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Coordinate autophagy and translation inhibition enhance cell death in melanoma

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Abstract: Melanoma treatments are necessary when surgically curable treatments are limited. The major challenge of targeted therapy for treating malignant melanoma is acquired drug resistance. Translation and autophagy pathways are interconnected and involved in developing cancer drug resistance.
We hypothesized that coordinate inhibition of autophagy and translation would lead to a better anticancer effect. In the present study, we used chloroquine combined with two translation inhibitors (NVP-BEZ235 and CGP57380) acting at different signaling pathway levels, activating the translation. Our study was conducted for human melanoma cell lines with similar genomic alteration (BRAFV600E and PTEN loss). The combination of the drugs suppresses cell invasiveness and growth by inducing apoptosis. We showed multiple direct and indirect interactions, indicating the overlap and interaction between the translation machinery and autophagy. These data suggest that coordinated inhibition of translation and autophagy promotes apoptosis and may be a new therapeutic model for melanoma treatment.

Keywords: mTOR, MNK, melanoma, eIF4E, eIF2α, chloroquine.

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Introduction

Accumulated genetic and epigenetic alterations activate signaling pathways critical for cell survival in cancer [1]. The progressive understanding of the molecular pathways in melanoma cells has enabled the development of multiple targeted therapies for unresectable melanoma. Frequently,

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one targeted therapy in melanoma activates other survival pathways, which develop an escape mechanism [2–5]. Autophagy is one of them known to play a complex role in cancer because of its dual role, i.e., suppressing or promoting tumorigenesis [6]. It has been shown that autophagy degrades tumor-promoting proteins, e.g., p62/SQSTM1, in addition to several other autophagic proteins (e.g., Beclin-1, UVRAG, or Bif-1) that can directly inhibit tumorigenesis [7]. Recycling intracellular elements can induce survival when autophagy is induced by agents that mimic starvation. Literature reports show that the consequences of defective autophagy in cancers essentially depend on the tumor development stage [8–10]. Inhibition of autophagy may increase the apoptotic efficiency of drugs by initiating self-destruction processes such as apoptosis (self-killing) or autophagy (self-eating). Both are regulated by their specific molecules (i.e., the autophagy regulators) that can act as apoptosis activators. Autophagy may also stimulate apoptosis by reducing endogenous inhibitors of this cell death pathway [11].

Melanoma is the most aggressive and drug-resistant form of skin cancer. In the early stages, it can be successfully cured with high survival rates with surgery, but after metastasis, there are no sufficient treatments, and survival rates decrease significantly. A high basal level of autophagy characterizes melanoma. Similarly to many cancers, autophagy is considered tumor-suppressive during the progression from benign to malignant state, but conversely, it becomes tumor-promoting in established melanoma [9, 12]. Approximately 50% and 20% of cutaneous melanoma have BRAF and N-RAS mutations, respectively. They lead to the activation of the MAPK signaling pathway. Also, the loss of tumor suppressor PTEN (phosphatase and tensin homolog on chromosome ten) correlates with melanoma aggressiveness. Mutated PTEN can lead to increased PI3K/AKT/mTOR pathway activity. Recently, we have shown that inhibiting the translation by PI3K/mTOR or MNK/eIF4 increases autophagy in melanoma cells [13].

Aggressive cancer with high levels of autophagy tends to develop resistance to the applied drug by using this process to escape from the induced stresses. Combining mTOR or MNK/eIF4 pathways inhibitors with simultaneous inhibition of autophagy seems to be an auspicious approach. Chloroquine (CQ), used in cancer therapy clinical trials [14], inhibits lysosomal hydrolase activity and, consequently, autophagy. We have previously shown that treating melanoma cells by CQ and silencing integrin-linked kinase has antitumor effects by reducing the translation of global and oncogenic proteins [15]. Our studies were conducted for human melanoma cells with genomic alteration BRAFV600E and PTEN loss. In the present study, we showed that coordinated autophagy and translation inhibition enhance cell death in melanoma.

Materials and Methods

Cell culture

Human melanoma cell lines with $BRAF^{V600E}$ and loss of PTEN used in this study were WM793 (from vertical growth phase, VGP) and 1205Lu (from lung metastatic). The latter was derived from a lung metastasis of WM793 cells after subcutaneous injection into immunodeficient mice. 1205Lu cells are highly invasive and exhibit spontaneous metastasis to the lung and liver. Cells were obtained from the ESTDAB Melanoma Cell Bank (Tubingen, Germany). All cell lines are tested for mycoplasma infection using the PCR method. Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The inhibitors were

added after 48 h in final concentration CGP5738 (10 μM), dactolisib (NVP-BEZ235) — (1 μM), and chloroquine — 50 μM, cells were incubated for 24 h before the analysis. DMSO was added to reference cells. The concentrations were selected based on our previous experience with CQ [15], CGP5738, and dactolisib (NVP-BEZ235) [13].

Proliferation

The proliferation of cells was assessed with the crystal violet test as previously described [16]; the absorbance, recorded at 450 nm, was measured for each sample by a microplate reader (BioKom Synergy, HTX). The assay was repeated thrice in duplicate.

Western blot analysis

Cell lysis, Western blot, detection, and visualization of blots were conducted as described elsewhere [17]. Primary antibodies used for analysis in dilution 1:1000 (from Cell Signaling Technology Inc) were: LC3A/B # 12741; AMPKα # 5832, AMPKα Y172 # 2535; ULK # 8054, ULK S757 # 6888, ULK S555 # 5869, 70S6K Y389 # 9234, pS6rp S235/236 # 4858, 4E-BP1 T37/46 # 2855, Akt S473 # 9271, eIF4E S209 # 9741, eIF2α S51 # 3398, Cyclin D1 # 2978, Cyclin D3 # 2936, p62/SQSTM1 # 8025, Caspase 3 # 9668, LaminA/C # 4777; from BD Transduction Laboratories (Akt # 610861) from Santa Cruz Biotechnology (70S6K sc-8418, eIF4E sc-9976, eIF2α sc-133132, 4EBP1 sc-9977, S6rp sc-74459), from Sigma (β-actin # A1978).

In vitro wound healing/migration assays

The *in vitro* wound healing assay was applied to evaluate the migration of melanoma cells after incubation with inhibitors combined with CQ, as previously described [13]. Culture media were used for Zymography analysis. Analyses of gelatinolytic activities of metalloproteinases MMP-2 and MMP-9 were described previously[18].

Mitochondrial membrane potential and reactive oxygen species production

Cells were plated at 2×10^4 cells/well in triplicate, in 24-well plates, and treated with CQ or CGP5738 or dactolisib or a combination of inhibitor and CQ the next day for 24 h. Rhodamine 123 (membrane-potential-dependent dye) was applied to monitor mitochondrial function. The mitochondria function generates a membrane potential that attracts and retains the dye. Loss of potential will result in loss of the dye and, therefore, the fluorescence intensity. Rhodamine was used as previously described [13].

Dihydrorhodamine 123 (DHR 123) is an uncharged and nonfluorescent indicator of reactive oxygen species (ROS), which is oxidized to cationic rhodamine 123 in the presence of reactive oxygen species. It is localized in the mitochondria and exhibits green fluorescence. DHR123 was prepared with a 1 mM stock solution in DMSO. Cells are loaded with DHR123 in the dark at a concentration of 10 μM in a culture medium without serum for 30 min at 37°C. After staining and rinsing the cells three times in PBS, fresh prewarmed media was added and measured on a plate reader (BioKom). The quantitative analysis measured the fluorescence of cells at excitation 485/20 nm/emission 528/20 nm, sensitivity 75.

Statistical analysis

Shapiro–Wilk W-test was applied to verify the normality distribution of each variable. Levene's test was applied to check the variance homogeneity. Statistical significance was obtained using one-way ANOVA, and Dunnett's post hoc comparison test was used to determine the difference between the samples and the control. Analyses were conducted using Statistica 13 software (StatSoft Inc., Tulsa, OK, USA). All results are presented as mean ± SD (notation: *p <0.05, **p <0.01, and ***p <0.001).

Results

Dactolisib and CGP5738 inhibit the survival of melanoma cells in combination with chloroquine

Previously, we found that PI3K/mTOR or MNK/eIF4E inhibitors effectively inhibited melanoma cell proliferation [13]. Chloroquine does not decrease the growth of melanoma cells in comparison to CGP5738 or dactolisib. The combination of CQ with dactolisib significantly reduced survival (about 38%) of metastatic cell lines 1205Lu and 28% in WM793 (Fig. 1A). The inhibitory effect of CQ and MNK/eIF4E axis is less visible, especially in VGP only 10% and 25% in the metastatic cell line. The expression of cyclins D_1 and D_3 is upregulated in melanoma and associated with a bad prognosis. For the studied melanoma cells, the expression of cyclin D_3 is downregulated after treatment with CQ in combination with CGP5738 or dactolisib. The latter is most efficient. The expression of cyclin D_1 is significantly lower in metastatic 1205Lu cells and has the most significant effect in the case of the combination of CQ with dactolisib. This combination also causes a decrease in cyclin D_1 expression in WM793 cells from vertical growth phases (Fig. 1B). In our previous research [13], we observed a slight upregulation of the expression of cyclin D_1 after treatment CGP5738 in WM793 cells, but in combination with CQ expression of D_1 is comparable to that in control cells. CQ alone does not change the expression of both cyclins in WM793 cells (Fig. 1B) but decreases significantly in metastatic 1205Lu cells. These results support the conclusion that metastatic melanoma depends more on autophagy than early-stage melanoma.

CQ together with inhibitors of PI3K/mTOR and MNK induces apoptosis

We previously reported that dactolisib or CGP57380 simultaneously induces autophagy and apoptosis in metastatic 1205Lu cells. In cells originating from the vertical growth phase, i.e., WM793 cells, autophagy induction is mainly caused by AMPK activation [13]. The basal level of autophagy is elevated in melanoma cells, and both signaling pathways, i.e., PI3K/mTOR and MNK/eIF4E, can regulate autophagy. Our previous research [13] showed that inhibiting these signaling pathways increased activity linked with autophagy in melanoma cells. Western blots of two canonical autophagy markers, i.e., LC3-II and p62/SQSTM1, examined its inhibition with CQ. The expression of LC3-II correlate with a number of autophagosomes and inversely with the expression of p62/SQSTM1. Expression of LC3-II undergoes degradation in autolysosomes when treating cells with CQ, which inhibits lysosomal activity, and arrests the latter step of autophagy, as observed by increased level of LC3-II and p62/SQSTM1. A combination of CQ with each of the inhibitors (dactolisib, CGP5738) was used slow down or stop autophagy (Fig. 2A). Apoptosis was observed as an increase the activity of caspase 3 and a decrease in the lamin A/C (Fig. 2B). Lamins are also

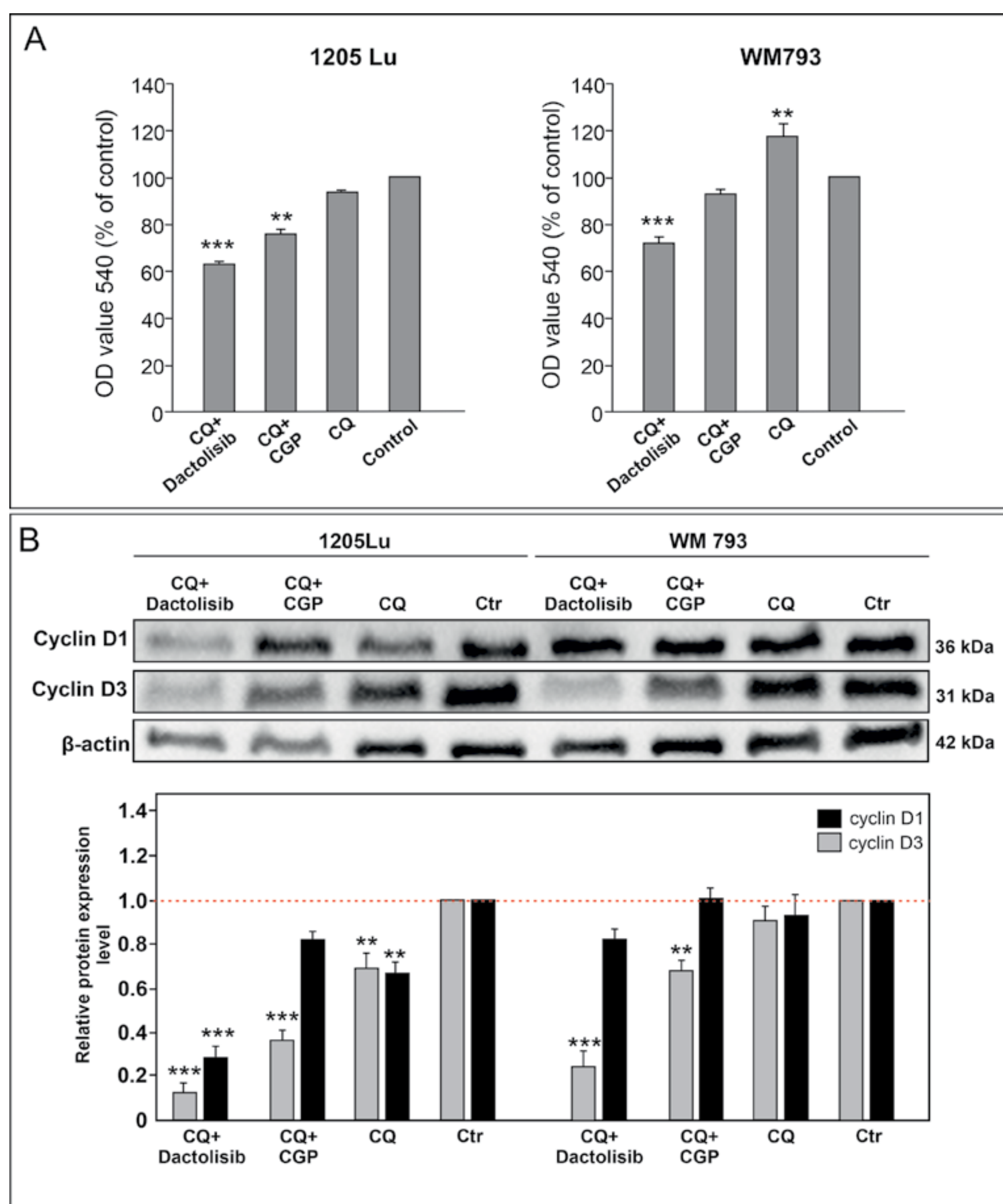

Fig. 1. The inhibitory effect of CGP5738 (CGP) or NVP-BEZ235 (dactolisib) in combination with chloroquine on the survival of melanoma cells. Values are expressed as mean ± standard deviation in 6 wells in three independent experiments; an asterisk (*) indicates a significant difference: *p <0.05, **p <0.01, ***p <0.001. (A) Cell proliferation was assessed with the crystal violet test. All results are presented as % of control (Ctr). (B) — the expression of cyclins detected by Western blotting. Representative blots and quantitative data representation after densitometry (mean ± SD) of three independent histogram experiments are displayed. Each data point was normalized against its corresponding β-actin data point. β-actin was used as a protein loading control.

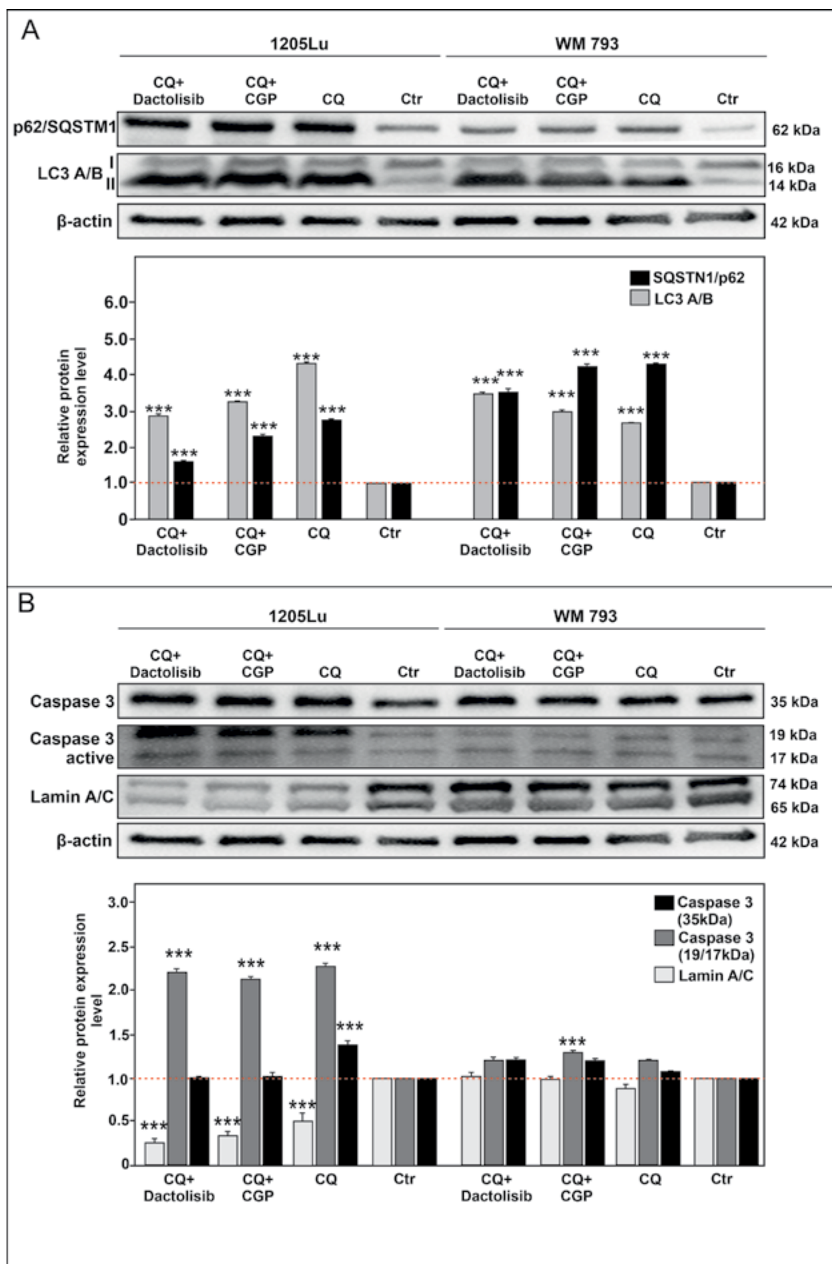

Fig. 2. Effect of PI3K/mTOR and Mnk inhibitors in combination with CQ on autophagy and apoptosis induction. The expression was determined by Western blotting of (A) — LC3I and-II, and p62/SQSTM1, (B) — caspase 3, both proteins, total and active, and Lamin A/C. Representative blots are displayed. The histograms quantitatively represent data after densitometry (mean ± SD) of three independent experiments. Each data point was normalized against its corresponding β-actin data point. β-actin was used as a protein loading control. Asterisks indicate significant differences from control cells. Values are denoted as *p <0.05, **p <0.01, and ***p <0.001.

apoptosis targets, and proteolytic degradation of lamin A/C is the results of activation of caspase-6. CQ alone induces apoptosis only in metastatic melanoma 1205Lu cells. Dactolisib or CGP57380, used in monotherapy, also causes apoptosis exclusively in the 1205Lu cells [13]. CQ, in combination with dactolisib or CGP57380, synergistically potentiates apoptotic effects of inhibitors in 1205Lu melanoma cell lines. Our results indicate that autophagy inhibition promotes apoptosis in metastatic melanoma cells, and a tendency toward apoptosis is also evident in the VGP stage.

Targeting the translational machinery in melanoma

Autophagy regulation affects the expression of proteins and thereby promotes or inhibits tumor growth. Both dactolisib and CGP57380 inhibited the pathways that regulate protein translation. CGP57380 inhibits the phosphorylation of eIF4E by MNK1/2, which reduces the translation of tumor-promoting proteins. The oncogenic BRAF mutation is responsible for higher MNK/eIF4E axis activity, especially in the studied melanoma cells. eIF4E was suggested to act as an oncogene, and the overexpression of eIF4E was observed in many tumors [19]. CQ used either alone but also especially in combination with dactolisib or CGP57380 decreased the basal level of eIF4E (Fig. 3A). Phosphorylation of eIF4E at the S209 site is the result of MNK kinase activity and plays a vital role in the cap-dependent translation. However, CQ alone reduces the level of eIF4E (S209) only in the VGP cells, i.e., in WM793 cells. However, the phosphoprotein to total protein ratio remains comparable to control cells. In metastatic 1205Lu cells, the phospho eIF4e level and the ratio of phosphoprotein to total protein remained elevated. CQ, in combination with both studied inhibitors, had inhibitory effects on the cap-dependent translation, showing a significant decrease in the basal level of eIF4E and phosphorylation of eIF4E in both studied melanoma cell lines. Cap-independent translation also plays an essential role in cancer. The selective translation of oncogene mRNAs is necessary not only for tumor growth, proliferation, and survival but also for global protein synthesis [20]. A primary mechanism of global translational control involves phosphorylation of the α-subunit of eIF2 on S51, which represses general translation initiation and paradoxically induces translation of protein involved in the integrated stress response [21]. An increase of phosphorylation of the eIF2α-subunit on S51 is visible for studied CQ combinations (Fig. 3B). However, the effect was not observed when dactolisib or CGP57380 were used in monotherapy [13]. Comparing the ratio of phospho- to total proteins, the synergistic effect of CQ with CGP57380 or dactolisib was observed. These results demonstrate that CQ combined with CGP57380 or dactolisib delivers better results in inhibiting oncoprotein and global protein translation.

Dactolisib, CGP57380, and CQ regulate the mTOR signaling pathway

Both studied melanoma cell lines show high PI3K/mTOR pathway activity due to mutations and attenuation of the PTEN phosphatase. A dual inhibitor of PI3K/mTOR, i.e., dactolisib causes decreased phosphorylation of downstream signaling molecules such ribosomal protein p70S6 kinase (p70S6K), eukaryotic translation initiation factor binding 4E-binding protein (4EBP1) and ribosomal protein 6 (S6rp) alone (what we described earlier [13]) and in the combination dactolisib with CQ also inhibits downstream signaling molecules in the studied melanomas (Fig. 4A).

Although CQ alone reduces levels of total protein and phosphorylated p70S6K, it maintains high levels of downstream mTOR signaling molecules, and in the case of 4EBP1, there is even an increase in total protein and phosphoprotein.

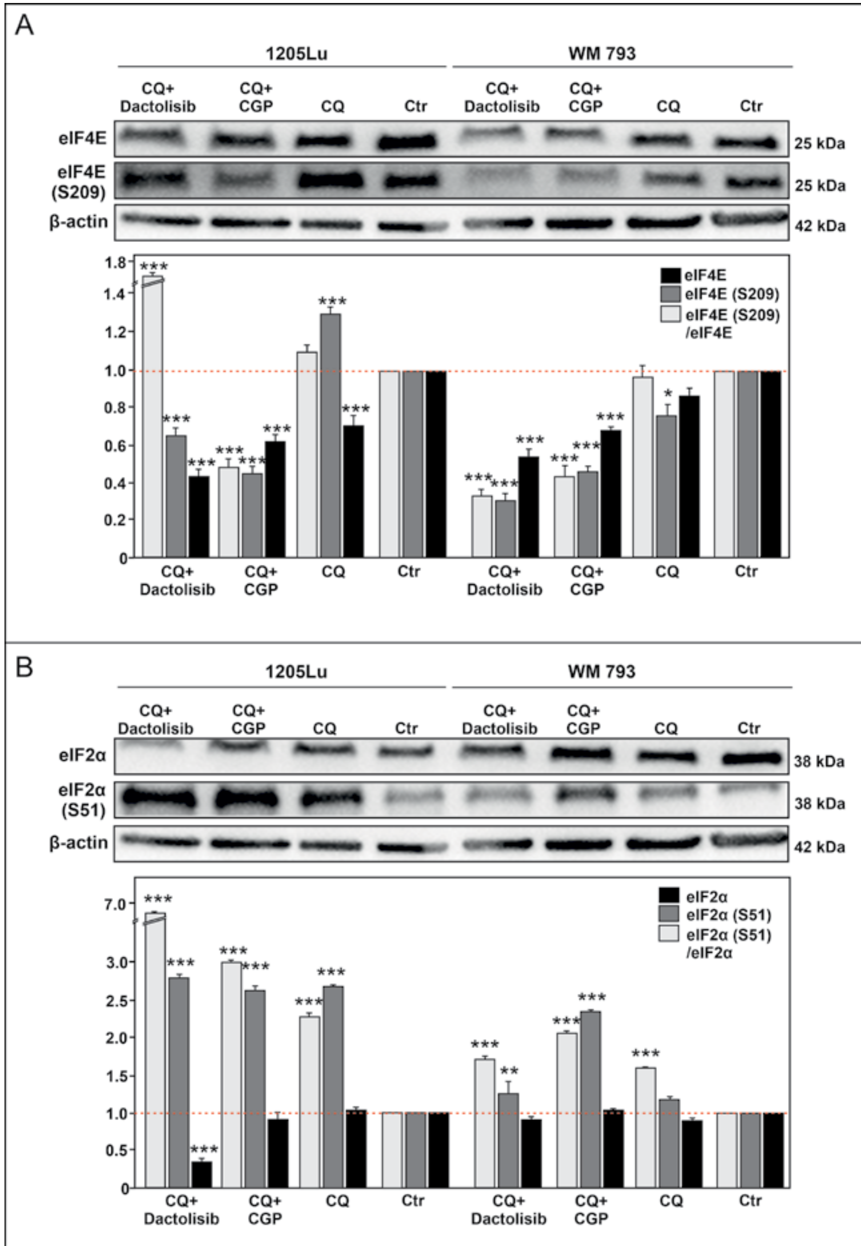

Fig. 3. The inhibitory effect on translation melanoma cells. The expression of phosphorylated and total proteins was determined by Western blotting, and representative blots were displayed. The histograms quantitatively represent data after densitometry (mean ± SD) of three independent experiments. Each data point was normalized against its corresponding β-actin data point. β-actin was used as a protein loading control. Asterisks indicate significant differences from control cells (Ctr). Values are denoted as *p <0.05, **p <0.01, and ***p <0.001. (A) — eukaryotic translational factor 4F (eIF4E), (B) — eukaryotic initiation translational factor eIF2α.

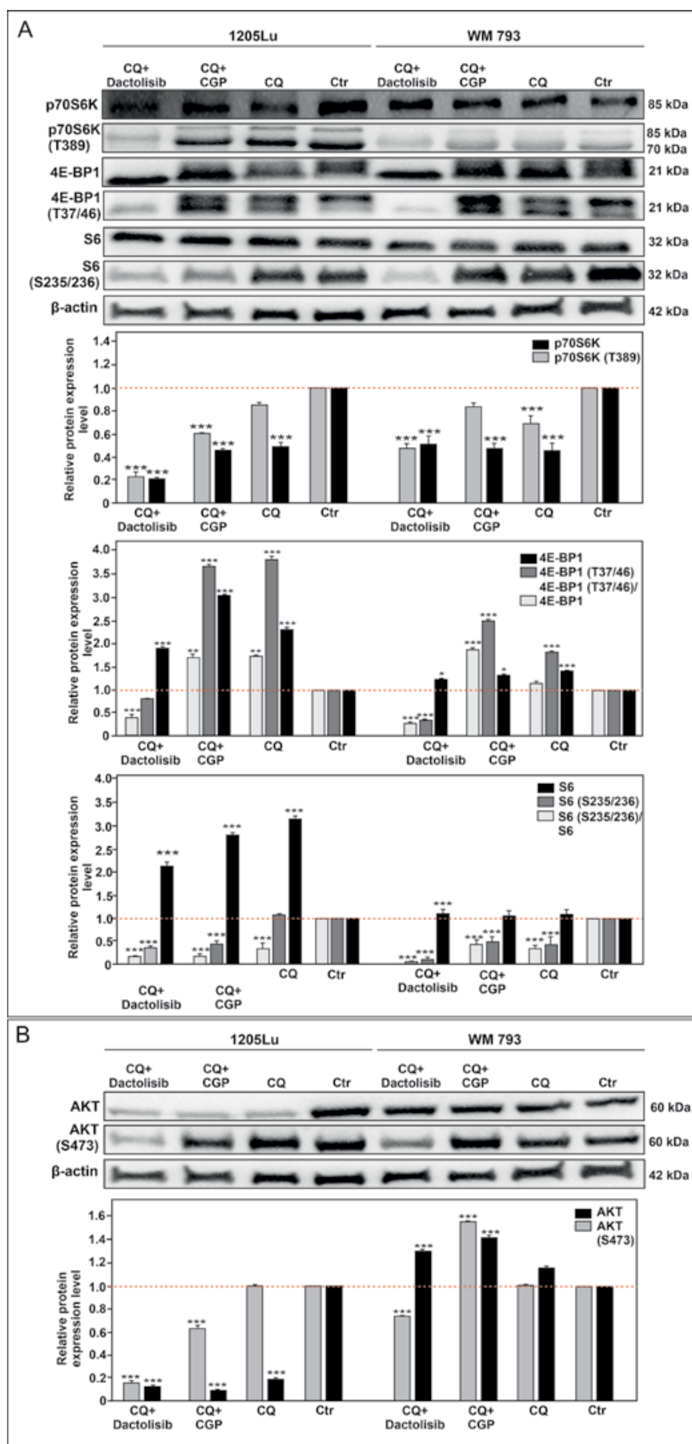

Fig. 4. The effect of upstream and downstream signaling molecules of mTOR pathways. Protein expression, both phosphorylated and total, was assessed by Western blotting. Representative blots are displayed. The histograms quantitatively represent data after densitometry (mean ± SD) of three independent experiments. Each data point was normalized against its corresponding β-actin data point. β-actin was used as a protein loading control. Asterisks indicate significant differences from control cells. Values are denoted as *p <0.05, **p <0.01, and ***p <0.001.

The phosphorylation level of p70S6 kinase and ribosomal proteins 6 are lower after treatment with CQ and CGP57380 than when CGP57380 was used alone [13]. We observed a synergistic effect when CGP57380 was combined with CQ. However, the phosphorylation of 4EBP1 and the level of total protein increased in combination with CGP57380 and CQ (Fig. 4A). PTEN loss is also responsible for a high activity of Akt monitored as a phosphorylation level on S473. Akt is an upstream element of mTORC1 and a downstream (target) molecule in the mTORC2 pathways. Inhibition of autophagy by CQ maintains high Akt activity in both cell lines. A decrease in the total amount of Akt in the metastatic 1205Lu melanoma line is observed in our study. The total protein to its phosphorylated form ratio is higher than in control (Fig. 4B). The results are consistent with our previous published study [15]. Dactolisib, a dual inhibitor of PI3K/mTOR, significantly inhibits the activity of Akt alone and also in combination with CQ in both melanoma cell lines (Fig. 4B). Combination CQ with CGP57380 maintains the high Akt activity in 1205Lu but also increases its activity in the WM793 cell line (Fig. 4B).

These data demonstrate that simultaneous inhibition of translation at various points in the signaling pathway (PI3K, mTORC1, and mTORC2) by dactolisib and autophagy by CQ produces a better result and does not induce potential drug resistance mechanisms.

The AMP-activated protein kinase and mTORC1 counteract the inhibition of autophagy by CQ

AMPK promotes autophagy as an energy sensor. AMPK is activated by phosphorylation at Thr 172, which regulates the activity of ULK1 by phosphorylation at S317, S777, and S555 for autophagy initiation. ULK1, a critical autophagy-initiating kinase, is regulated in a phosphorylation-dependent manner by AMPK and mTORC1 [22]. The active mTORC1 phosphorylates S757 and prevents ULK1 initiation of autophagy. ULK1 may be activated by AMPK only when mTORC1 phosphorylation of S757 is decreased. In our previous study, an increase in AMPK activation in vertical growth melanoma (WM793) was observed during the initiation of autophagy [13, 15]. The increased phosphorylation of AMPK was also visible after CQ treatment in WM793 cells [13]. CQ phosphorylates AMPK, and high AMPK activity in WM793 cells is observed in all combinations of CQ with the inhibitors used. Consequently, we see increased phosphorylation at ULK S555 (Fig. 5). AMPK activation appears to be a feedback mechanism of autophagy inhibition by CQ in the WM793 melanoma cells. In metastatic melanoma 1205Lu cells, we find a significant increase of phosphorylation on S757 in ULK1 due to mTORC1 activity after CQ treatment. Only in combination CQ with PI3K/mTOR inhibitor, dactolisib, phosphorylation on S757 drops significantly in both cell lines (Fig. 5B).

CQ by Akt/mTOR controls mitochondrial function

Mitochondria are key organelles for energy production and regulate cellular redox signaling pathways and programmed cell death [23]. To evaluate the effects of CQ and inhibitors on mitochondrial function, we checked the production of the mitochondrial membrane potential and reactive oxygen species (ROS). The interaction of ROS and autophagy plays an essential role in both cell damage and cell survival. A small quantity of ROS is necessary to maintain normal physiology in cells, but there is strong evidence that excessive generation of ROS is implicated in mitochondrial dysfunction [24]. In our study, CQ increased the normal mitochondria functionality and

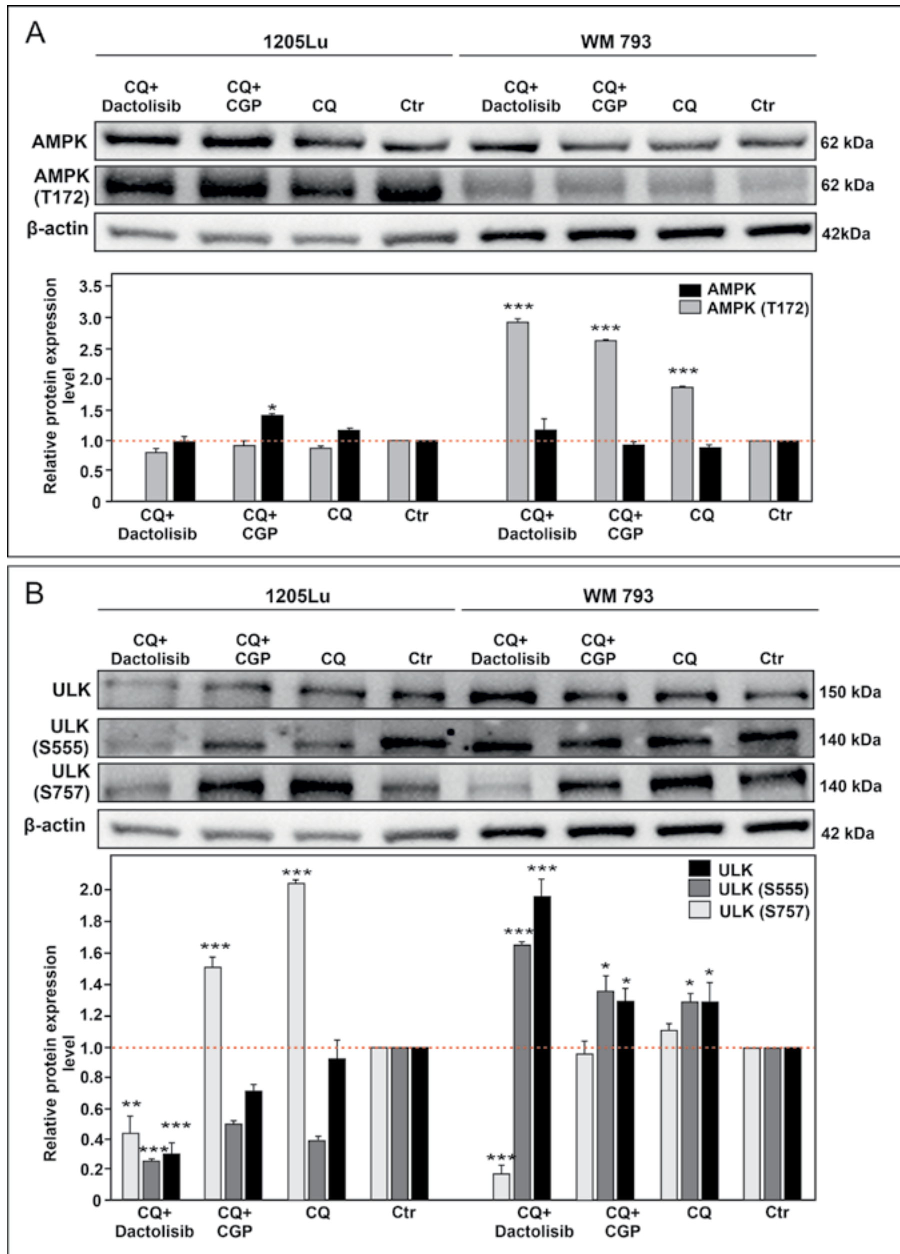

Fig. 5. AMPK and mTOR regulate autophagy through direct phosphorylation of ULK1. The expression of phosphorylated and total proteins was determined by Western blotting, and representative blots were displayed. The histograms quantitatively represent data after densitometry (mean ± SD) of three independent experiments. Each data point was normalized against its corresponding β-actin data point. β-actin was used as a protein loading control. Asterisks indicate significant differences from control cells (Ctr). Values are denoted as *p <0.05, **p <0.01, and ***p <0.001.

suppressed the generation of ROS in both cell lines. We observed a slight decrease mitochondrial membrane potential only in metastatic 1205Lu cells (Fig. 6A). The protective actions of CQ on mitochondrial membrane potential were also visible in its combination with dactolisib or CGP57380 when compared to the monotherapy. However, the generation of ROS in CQ in combination with inhibitors seems to increase (Fig. 6B).

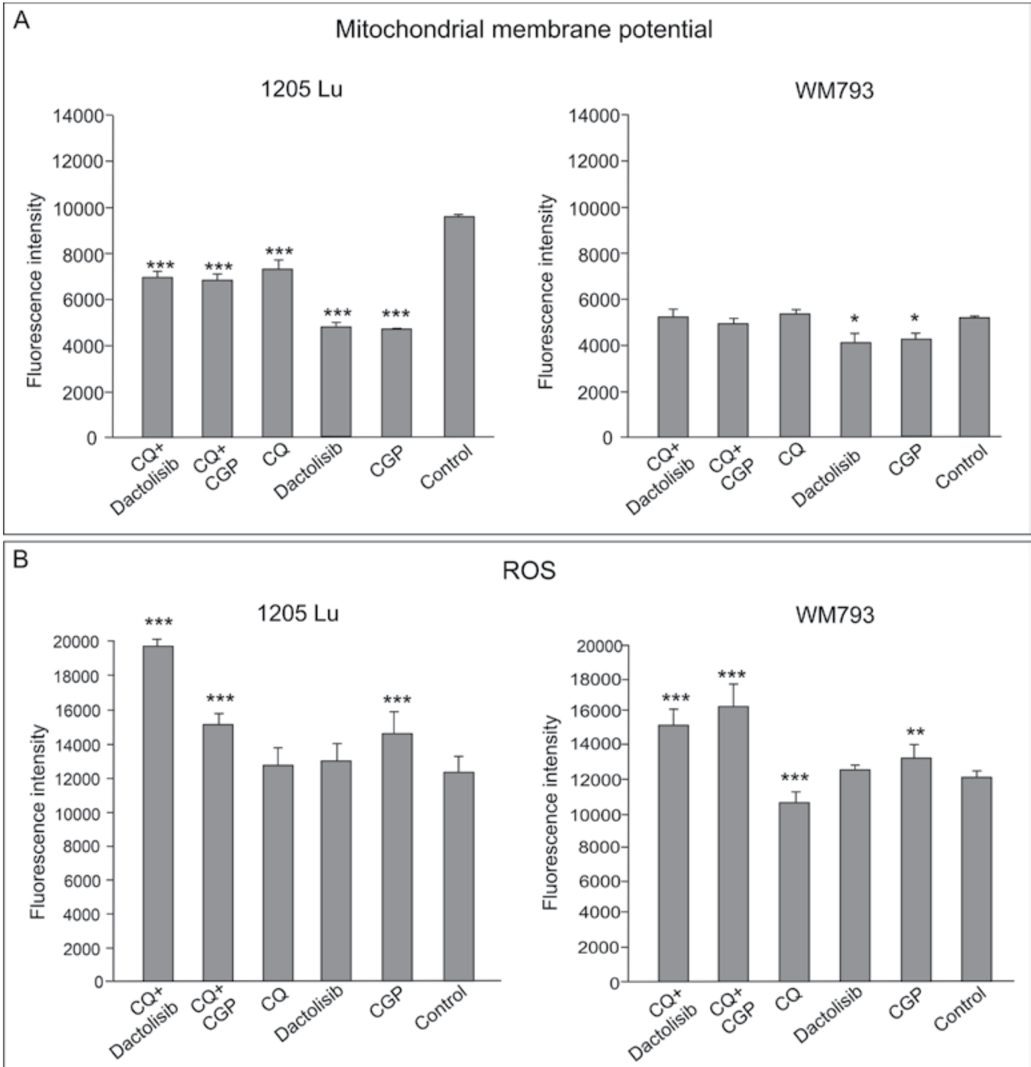

Fig. 6. Effect of combination CQ with CGP 5738 or dactolisib on mitochondrial condition. (A) — Mitochondrial membrane potential quantification using Rhodamine 123. (B) — Dihydrorhodamine 123 (DHR 123) is a reactive oxygen species (ROS) indicator. The quantitative analysis measured the 'sample's fluorescence at excitation 485/20 nm/ emission 528/20 nm, sensitivity 75.

Inhibition of autophagy and translation limits the invasiveness of melanoma cells

We performed a scratch wound healing assay and zymography to evaluate the effects of simultaneous inhibition of autophagy and translation on melanoma invasiveness. The scratch wound healing assay was used to evaluate the migration of cells (Fig. 7A, 7C). The invasive ability was verified by MMP activities tested in zymography (Fig. 7B, 7D). The results suggest that both invasive ability and migration decrease synergistically after inhibition of autophagy and translation. It seems autophagy is required for melanoma cell motility, as autophagy inhibition blocks the invasiveness of melanoma.

Discussion

Many anticancer agents can induce autophagy, and consequently, they can either suppress or promote cancer progression. We often find evidence in the literature that the optimal combination of autophagy inhibition with other conventional therapies can effectively improve their anticancer effects [7, 25–30]. Cancer often results from disrupting the interconnections between signaling pathways that regulate translation, autophagy, or apoptosis. Cancer cells depend on protein synthesis, and dysregulation of translational control is a common feature of all types of cancer [20]. We have previously described that inhibition of translation at various signaling points by dactolisib (NVP-BEZ235) or CGP57380 (an inhibitor of Mnk/eIF4E) induces autophagy in melanoma [13]. Here, we demonstrated that inhibition of autophagy and translation simultaneously trigger apoptosis. We previously identified that inhibition of the PI3K/mTOR pathway, a signaling cascade shared by both translation and autophagy, was insufficient to reduce the phosphorylation level of eIF4E protein on S209 [13]. eIF4E phosphorylation is important in promoting the expression of oncogenic proteins needed for the proliferation and survival of cancer cells [19]. But eIF4e can also regulate ATF4 (Activating Transcription Factor 4) binding to some promoters ATG (autophagy-related genes) like MAP1LC3B (gene coding LC3) and leading to autophagy induction [31]. Autophagy induced by mTOR inhibition may be a mechanism of death or tumor survival. Interestingly, dactolisib, in combination with chloroquine, has a powerful inhibitory effect on the phosphorylation of eIF4E on S209 in both melanomas studied here. Our work shows that simultaneous inhibition of the PI3K/mTOR pathway and autophagy reduce the risk of possible drug resistance. mTOR1 activity decreases the binding affinity of 4E-BP1(eukaryotic translation factor 4E-binding protein) to eIF4E and facilitates cap-dependent translation. Acevo-Rodríguez and colleagues described 4E-BP1 as a regulator of autophagy, which increased expression leads to a decrease in autophagy [31]. We show that CQ alone increased the expression of 4E-BP1 and phosphorylation. It is not elucidated what is needed to regulate autophagy. Whether the inhibitory role 4E-BP1 over autophagy results from its phosphorylation or from binding to eIF4E. Morita and colleagues have reported that 4E-BPs act as major mediators of the effects of mTORC1 on mitochondrial biogenesis and function [32]. The authors propose a feed-forward mechanism in which the translation of nucleus-encoded mitochondria-related mRNAs is modulated via the mTORC1/4E-BP/eIF4 pathway. CQ seems to contribute to increasing the normally functional mitochondria by inhibiting mitochondrial degradation and protective actions on mitochondrial membrane potential.

Translational control of autophagy also involves eIF2α subunits. Phosphorylation on S51 α-subunit eIF2 inhibits global protein synthesis while facilitating the translation of selected autophagy proteins [31]. The decrease in global protein translation was associated with reduced proliferation and expression of cyclins D.

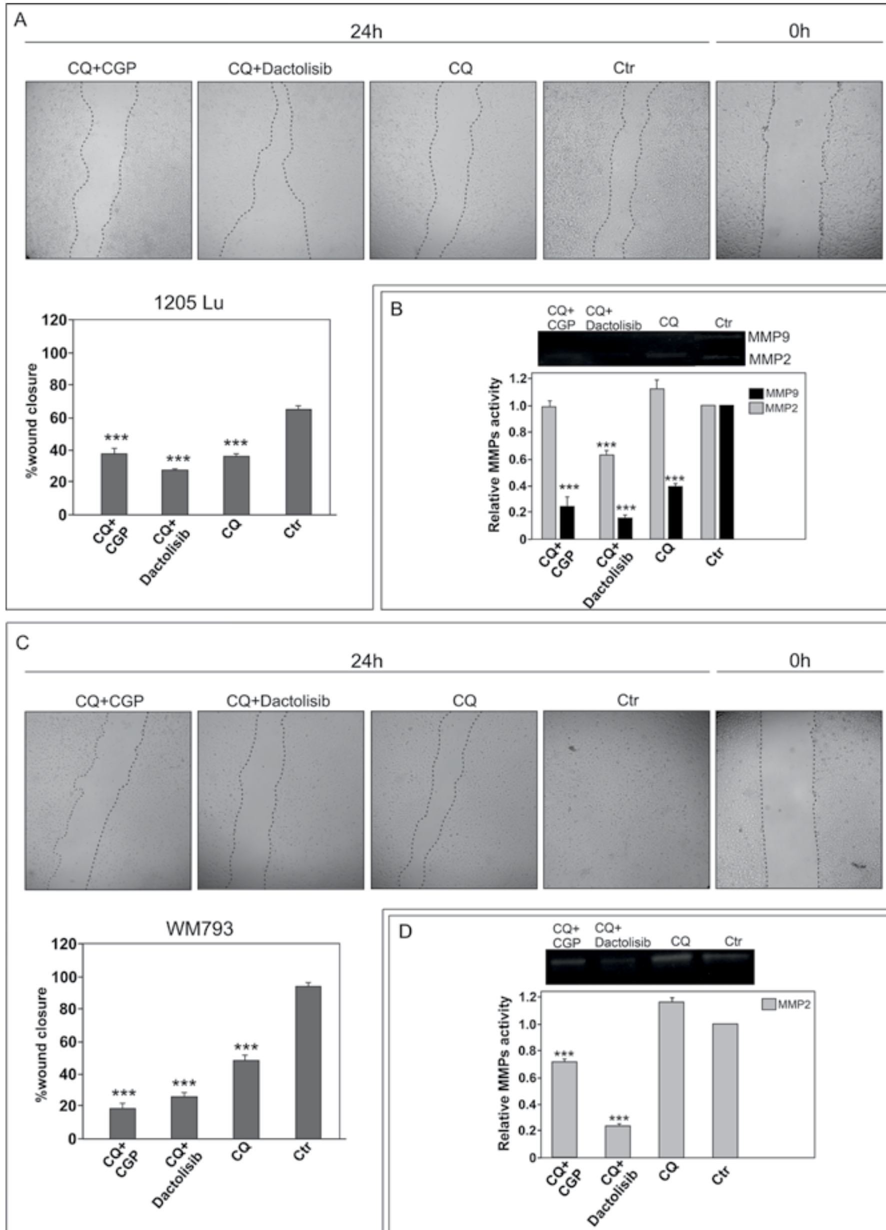

Fig. 7. Inhibition of autophagy and translation limits the invasiveness of melanoma cells. In vitro wound healing/migration assay, after 24 h, the confluent cell monolayer was wounded, and wound closure was captured by a digital camera connected to the inverted microscope. The assay was repeated thrice in duplicate. The wound closure area was measured using ImageJ and expressed as the percentage of wound closure. Asterisks indicate significant differences from control cells. The histograms are a quantitative representation of data (mean ± SD) from three independent experiments. Values are denoted as *p <0.05, **p <0.01, and ***p <0.001. MMP2 and MMP9 activities were shown as zymograms.

Inhibition of translation by different inhibitors in different ways produces different feedback effects when combined with CQ. The increase in Akt phosphorylation on serine in position 473, which is a target for mTORC2, may be a feedback mechanism to enhance cell survival after autophagy inhibition by CQ or inhibition of translation on eIF4E level by CGP57380. We did not observe Akt activation when we used dactolisib, which can inhibit translation simultaneously on different points of the signaling pathway (PI3K, mTORC1, and mTORC2).

Differences between melanoma lines (VGP WM793 and lung metastasis 1205Lu cells) with the same genetic background but different phenotypes may also explain the different regulation of pathways during treatment with the same drugs. However, the final response to the treatment is comparable in both cell lines. The observed increase in Akt activity after treatment with CQ alone or in combination with CGP57380 in the metastatic 1205Lu cells may be the reason for the increase in ULK phosphorylation on S757 mediated by mTORC1. Increased AMPK activation in vertical growth melanoma (WM793 cells) after CQ treatment negatively regulates the activity of the mTORC1, which is visible as increased phosphorylation at S555 in ULK and decreased phosphorylation at S757 in ULK. Our results are consistent with Panwar and colleagues' observation that AMPK negatively regulates the activity of the mTORC1 [33]. Additionally, AMPK and Akt can carry out counteracting actions on each other [34].

Although various upstream signals control autophagy and apoptosis, these processes also cross-regulate each other, mainly in an inhibitory manner. Autophagy decreases the likelihood of cells undergoing apoptosis, and the initiation of the apoptotic program is associated with the inhibition of autophagy [35]. Autophagy mediates cell death as an effector mechanism in a process known as autophagic cell death, blocking basal autophagy and promoting apoptosis. Numerous direct and indirect interactions have been identified, indicating a mechanistic overlap and interplay between the apoptosis machinery and autophagy proteins. The autophagy protein in autophagy-apoptosis interactions is p62 (p62/SQSTM1), a protein essential for melanoma progression [36]. p62 is essential for the selective autophagic degradation of various proteins and mitochondria. It directly interacts with several proteins involved in apoptotic and survival pathways, such as Caspase-8, TRAF6 (which modulates NF-kB survival pathways), and ERK [35]. Degradation of p62 as autolysosome cargo during autophagy affects apoptosis. Inhibition of autophagy by CQ stopped p62 degradation and allowed for interaction with apoptotic proteins. Modulating autophagy by pharmacological agents has become an attractive strategy for cancer treatment [10, 37, 38]. Our data show that CQ promotes apoptosis by inhibiting autophagy. Accumulation of autophagosomes apparently activates apoptosis. Another cause of apoptosis induction described in the literature may be the regulation of mitochondrial dysfunction [29]. Autophagy impairs mitochondrial functional capacity when mitochondrial permeability is increased.

The observed reduction in mitochondrial membrane potential may increase mitochondrial permeability after treatment of translation inhibitors, i.e., dactolisib or CGP57380. A reduction in mitochondrial capacity is causally linked to a shortened lifespan. Inhibiting autophagy restores lifespan by improving mitochondrial function [24]. The result is consistent with the findings from our study. We also observed that CQ did not lower mitochondrial membrane potential significantly and did not increase ROS generation. In our data, CQ also increased the expression of 4E-BP1 protein, which is essential for mitochondrial biogenesis and function. However, inhibiting autophagy results in no increase in the overall oxidative capacity of the mitochondria and thus can no longer restore the expected lifespan. It begins to cause apoptosis [24]. We also saw that CQ, combined with inhibitors, increased ROS production. Our observations confirm that inhibition of autophagy with an increase

in ROS production and a decrease in potential further increases ROS production and causes apoptosis. However, we have not sufficiently explained this aspect because it requires separate research.

The combined inhibition of translation and autophagy is superior to inhibiting only translation alone to trigger apoptosis. Inhibitors in cancer treatment often limit cell growth through autophagy and subsequent apoptosis, similar to dactolisib or CGP57380.

For autophagy to promote apoptosis, it is necessary to provide a mechanism to turn off autophagy, which allows the cell to decide to undergo apoptosis (Fig. 8).

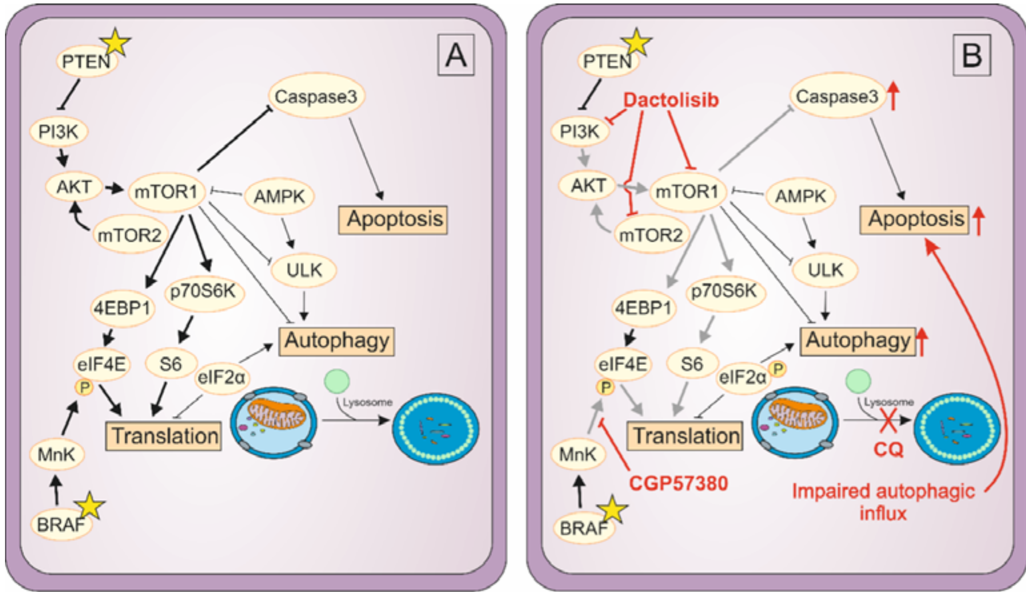

Fig. 8. Schematic representation of signaling pathways in tested melanoma cells. A. Mechanism of translation regulation and autophagy. B. Combining translation inhibitors (dactolisib and CGP57380) and chloroquine–autophagy inhibitors.

Conclusion

In summary, our study demonstrated that enhancement of autophagy by translation inhibition and simultaneous its inhibition by chloroquine may lead to apoptosis. We also showed multiple direct and indirect interactions, confirming the overlap and interaction between the translation machinery and autophagy. Our data suggest that coordinated inhibition of translation and autophagy promotes apoptosis and maybe a new therapeutic model for melanoma treatment.

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

D.G. conceived the original idea, designed the study, performed the experiments, and wrote the manuscript; M.Z. performed the graphical abstract and statistical data analysis and helped prepare figures; M.L. reviewed and edited. All authors have read and approved the final version of the manuscript.

Ethical approval

This article does not contain any studies with human participants or animals performed by any authors.

Data availability

Data will be made available on request.

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