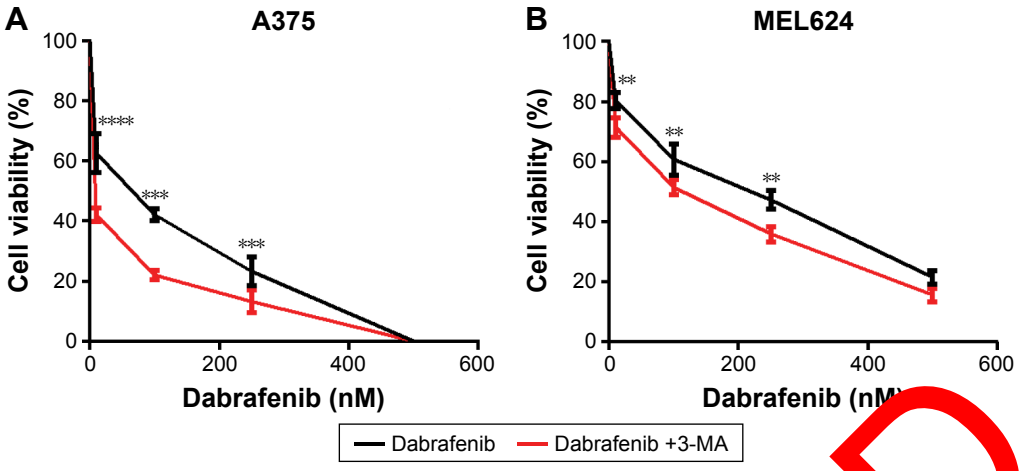

Figure 5 Targeting autophagy enhances the dabrafenib efficacy to melanoma cells.
Notes: A375 (**A**) and MEL624 (**B**) cells were treated with the indicated concentrations of dabrafenib for 24 hours in the presence or absence of 3-MA (2 mM). At the end of treatment, cell viability was measured by the Cell Counting Kit-8. The data are presented as the mean ± standard deviation of at least three independent experiments (***P*<0.01, ****P*<0.001, *****P*<0.0001, two-way analysis of variance with Bonferroni correction).
Abbreviation: 3-MA, 3-methyladenine.

Whereas in some studies of B-Raf mutant melanoma, the activation of autophagy has been associated with drug resistance and provides a critical nutrient supplement that aids in cell survival in melanoma,[28] In other studies, drug-induced autophagy was indicated to increase the level of senescence marker, which shows its role as a tumor suppresso... date, in dabrafenib-based chemotherapy the role of autophagy has not been well characterized. Our data demonstrated ... autophagy was induced by dabrafenib in a dose-dependent pattern and targeting dabrafenib-induced autophagy overcame the drug resistance in melanoma cells.

Emerging evidence suggests that ER stress contributes to the activation of autophagy related to anticancer drug resistance in many types of cancers.[21] However, less is known about the link between autophagy and ER stress in dabrafenib-induced drug resistance in melanoma. We have previously reported that autophagy modulates the sensitivity of colorectal cancer cells to oxaliplatin[21] and the activation of autophagy is related to drug-induced ER stress response (not published). In our study on melanoma, we found that dabrafenib can induce ER stress and further activate autophagy. The PERK signaling plays a critical role in this mechanism since PERK knockdown can largely impair the level of autophagy induced. Therefore, our current studies support that dabrafenib-induced ER stress can further activate autophagy and, therefore, provide multiple potential targets in the ER stress–autophagy link in melanoma treatment.

In conclusion, our studies support that both ER stress and autophagy are induced in the melanoma cells after the treatment with dabrafenib and ER stress-induced autophagy protects melanoma cells to dabrafenib. Moreover, inhibition of both ER stress and autophagy promote the sensitivity of melanoma cells to dabrafenib. These results provide us with promising evidence that the inhibition of autophagy and ER stress could have a therapeutic effect to the conventional dabrafenib chemotherapy. Further studies and clinical trials are needed to determine whether autophagy manipulation in B-Raf-mutant melanomas along with other anticancer drugs is beneficial for the patient.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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