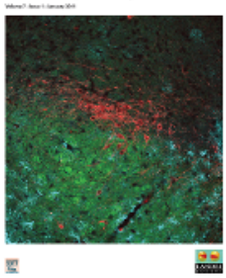
Autophagy



Autophagy

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Compound C induces protective autophagy in cancer cells through AMPK inhibition-independent blockade of Akt/mTOR pathway

Ljubica Vucicevic,[1,2] Maja Misirkic,[1,2] Kristina Janjetovic,[1,2] Urosh Vilimanovich,[3] Emina Sudar,[4] Esma Isenovic,[4] Marko Prica,[1] Ljubica Harhaji-Trajkovic,[2] Tamara Kravic-Stevovic,[3] Vladimir Bumbasirevic[3] and Vladimir Trajkovic[1,*]

[1]Institute of Microbiology and Immunology; [3]Institute of Histology and Embryology; School of Medicine; [2]Institute for Biological Research "Sinisa Stankovic"; [4]Laboratory for Molecular Genetics and Radiobiology; Vinca Institute of Nuclear Sciences; University of Belgrade; Belgrade, Serbia

Key words: Akt, AMPK, apoptosis, autophagy, cancer, compound C, mTOR

Abbreviations: AMPK, adenosine monophosphate-activated protein kinase; ANOVA, analysis of variance; LDH, lactate dehydrogenase; mTOR, mammalian target of rapamycin; AICAR, aminoimidazole carboxamide ribonucleotide; DEBC, 10-DEBC hydrochloride; p70S6K, p70S6 kinase; PI3K, phosphoinositide 3-kinase; siRNA, small interfering RNA; TEM, transmission electron microscopy; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand

In the present study, we report that compound C, an inhibitor of a key intracellular energy sensor AMP-activated protein kinase (AMPK), can induce autophagy in cancer cells. The induction of autophagy in U251 human glioma cell line was demonstrated by acridine orange staining of intracellular acidic vesicles, Beclin 1 induction, p62 decrease and conversion of LC3-I to autophagosome-associated LC3-II in the presence of proteolysis inhibitors. The presence of autophagosome-like vesicles was confirmed by transmission electron microscopy. Compound C-mediated inhibition of AMPK and raptor in U251 cells was associated with paradoxical decrease in phosphorylation of AMPK/raptor-repressed mTOR, a major negative regulator of autophagy, and its downstream target p70S6K. The phosphorylation of an mTOR activator Akt and the PI3K-activating kinase Src was also impaired in compound C-treated cells. The siRNA-mediated AMPK silencing did not reduce the activity of the Akt/mTOR/p70S6K pathway and AMPK activators metformin and AICAR failed to block compound C-induced autophagy. Autophagy inhibitors bafilomycin and chloroquine significantly increased the cytotoxicity of compound C towards U251 cells, as confirmed by increase in lactate dehydrogenase release, DNA fragmentation and caspase-3 activation. Similar effects of compound C were also observed in C6 rat glioma, L929 mouse fibrosarcoma and B16 mouse melanoma cell lines. Since compound C has previously been reported to suppress AMPK-dependent autophagy in different cell types, our findings suggest that the effects of compound C on autophagy might be dose-, cell type- and/or context-dependent. By demonstrating the ability of compound C to induce autophagic response in cancer cells via AMPK inhibition-independent downregulation of Akt/mTOR pathway, our results warrant caution when using compound C to inhibit AMPK-dependent cellular responses, but also support further exploration of compound C and related molecules as potential anticancer agents.

Introduction

Macroautophagy (referred to hereafter as autophagy) is a self-cannibalization process involving sequestration of cell structures in double-membraned organelles, called autophagosomes.[1] This is followed by fusion of autophagosomes with lysosomes and formation of autolysosomes in which internal content is degraded by acidic lysosomal hydrolases. The physiological role of autophagy is to remove long-lived proteins and damaged organelles, but when it is extensive, activated inappropriately or in cells which are unable to die by apoptosis, autophagy acts as an alternative cell-death pathway called programmed cell death type II.[2,3] Induction of autophagy by hypoxia or anticancer agents such as arsenic trioxide, temozolomide or fenretinide, appears to be deleterious for tumor cells[4-7] and autophagy-deficient mice are more susceptible to tumorigenesis.[8] Accordingly, autophagy has been proposed as a "magic bullet" in fighting apoptosis-resistant cancers.[9] On the other hand, a large body of evidence indicates that autophagy can also act as a survival mechanism that provides energy during metabolic stress and protects cancer

*Correspondence to: Vladimir Trajkovic; Email: vtrajkovic@eunet.rs
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cells from apoptotic or necrotic cell death induced by various anticancer treatments.[10]

The serine/threonine kinase mammalian target of rapamycin (mTOR) is a major negative regulator of autophagy.[11,12] The phosphoinositide 3-kinase (PI3K)-activated serine/threonine kinase Akt phosphorylates the mTOR repressor tuberous sclerosis complex 2,[13] thus leading to activation of mTOR and subsequent blockade of expression and function of autophagy-inducing Atg proteins.[11,12] In addition to Akt, one of the main mTOR regulators is AMP-activated protein kinase (AMPK), a principal energy-sensing intracellular enzyme activated in various cellular and environmental stress conditions. In response to increase in AMP/ATP ratio, AMPK preserves energy by switching off ATP-requiring processes, while switching on ATP-generating catabolic pathways.[14] AMPK maintains energy homeostasis by inducing autophagy and blocking protein synthesis and cell proliferation mainly through phosphorylation of its downstream target raptor and consequent inhibition of mTOR.[15] In addition to a major role of AMPK/mTOR pathway in regulation of intracellular and whole body metabolism, recent findings point to its potential involvement in controlling proliferation, survival and death of cancer cells.[16] Namely, pharmacological activation or overexpression of AMPK in a variety of cancer cell types caused an mTOR inhibition-associated cell cycle arrest and apoptotic death both in vitro and in vivo.[17-20] On the other hand, AMPK activity in certain conditions protected normal and cancer cells from metabolic stress and/or chemotherapy-induced apoptosis.[21-24] Autophagic digestion of intracellular proteins triggered by mTOR-down-regulation in tumor cells seems to play an important role both in AMPK-mediated cytotoxicity[25,26] and cytoprotection,[27,28] the final outcome possibly depending on the nature of the cytotoxic stimulus.

Compound C (6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine), also known as dorsomorphin, has recently been described as a pharmacological AMPK inhibitor that efficiently blocks metabolic actions of AMPK.[29] In addition, compound C inhibited pro-apoptotic and anti-apoptotic actions of AMPK in various experimental settings[18,24,25,30-36] and blocked AMPK-dependent autophagy in different types of normal and cancer cells.[25,37-39] In the present study, contrary to expectations, we demonstrate for the first time the ability of compound C to induce autophagic response in different cancer cell lines. The observed effect was independent of AMPK inhibition and mediated through suppression of Akt and subsequent down-regulation of mTOR, ultimately leading to autophagy-dependent interference with compound C-induced apoptosis.

Results

Compound C induces autophagy in U251 glioma cells. In preliminary experiments, we have observed that treatment of U251 glioma cells with compound C led to accumulation of acridine orange-stained orange-red acidic vesicles resembling autolysosomes (**Fig. 1A**, upper part), resulting in the increase in cellular red fluorescence measured by flow cytometry (**Fig. 1A**, lower part). The quantitative analysis of acridine orange-stained U251 cells confirmed that compound C caused both dose- and time-dependent increase in red/green (FL3/FL1) fluorescence ratio (**Fig. 1B**). Ultrastructural TEM analysis (**Fig. 1C**) was consistent with the induction of autophagy by compound C, showing an extensive cytoplasmic vacuolization (middle part) with many autolysosome-like vesicles containing cellular debris (right part). An immunoblot analysis revealed a time-dependent conversion of microtubule-associated protein 1 light-chain 3 (LC3)-I isoform to autophagosome-associated LC3-II, as well as an increase in the expression of the pro-autophagic protein Beclin 1 in compound C-treated U251 cells (**Fig. 1D**). However, Beclin 1 increase is not specific for autophagy induction, as it can also play a role in apoptosis,[40] and the increase in LC3-II may also occur as a consequence of reduced autophagic proteolysis.[41] Therefore, we assessed the influence of compound C on LC3-II levels in the presence of lysosomal acidification blockers that inhibit proteolysis: bafilomycin A1, chloroquine and ammonium chloride. The concentrations of proteolysis inhibitors saturating for LC3-II increase were determined in preliminary experiments (data not shown). While proteolysis inhibition expectedly increased LC3-II levels in U251 cells, treatment with compound C additionally enhanced LC3 conversion (**Fig. 1E**). These findings indicate that compound C increased autophagic flux, rather than merely prevented autophagic proteolysis in U251 glioma cells. Accordingly, treatment with compound C reduced the levels of p62 (**Fig. 1D**), a protein selectively degraded by autophagy.[42] Similar results were obtained in cell culture medium without glutamine (data not shown), thus eliminating the possibility that autophagy induction was mediated by ammonia derived from glutaminolysis.[43]

Compound C-mediated autophagy is associated with down-regulation of AMPK and Akt/mTOR signaling. To get some insight into the molecular mechanisms of compound C-mediated autophagy induction in U251 cells, we analyzed the activation status of the key members of autophagy-related AMPK/mTOR and Akt/mTOR pathways. As expected, compound C caused a potent downregulation of AMPK activation, almost completely blocking its phosphorylation after 1 h, which was accordingly accompanied by a marked reduction in phosphorylation of the AMPK target raptor (**Fig. 2A**). However, contrary to the role of raptor in mTOR inhibition, phosphorylation of mTOR, as well as that of the direct mTOR downstream target p70S6K, was also reduced by compound C (**Fig. 2A**). The discrepancy between the activation status of AMPK/raptor on one side and mTOR/p70S6K on the other side prompted us to assess the influence of compound C on Akt, a well-known mTOR activator. Indeed, compound C in a time-dependent manner reduced the phosphorylation of Akt, which was almost completely abolished after 18 h of treatment (**Fig. 2A**). It should be noted that the apparent rebound and mTOR activation at the 18th hour was not associated with the increase of S6K and Akt phosphorylation, which is consistent with the fact that mTOR and S6K might also be regulated independently of Akt and mTOR, respectively.[44,45] Interestingly, the phosphorylation of tyrosine kinase Src at Y418 (Y424 in mouse), which triggers PI3K activation and subsequent phosphorylation of Akt,[46] was also impaired in compound C-exposed cells (**Fig. 2A**). Treatment with Akt inhibitor

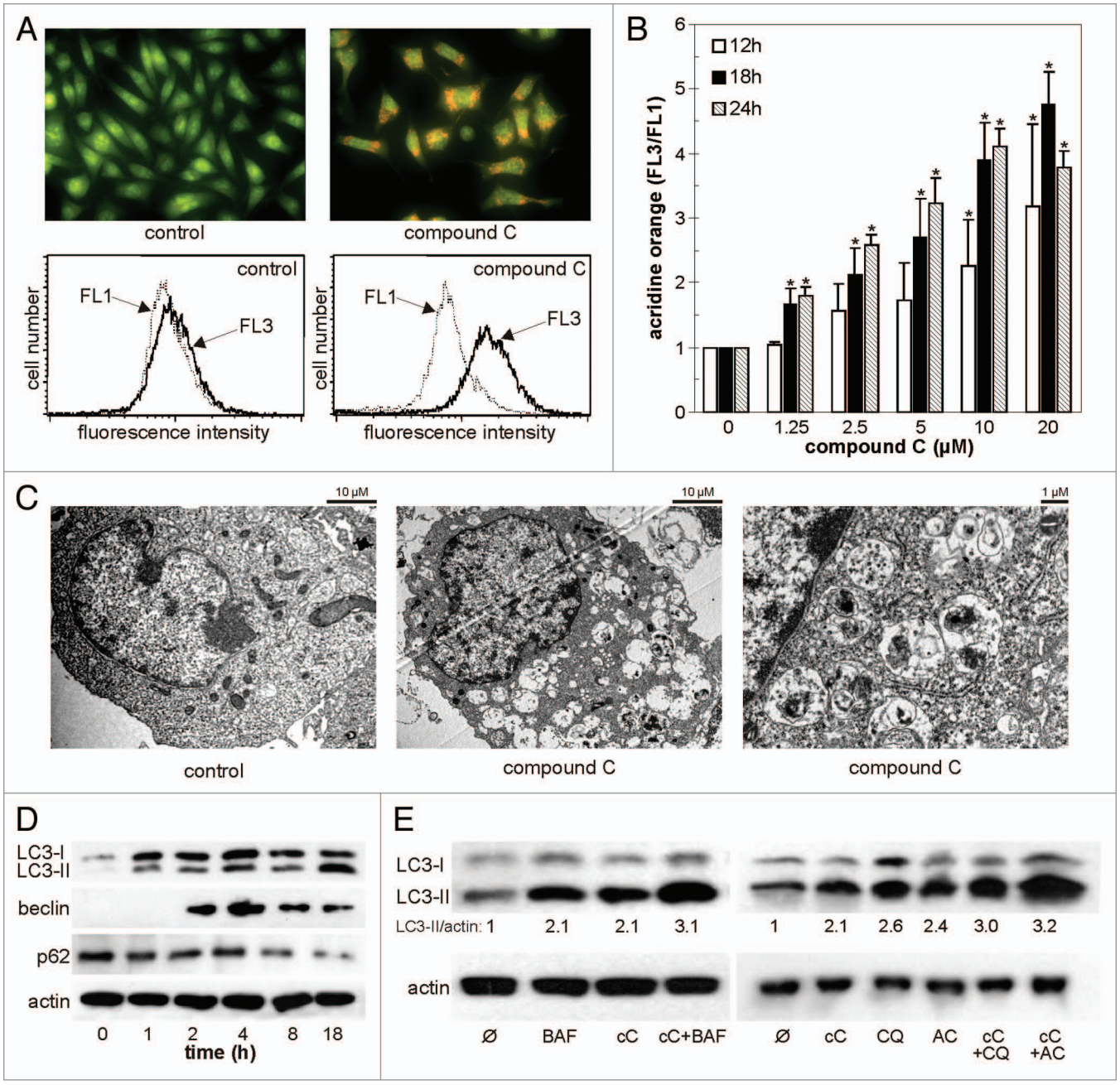

Figure 1. Compound C induces autophagy in U251 glioma cells. (A and B) U251 cells were incubated with or without 10 μM of compound C for 24 h (A) or different concentrations of compound C for the indicated time periods (B). The presence of acridine orange-stained intracellular vesicles was demonstrated by fluorescent microscopy (A; upper panel) and flow cytometry, showing an increase in red-to-green (FL3/FL1) fluorescence intensity (A; lower panel). (B) The autophagy was quantified by calculating FL3/FL1 geomean fluorescence ratio (the data are mean + SD values from three experiments, *$p < 0.05$). (C) The TEM analysis of the compound C-treated cells (10 μM, 24 h) showing intensive vacuolization (middle panel) and autophagolysosome-like vesicles in which cellular content is degraded (right panel). (D and E) Immunoblot analysis of (D) LC3 conversion, beclin-1 and p62 levels in U251 cells treated with compound C (10 μM) for the indicated times or with (E) compound C (10 μM) and/or proteolysis inhibitors bafilomycin A1 (BAF; 50 nm), chloroquine (CQ; 50 μM) and amonium chloride (AC; 25 mM) for 6 h (the results from one of two experiments with similar results are shown).

10-DEBC hydrochloride, PI3 kinase/Akt inhibitor LY294002 or mTOR inhibitor rapamycin mimicked the compound C-induced increase in acridine orange red/green fluorescence ratio in U251 cells (**Fig. 2B**). Thus, it appears that downregulation of Src/Akt/mTOR pathway might be responsible for compound C-mediated autophagy in U251 glioma cells.

AMPK inhibition is not involved in compound C-induced Akt/mTOR blockade and autophagy. To investigate if the AMPK inhibition was involved in the observed blockade of Akt/mTOR signaling by compound C, we transfected U251 cells with siRNA directed against both α1 and α2 catalytic subunits of AMPK. The efficiency of siRNA-mediated AMPK downregulation was

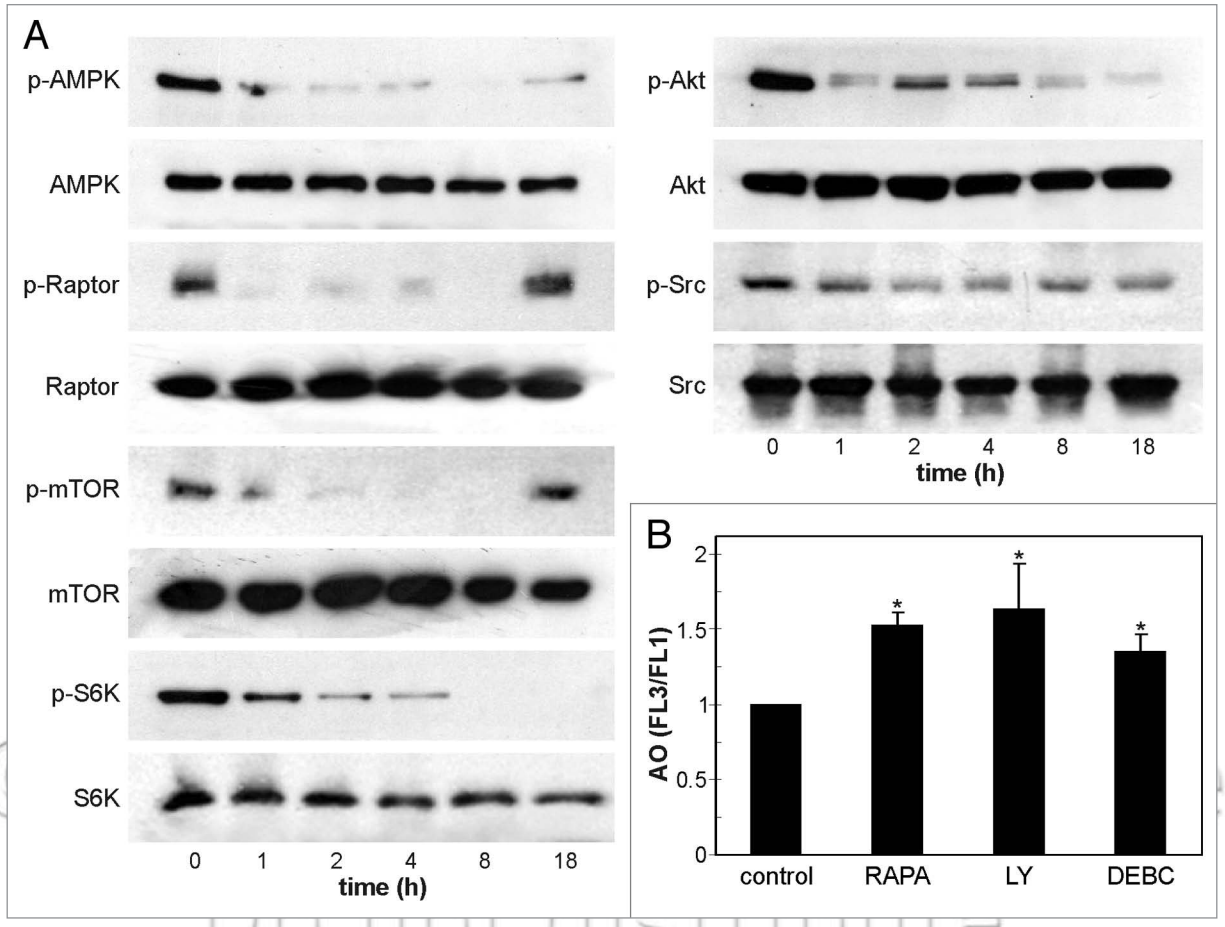

Figure 2. Compound C-mediated downregulation of AMPK/raptor and Akt/mTOR pathways and the role of Akt/mTOR blockade in autophagy induction. (A) U251 cells were treated with 10 μM of compound C for the indicated times and the activation status of AMPK/Akt/mTOR pathways was assessed by immunoblotting (the results from one of two experiments with similar results are shown). (B) U251 cells were incubated for 24 h with mTOR inhibitor rapamycin (RAPA; 10 nM), PI3K/Akt inhibitor LY294002 (LY; 50 μM) or 10-DEBC hydrochloride (DEBC; 20 μM) and the size of the intracellular acidic compartment was estimated by flow cytometric analysis of red/green (FL3/FL1) fluorescence ratio (the data are mean ± SD values from three experiments, *$p < 0.05$).

confirmed by immunoblot showing reduced levels of both total and phosphorylated AMPK in siRNA-transfected cells (**Fig. 3A**). Accordingly, AMPK-controlled phosphorylation of raptor was also reduced (**Fig. 3A**). However, siRNA-mediated AMPK inhibition completely failed to mimic compound C-induced suppression of Akt activation, as Akt phosphorylation was unaltered in AMPK siRNA-transfected cells (**Fig. 3A**). Furthermore, in contrast to treatment with compound C, transfection with AMPK-directed siRNA caused an increase in phosphorylation of both mTOR and its target, p70S6K (**Fig. 3A**). However, no significant change in LC3 conversion was observed (**Fig. 3A**), indicating that mTOR-repressed basal autophagy could not be further inhibited by additional mTOR activation. We have previously shown that AMPK activators metformin and AICAR reduced oxidative stress and apoptosis triggered by high concentration (20 μM) of compound C.[33] However, although metformin and AICAR at concentrations efficient in preventing apoptosis (2 mM and 0.25 mM, respectively) restored AMPK activation, they were unable to affect Akt and S6K inhibition or decrease LC3 conversion and intracellular acidification in compound C-treated cells (**Fig. 3B and C**). It therefore appears that compound C-triggered Akt/mTOR blockade and subsequent autophagy, unlike apoptosis, are totally independent of AMPK inhibition. Reduction of the intracellular levels of ATP, a known activator of Akt,[47] was also not responsible for the observed Akt inhibition and autophagy, as treatment with compound C for 6 h, when Akt suppression and autophagy were evident (**Fig. 1D and E and Fig. 2A**), completely failed to decrease ATP concentration in U251 cells (100 ± 18% vs. 131 ± 24% in control and compound C-treated cells, respectively; n = 3). No cell death was observed at this time-point (data not shown).

Autophagy inhibition increases cytotoxicity of compound C. During the autophagy studies, we have consistently observed that a proton-pump blocker bafilomycin A1, along with an expected reduction in autophagosome acidification in compound C-treated cells, caused a clear increase in number of cells displaying pyknotic (apoptotic) nuclei (**Fig. 4A**). This finding prompted us to explore if compound C-triggered autophagy might have a cytoprotective effect in the experimental setting employed. Indeed, the inhibition of autophagy by bafilomycin

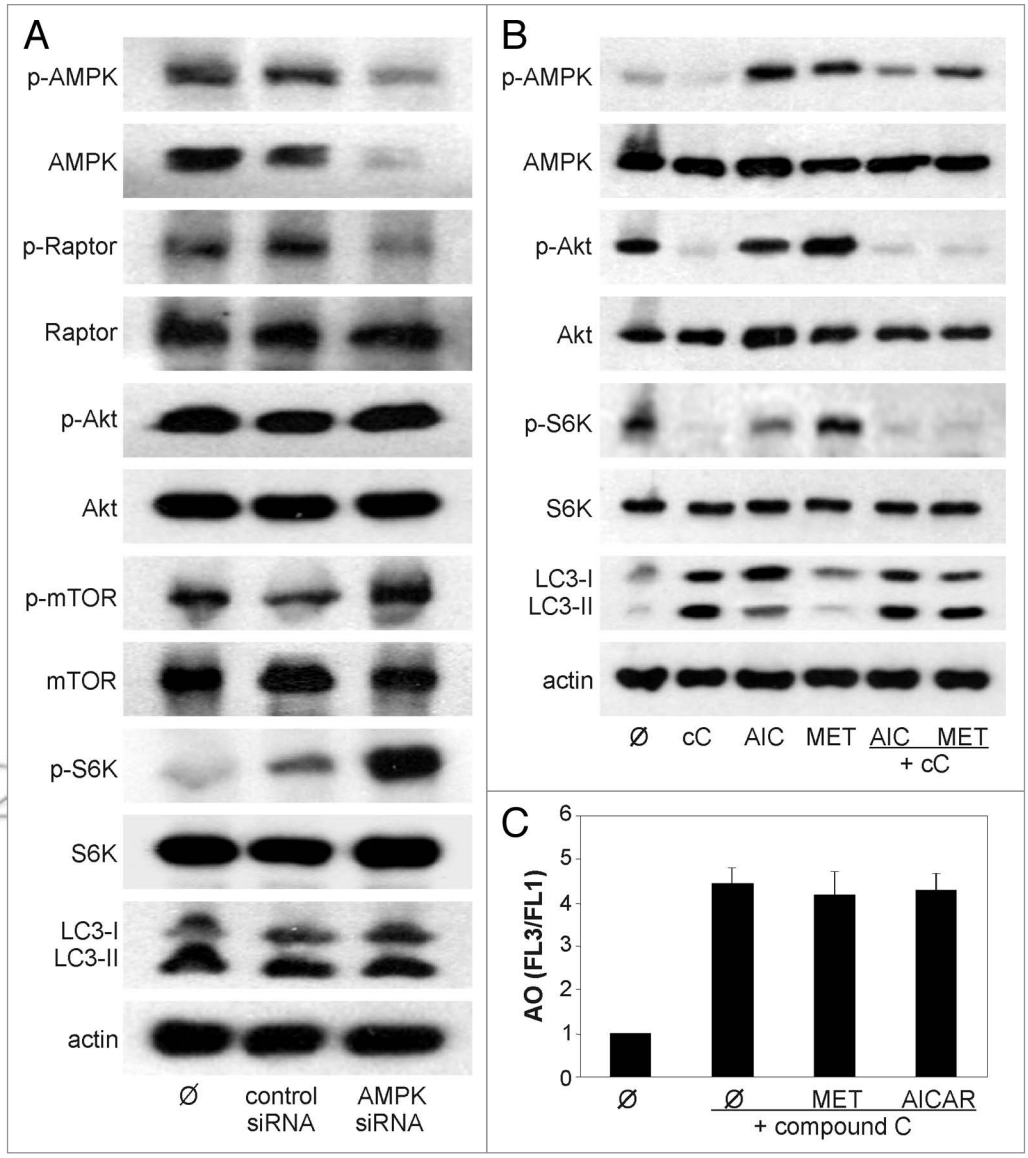

Figure 3. AMPK inhibition does not mediate compound C-induced Akt/mTOR blockade and autophagy. (A) The activation status of AMPK/Akt/mTOR pathways and LC3 conversion were evaluated by immunoblotting in nontransfected, control siRNA-transfected and AMPK-siRNA-transfected U251 cells 48 h after transfection. (B and C) U251 cells were pretreated for 2 h with AMPK activators metformin (MET; 2 mM) or AICAR (0.25 mM) and then incubated with compound C (10 μM). The activation status of AMPK/Akt/mTOR pathways and LC3 conversion were evaluated after 8 h (B; the data from one of two experiments with similar results are shown), while the accumulation of acridine orange-stained acidic vesicles was assessed by flow cytometry after 24 h (C; the data are mean ± SD values from three experiments).

A1 or lysosomal inhibitor chloroquine (**Fig. 4B**, left) significantly increased the LDH release from compound C-treated U251 cells (**Fig. 4B**, right). In accordance with the LDH release data, compound C alone induced only marginal DNA fragmentation, which was markedly augmented by pharmacological inhibition of autophagy with bafilomycin or chloroquine (**Fig. 4C**). Of note, the autophagy blockers did not cause a significant increase in LDH release or DNA fragmentation when applied alone (data not shown). Accordingly, an immunoblot analysis of caspase-3 cleavage fragment has demonstrated that bafilomycin A1 markedly increased caspase-3 activation induced by compound C (**Fig. 4D**). These data indicate that compound C-triggered autophagic response protects U251 cells from concomitant induction of apoptotic death.

Compound C induces protective autophagic response in different cancer cell lines. We next assessed whether the autophagy-inducing capacity of compound C was a unique feature of U251 glioma cells, or it could also be observed in other cancer cell lines. The latter was apparently true, as the treatment with compound C caused a clear dose-dependent increase in FL3/FL1 ratio in acridine orange-stained C6 rat glioma, L929 mouse fibrosarcoma and B16 mouse melanoma cell lines (**Fig. 5A**). The induction of autophagy in compound C-treated L929 cells was further confirmed by an increase in LC3 conversion in the

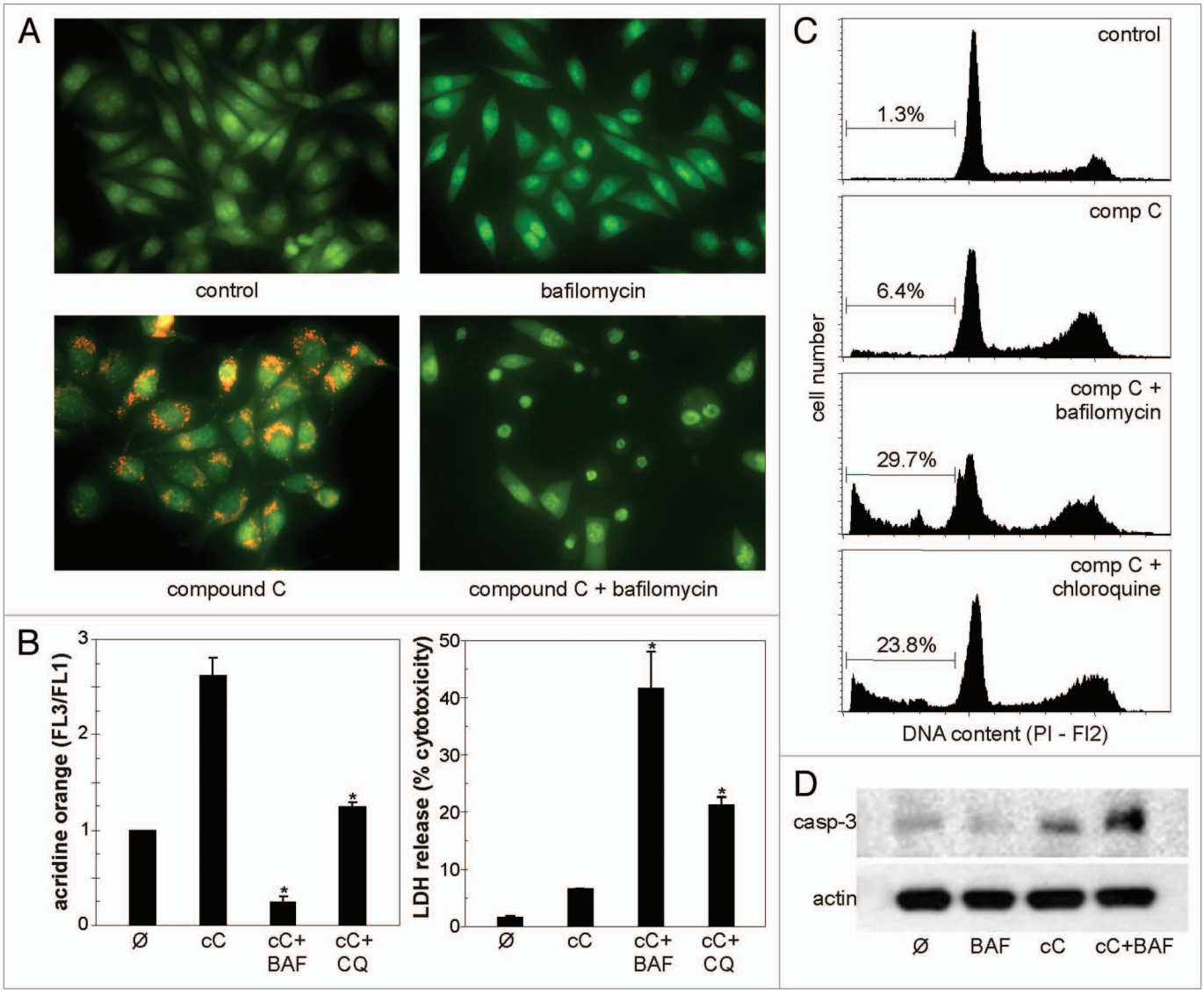

Figure 4. Inhibition of autophagy increases cytotoxicity of compound C towards U251 cells. (A) U251 cells were treated with compound C (10 μM) and/or autophagy inhibitor bafilomycin A1 (50 nM). After 24 h, cells were stained with acridine orange for fluorescent microscopy evaluation of the intracellular acidic compartment and nuclear morphology. (B and C) U251 cells were incubated for 24 h with compound C (cC; 10 μM), in the presence or absence of autophagy inhibitors bafilomycin A1 (BAF; 50 nm) or chloroquine (CQ; 50 μM). The presence of intracellular acidic vesicles was assessed by flow cytometric analysis of acridine orange-stained cells (B, left; the data are mean ± SD values from three experiments, *p < 0.05), while the cell death was determined by LDH release assay (B, right; the data are mean ± SD values of triplicates from a representative of three experiments, *p < 0.05). The representative flow cytometry histograms (from one of two experiments with similar results) showing the percentage of PI-stained cells with fragmented DNA (sub-G) are presented in (C). (D) U251 cells are treated as in (A) and the presence of caspase-3 cleavage fragment was assessed by immunoblotting.

presence of proteolysis inhibitors (**Fig. 5B**), reduction of p62 levels and increase in Beclin 1 expression (**Fig. 5C**). Similarly to results obtained in U251 cells, these effects were associated with downregulation of AMPK, Akt, p70S6K and Src phosphorylation (**Fig. 5C**). Finally, autophagy inhibition with bafilomycin or chloroquine significantly augmented LDH release in compound C-treated cultures of C6, L929 and B16 cells (**Fig. 5D**). Neither bafilomycin nor chloroquine significantly affected LDH release when applied alone (data not shown). Therefore, the ability of compound C to induce protective autophagic response in tumor cells was apparently not cell type- or species-specific.

Discussion

The present study for the first time demonstrates the ability of compound C, a well-known AMPK inhibitor, to induce autophagy in cancer cell lines via AMPK inhibition-independent downregulation of Akt/mTOR pathway. The autophagy triggered by compound C apparently protected tumor cells from concomitant induction of apoptotic cell death.

In accordance with the key role of mTOR as a repressor of autophagy, downregulation of mTOR activity efficiently triggered an autophagic response in mammalian cells,[48,49] which has

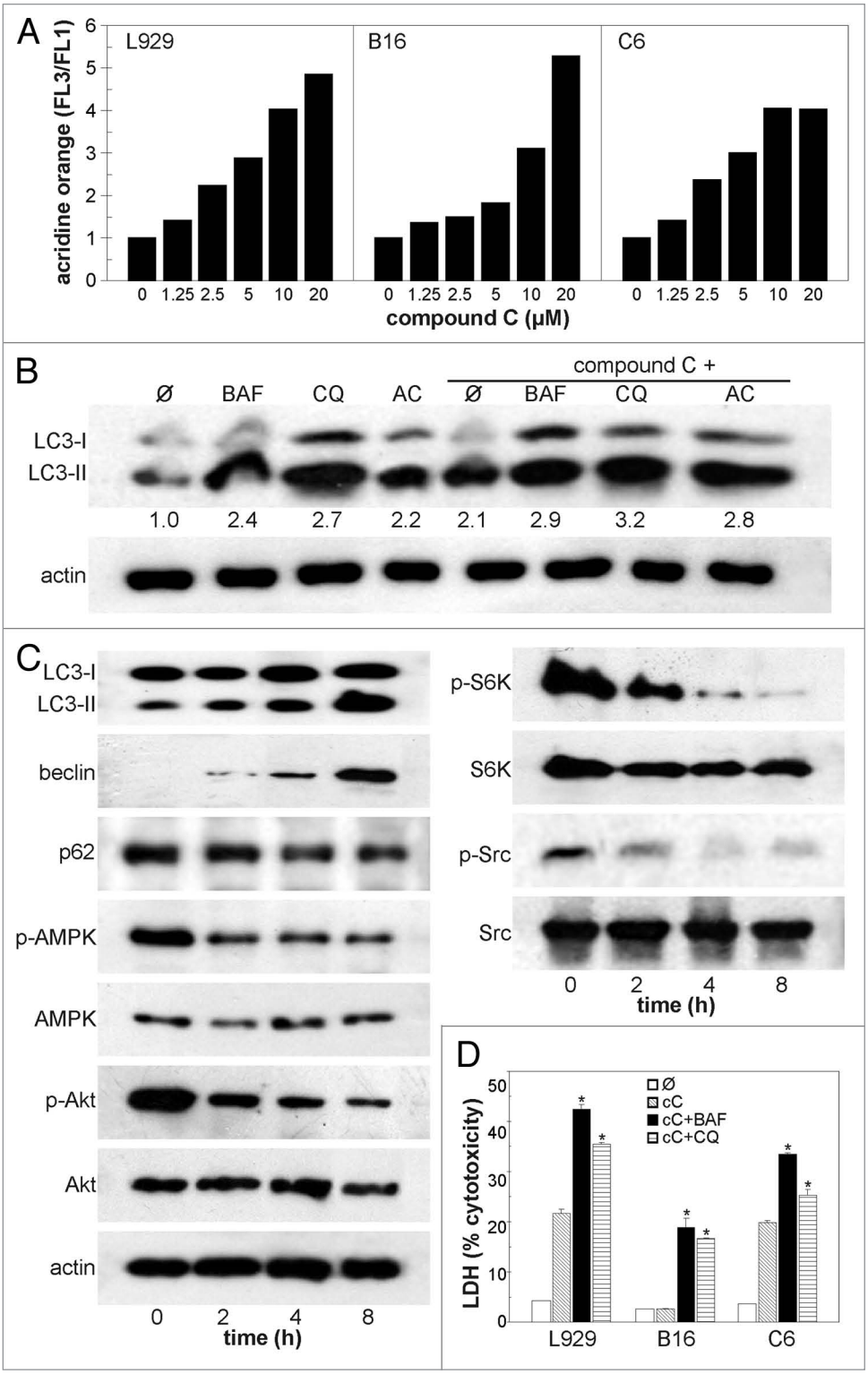

Figure 5. Compound C induces Akt/mTOR blockade-dependent protective autophagy in different cancer cell lines. (A) L929 mouse fibrosarcoma, B16 mouse melanoma and C6 rat glioma cells were incubated for 24 h with different concentrations of compound C and the presence of intracellular acidic vesicles was assessed by flow cytometric analysis of acridine orange red/green (FL3/FL1) fluorescence ratio (the data from one of two experiments with similar results are shown). (B) Immunoblot analysis of LC3 conversion in L929 cells treated for 6 h with compound C (10 μM) and/or proteolysis inhibitors bafilomycin A1 (BAF; 50 nm), chloroquine (CQ; 50 μM) and amonium chloride (AC; 25 mM). (C) Immunoblot analysis of LC3 conversion, Beclin1 levels, p62 levels and AMPK/Akt/mTOR pathways in cells treated with compound C (10 μM) for the indicated times (the results from one of two experiments with similar results are shown). (D) L929, B16 and C6 cells were incubated for 24 h with compound C (cC; 10 μM), in the presence or absence of autophagy inhibitors bafilomycin A1 (BAF; 50 nm) or chloroquine (CQ; 50 μM) and the cell death was determined by LDH release assay (the data are mean ± SD values of triplicates from a representative of three experiments; *$p < 0.05$).

been confirmed in the present study by using mTOR inhibitor rapamycin. Alternatively, autophagy can be readily induced by interfering with the activation of Akt,[50,51] one of the key upstream positive regulators of mTOR, as seen in our experiments with Akt inhibitor 10-DEBC hydrochloride and PI3K/Akt inhibitor LY294002. The induction of autophagy by LY294002 might seem surprising, having in mind the ability of PI3K inhibitors to inhibit autophagy induced by different stimuli.[52] It has been shown, however, that PI3K inhibitors including LY294002 could also induce autophagy[53,54] despite being able to suppress autophagic response to other stimuli. This dual role is probably mediated via different temporal patterns of inhibition of class I and class III PI3K involved in Akt activation and autophagosome formation, respectively.[55] Therefore, it seems conceivable to assume that the autophagy induction in compound C-exposed tumor cell lines in our study was mediated via downregulation of the Akt/mTOR pathway. This hypothesis is consistent with the ability of compound C to suppress vascular endothelial growth factor-triggered phosphorylation of Akt in endothelial cells,[56] as well as insulin-dependent Akt activation in skeletal muscle cells.[57] Moreover, it should be noted that compound C belongs to a pyrazolopirymidine class of molecules, some of which are known to potently reduce Akt phosphorylation through blockade of Src kinase family.[58] Accordingly, our demonstration of the ability of

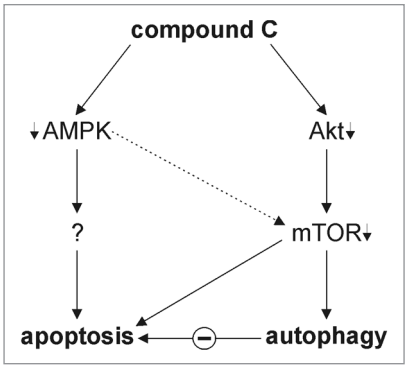

Figure 6. A hypothetical mechanism of signaling and apoptosis/autophagy interactions in compound C-treated cancer cells. The downward arrows denote inhibition of AMPK, Akt and mTOR. The dashed line indicates that the expected upregulation of mTOR by AMPK blockade is overcome by concomitant downregulation of mTOR via Akt blockade.

compound C to reduce phosphorylation of Src indicates that the inhibition of this tyrosine kinase might be at least partly involved in the observed downregulation of Akt and subsequent induction of autophagy.

Although AMPK and Akt activation are generally thought to be independently regulated,[44,59] some recent data indicate a link between the two signaling routes. Namely, adiponectin-activated AMPK was responsible for Akt dephosphorylation in breast cancer cells,[60] while, on the other hand, adiponectin- or vascular growth endothelial factor-mediated Akt activation in endothelial cells was AMPK-dependent.[61] These findings suggest that in certain conditions AMPK could regulate Akt activation in a context-dependent manner. This, however, was apparently not the case in our experiments, as siRNA knockdown of AMPK, while expectedly causing raptor inactivation and subsequent upregulation of mTOR/p70S6K, completely failed to affect Akt phosphorylation in U251 glioma cells. Accordingly, transfection with AMPK siRNA could not mimic Akt inhibition-dependent induction of autophagy by compound C. The finding that AMPK activators metformin and AICAR restored AMPK activity, but failed to increase Akt/S6K phosphorylation and reduce LC3 conversion in compound C-treated cells, further confirms that AMPK inhibition was not required for autophagy induction by compound C. Therefore, compound C-mediated Akt downregulation leading to autophagic response was completely independent of concomitant AMPK suppression and able to overcome the stimulatory effect of AMPK/raptor blockade on the activity of mTOR. This is not surprising, as compound C has previously been reported to exert AMPK-independent biological effects, including inhibition of bone morphogenetic protein signaling,[62] blockade of hypoxia inducible factor-1 activation,[63] and induction of cell cycle regulator p21.[64]

In contrast to expectations that induction of autophagy might be used as a "magic bullet" for the treatment of apoptosis-resistant cancers,[9] a large body evidence supports the hypothesis that tumor cells in certain conditions might actually use autophagy to evade therapy-induced death. Accordingly, inhibition of autophagy, particularly at a late stage, sensitized cancer cells to apoptosis induced by ionizing irradiation, hyperthermia, DNA-damaging agents, histone deacetylase inhibitors, imatinib, tamoxifen, TNF or TRAIL.[27,28,65-73] Consistent with these findings, pharmacological inhibition of autophagy in compound C-treated U251 cells markedly increased caspase activation and ensuing apoptotic cell death in our experiments. We have previously reported that cytoprotective effects of autophagy might involve interference with the proapoptotic shift in the expression of Bcl-2 family proteins and prevention of oxidative stress.[27] Similar mechanisms might contribute to autophagy-mediated protection from compound C, keeping in mind that compound C-triggered apoptosis was associated both with the increase in Bax/Bcl-2 ratio and induction of oxidative stress.[33] However, the exact mechanisms underlying the anti-apoptotic action of autophagy in compound C-exposed cancer cells remain to be established.

Our previous data suggest that AMPK inhibition is required, but not sufficient for the compound C-mediated apoptosis induction in U251 glioma cells, thus implying an AMPK-independent component in the observed effect.[33] The inhibition of Akt, a known prosurvival signal,[59] seems a plausible candidate for the putative AMPK blockade-independent proapoptotic action of compound C. We therefore propose a model in which both AMPK suppression and AMPK inhibition-independent Akt blockade participate in compound C-mediated apoptosis, while the latter mechanism and subsequent mTOR downregulation are responsible for the concomitant induction of autophagy that limits progression of apoptosis (**Fig. 6**). On the other hand, it should be noted that compound C inhibited AMPK-dependent autophagy in rat hepatocytes, porcine endothelial cells, human osteosarcoma cells, HT-29 human colon cancer cells, HeLa human cervical carcinoma cells and MCF-7 breast cancer cells exposed to starvation, avicin D, troglitazone or alisol B.[25,37-39] Accordingly, we have observed that lower concentrations of compound C (≤1 μM), unlike higher concentrations (≥5 μM), can actually suppress AMPK-dependent autophagy in UV-irradiated U251 cells (Vucicevic T, et al. unpublished). While these findings suggest that the effects of compound C on autophagy might be dose-, cell-type- and/or context-dependent, the reasons for the observed discrepancies are still to be fully clarified. Nevertheless, our data warrant caution when using compound C to inhibit AMPK-dependent cellular responses. In addition, they reinforce the concept of autophagy inhibition as a strategy for cancer cell sensitization and support further exploration of compound C and related molecules as potential anticancer agents.

Materials and Methods

Cells and cell cultures. The human glioma cell line U251 and rat glioma cell line C6 were kindly donated by Dr. Pedro Tranque (Universidad de Castilla-La Mancha, Albacete, Spain). The mouse fibrosarcoma L929 was obtained from the European Collection of Animal Cell Cultures (85011425), while B16 mouse melanoma cell line was a kind gift from Dr. Sinisa Radulovic (Institute for Oncology and Radiology of Serbia, Belgrade, Serbia). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2, in a HEPES-buffered RPMI 1640

cell culture medium with L-glutamine (Sigma-Aldrich, R7388) supplemented with 5% fetal bovine serum (Sigma-Aldrich, F2442), 1 mM sodium pyruvate (Sigma-Aldrich, S8636) and 10 ml/L penicillin/streptomycin (Sigma-Aldrich, P0781). The cells were incubated in 96-well flat bottom plates (1 x 10^4 cells/well) for the cell viability assays, 24-well plates (1 x 10^5 cells/well) for the flow cytometry analysis or 90 mm Petri dishes (2 x 10^6 cells) for the western blotting. Cells were rested for 24 h and then treated with compound C (Sigma-Aldrich, P5499) and/or Akt inhibitor 10-DEBC hydrochloride (Tocris Bioscience, 2558), PI3K inhibitor LY294002 (Tocris Bioscience, 1130), mTOR inhibitor rapamycin (Sigma-Aldrich, R8781), AMPK activators metformin hydrochloride (Gluformin®, Hemofarm) and aminoimidazole carboxamide ribonucleotide (AICAR; Sigma-Aldrich, A9978) or lysosomal inhibitors bafilomycin A1 (Sigma-Aldrich, 11711), ammonium chloride (Sigma-Aldrich, A0171) and chloroquine (Sigma-Aldrich, C6628). The incubation times and concentrations of agents are stated in figure legends and/or figures.

Autophagy detection by acridine orange staining and transmission electron microscopy (TEM). The acidic intracellular compartments were visualized by supravital acridine orange staining. After incubation, cells were washed with phosphate-buffered saline and stained with 1 μM acridine orange (Sigma-Aldrich, 318337) for 15 min at 37°C. Subsequently, cells were washed and analyzed under the inverted fluorescent microscope. Depending on their acidity, autophagic lysosomes appeared as orange/red fluorescent cytoplasmic vesicles, while nuclei were stained green. Alternatively, acridine orange-stained cells were trypsinized, washed and analyzed on a FACSCalibur flow cytometer (Becton, Dickinson and Company) using Cell Quest Pro software. The autophagy was quantified as a ratio between geomean fluorescence intensity of red vs. green fluorescence (FL3/FL1). For the TEM analysis, trypsinized cells were fixed with 2.5% glutaraldehyde in phosphate-buffered saline, followed by 2% OsO_4. After dehydration, thin sections were stained with uranyl acetate and lead citrate for observation under a Morgagni 268(D) electron microscope (FEI).

Western blot analysis of autophagy, AMPK/Akt/mTOR signaling and caspase-3 activation. Cells were lysed in lysis buffer (30 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40) containing 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, P7626) and protease inhibitor cocktail (Sigma-Aldrich, P8340) on ice for 30 min, centrifuged at 14,000 g for 15 min at 4°C, and the supernatants were collected as the cell lysates. Equal amounts of protein from each sample was separated by SDS-PAGE on 6–15% gels and transferred to nitrocellulose membranes (Bio-Rad, 162-0115). Following incubation with primary antibodies against LC3B (2775), phospho-AMPK (2535), AMPK (2603), phospho-Akt (4058), Akt (9272), phospho-mTOR (2971), mTOR (2983), phospho-raptor (2083), raptor (2280), phospho-p70S6K (9205), p70S6K (2708), Beclin 1 (3495), caspase-3 cleavage fragment (9664), actin (4968; Cell Signaling Technology), phospho-Src (ab4816), Src (ab47405; Abcam) or p62 (647702; Biolegend) and peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, 111-035-003) as the secondary antibody, specific protein bands were visualized using Amersham ECL reagent (GE Healthcare, RPN2109). The levels of LC3-II were quantified by densitometry using ImageJ software and expressed relative to actin signals.

Transfection with small interfering RNA (siRNA). The siRNA targeting human AMPKα1/2 and scrambled control siRNA were obtained from Santa Cruz Biotechnology (sc-45312). Subconfluent U251 cells in 90 mm Petri dishes were transfected with AMPK and control siRNA according to the manufacturer's protocol. Cells were allowed to grow 48 h following transfection and then were lysed for western blot analysis.

Determination of cell viability and apoptosis. The release of cytosolic lactate dehydrogenase (LDH) into the supernatant of cultured cells indicates the loss of membrane integrity that occurs in dead cells. The assay was performed exactly as previously described[74] and the pyruvate-mediated conversion of 2,4-dinitrophenylhydrazine into visible hydrazone precipitate, correlating with the extent of cell death, was measured at 492 nm. The results were presented as % cytotoxicity according to the formula: 100 x (a - b)/(c - b), where a, b and c are the absorbances of the experimental sample, negative control (cell culture medium without cells) and positive control (cells lysed with 3% Triton-X100), respectively. DNA fragmentation as a marker of apoptosis was assessed by flow cytometric analysis of cells stained with DNA-binding dye propidium iodide. Briefly, cells were fixed in 70% ethanol and then incubated with 50 μg/ml RNAse (Sigma-Aldrich, R6513) and 40 μg/ml propidium iodide (Sigma-Aldrich, P4170) for 30 min at 37°C in the dark. Red fluorescence was analyzed at FACSCalibur flow cytometer, using FL2-W vs. FL2-A dot plot to exclude cell aggregates. The number of hypodiploid apoptotic cells in sub-G_0/G_1 compartment was analyzed using Cell Quest Pro software.

Intracellular ATP measurement. The concentration of intracellular ATP was determined using a luminescence-based ATP detection assay system (ATPLite, Perkin Elmer, 6016943) according to the manufacturer's instructions.

Statistical analysis. The statistical significance of the differences between treatments was assessed using one-way ANOVA followed by Student-Neuman-Keuls test for multiple comparisons. The value of $p < 0.05$ was considered significant.

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References

1. Yorimitsu T, Klionsky DJ. Autophagy: molecular machinery for self-eating. Cell Death Differ 2005; 12:1542-52.
2. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature 2008; 451:1069-75.
3. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol 2007; 8:741-52.
4. Azad MB, Chen Y, Henson ES, Cizeau J, McMillan-Ward E, Israels SJ, Gibson SB. Hypoxia induces autophagic cell death in apoptosis-competent cells through a mechanism involving BNIP3. Autophagy 2008; 4:195-204.
5. Kanzawa T, Kondo Y, Ito H, Kondo S, Germano I. Induction of autophagic cell death in malignant glioma cells by arsenic trioxide. Cancer Res 2003; 63:2103-8.
6. Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. Cell Death Differ 2004; 11:448-57.
7. Fazi B, Bursch W, Fimia GM, Nardacci R, Piacentini M, Di Sano F, Piredda L. Fenretinide induces autophagic cell death in caspase-defective breast cancer cells. Autophagy 2008; 4:435-41.
8. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, et al. Promotion of tumorigenesis by heterozygous disruption of the *beclin 1* autophagy gene. J Clin Invest 2003; 112:1809-920.
9. Gozuacik D, Kimchi A. Autophagy as a cell death and tumor suppressor mechanism. Oncogene 2004; 23:2891-906.
10. White E, Autophagic cell death unraveled: Pharmacological inhibition of apoptosis and autophagy enables necrosis. Autophagy 2008; 4:399-401.
11. He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet 2009; 43:67-93.
12. Meijer AJ, Codogno P. Regulation and role of autophagy in mammalian cells. Int J Biochem Cell Biol 2004; 36:2445-62.
13. Hay N. The Akt-mTOR tango and its relevance to cancer. Cancer Cell 2005; 8:179-83.
14. Kahn BB, Alquier T, Carling D, Hardie DG. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metab 2005; 1:15-25.
15. Shaw RJ. LKB1 and AMP-activated protein kinase control of mTOR signalling and growth. Acta Physiol (Oxf) 2009; 196:65-80.
16. Luo Z, Saha AK, Xiang X, Ruderman NB. AMPK, the metabolic syndrome and cancer. Trends Pharmacol Sci 2005; 26:69-76.
17. Rattan R, Giri S, Singh AK, Singh I. 5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside inhibits cancer cell proliferation in vitro and in vivo via AMP-activated protein kinase. J Biol Chem 2005; 280:39582-93.
18. Jin Q, Feng L, Behrens C, Bekele BN, Wistuba II, Hong WK, Lee HY. Implication of AMP-activated protein kinase and Akt-regulated survivin in lung cancer chemopreventive activities of deguelin. Cancer Res 2007; 67:11630-9.
19. Memmott RM, Gills JJ, Hollingshead M, Powers MC, Chen Z, Kemp B, et al. Phosphatidylinositol ether lipid analogues induce AMP-activated protein kinase-dependent death in LKB1-mutant non small cell lung cancer cells. Cancer Res 2008; 68:580-8.
20. Okoshi R, Ozaki T, Yamamoto H, Ando K, Koida N, Ono S, et al. Activation of AMP-activated protein kinase induces p53-dependent apoptotic cell death in response to energetic stress. J Biol Chem 2008; 283:3979-87.
21. Laderoute KR, Amin K, Calaoagan JM, Knapp M, Le T, Orduna J, et al. 5'-AMP-activated protein kinase (AMPK) is induced by low-oxygen and glucose deprivation conditions found in solid-tumor microenvironments. Mol Cell Biol 2006; 26:5336-47.
22. Williams T, Forsberg LJ, Viollet B, Brenman JE. Basal autophagy induction without AMP-activated protein kinase under low glucose conditions. Autophagy 2009; 5:1155-65.
23. Kim HS, Hwang JT, Yun H, Chi SG, Lee SJ, Kang I, et al. Inhibition of AMP-activated protein kinase sensitizes cancer cells to cisplatin-induced apoptosis via hyper-induction of p53. J Biol Chem 2008; 283:3731-42.
24. Park HU, Suy S, Danner M, Dailey V, Zhang Y, Li H, et al. AMP-activated protein kinase promotes human prostate cancer cell growth and survival. Mol Cancer Ther 2009; 8:733-41.
25. Xu ZX, Liang J, Haridas V, Gaikwad A, Connolly FP, Mills GB, Gutterman JU. A plant triterpenoid, avicin D, induces autophagy by activation of AMP-activated protein kinase. Cell Death Differ 2007; 14:1948-57.
26. Papandreou I, Lim AL, Laderoute K, Denko NC. Hypoxia signals autophagy in tumor cells via AMPK activity, independent of HIF-1, BNIP3 and BNIP3L. Cell Death Differ 2008; 15:1572-81.
27. Harhaji-Trajkovic L, Vilimanovich U, Kravic-Stevovic T, Bumbasirevic V, Trajkovic V. AMPK-mediated autophagy inhibits apoptosis in cisplatin-treated tumour cells. J Cell Mol Med 2009; 13:3644-54.
28. Filomeni G, Desideri E, Cardaci S, Graziani I, Piccirillo S, Rotilio G, Ciriolo MR. Carcinoma cells activate AMP-activated protein kinase-dependent autophagy as survival response to kaempferol-mediated energetic impairment. Autophagy 2010; 6:202-16.
29. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 2001; 108:1167-74.
30. Isakovic A, Harhaji L, Stevanovic D, Markovic Z, Sumarac-Dumanovic M, Starcevic V, et al. Dual antiglioma action of metformin: cell cycle arrest and mitochondria-dependent apoptosis. Cell Mol Life Sci 2007; 64:1290-302.
31. Shaw MM, Gurr WK, McCrimmon RJ, Schorderet DF, Sherwin RS. 5'AMP-activated protein kinase alpha deficiency enhances stress-induced apoptosis in BHK and PC12 cells. J Cell Mol Med 2007; 11:286-98.
32. Zhou W, Han WF, Landree LE, Thupari JN, Pinn ML, Bililign T, et al. Fatty acid synthase inhibition activates AMP-activated protein kinase in SKOV3 human ovarian cancer cells. Cancer Res 2007; 67:2964-71.
33. Vucicevic L, Misirkic M, Janjetovic K, Harhaji-Trajkovic L, Prica M, Stevanovic D, et al. AMP-activated protein kinase-dependent and -independent mechanisms underlying in vitro antiglioma action of compound C. Biochem Pharmacol 2009; 77:684-93.
34. Han JH, Ahn YH, Choi KY, Hong SH. Involvement of AMP-activated protein kinase and p38 mitogen-activated protein kinase in 8-Cl-cAMP-induced growth inhibition. J Cell Physiol 2009; 218:104-12.
35. Chiang PC, Lin SC, Pan SL, Kuo CH, Tsai IL, Kuo MT, et al. Antroquinonol displays anticancer potential against human hepatocellular carcinoma cells: a crucial role of AMPK and mTOR pathways. Biochem Pharmacol 2010; 79:162-71.
36. Hsu YC, Meng X, Ou L, Ip MM. Activation of the AMP-activated protein kinase-p38 MAP kinase pathway mediates apoptosis induced by conjugated linoleic acid in p53-mutant mouse mammary tumor cells. Cell Signal 2010; 22:590-9.
37. Meley D, Bauvy C, Houben-Weerts JH, Dubbelhuis PF, Helmond MT, Codogno P, Meijer AJ. AMP-activated protein kinase and the regulation of autophagic proteolysis. J Biol Chem 2006; 281:34870-9.
38. Yan J, Yang H, Wang G, Sun L, Zhou Y, Guo Y, et al. Autophagy augmented by troglitazone is independent of EGFR transactivation and correlated with AMP-activated protein kinase signaling. Autophagy 2010; 6:67-73.
39. Law BY, Wang M, Ma DL, Al-Mousa F, Michelangeli F, Cheng SH, et al. Alisol B, a novel inhibitor of the sarcoplasmic/endoplasmic reticulum Ca(2+) ATPase pump, induces autophagy, endoplasmic reticulum stress and apoptosis. Mol Cancer Ther 2010; 9:718-30.
40. Djavaheri-Mergny M, Maiuri MC, Kroemer G. Cross talk between apoptosis and autophagy by caspase-mediated cleavage of Beclin 1. Oncogene 2010; 29:1717-9.
41. Rubinsztein DC, Cuervo AM, Ravikumar B, Sarkar S, Korolchuk V, Kaushik S, Klionsky DJ. In search of an "autophagomometer". Autophagy 2009; 5:585-9.
42. Ichimura Y, Kumanomidou T, Sou YS, Mizushima T, Ezaki J, Ueno T, et al. Structural basis for sorting mechanism of p62 in selective autophagy. J Biol Chem 2008; 283:22847-57.
43. Eng CH, Yu K, Lucas J, White E, Abraham RT. Ammonia derived from glutaminolysis is a diffusible regulator of autophagy. Sci Signal 2010; 3:31.
44. Memmott RM, Dennis PA. Akt-dependent and -independent mechanisms of mTOR regulation in cancer. Cell Signal 2009; 21:656-64.
45. Jaeschke A, Hartkamp J, Saitoh M, Roworth W, Nobukuni T, Hodges A, et al. Tuberous sclerosis complex tumor suppressor-mediated S6 kinase inhibition by phosphatidylinositide-3-OH kinase is mTOR independent. J Cell Biol 2002; 159:217-24.
46. Koga F, Xu W, Karpova TS, McNally JG, Baron R, Neckers L. Hsp90 inhibition transiently activates Src kinase and promotes Src-dependent Akt and Erk activation. Proc Natl Acad Sci USA 2006; 103:11318-22.
47. Gerasimovskaya EV, Tucker DA, Weiser-Evans M, Wenzlau JM, Klemm DJ, Banks M, Stenmark KR. Extracellular ATP-induced proliferation of adventitial fibroblasts requires phosphoinositide 3-kinase, Akt, mammalian target of rapamycin and p70 S6 kinase signaling pathways. J Biol Chem 2005; 280:1838-48.
48. Iwamaru A, Kondo Y, Iwado E, Aoki H, Fujiwara K, Yokoyama T, et al. Silencing mammalian target of rapamycin signaling by small interfering RNA enhances rapamycin-induced autophagy in malignant glioma cells. Oncogene 2007; 26:1840-51.
49. Kim KW, Mutter RW, Cao C, Albert JM, Freeman M, Hallahan DE, Lu B. Autophagy for cancer therapy through inhibition of pro-apoptotic proteins and mammalian target of rapamycin signaling. J Biol Chem 2006; 281:36883-90.
50. Degtyarev M, De Mazière A, Klumperman J, Lin K. Autophagy, an Achilles' heel AKTing against cancer? Autophagy 2009; 5:415-8.
51. Fujiwara K, Iwado E, Mills GB, Sawaya R, Kondo S, Kondo Y. Akt inhibitor shows anticancer and radiosensitizing effects in malignant glioma cells by inducing autophagy. Int J Oncol 2007; 31:753-60.
52. Proikas-Cezanne T, Ruckerbauer S, Stierhof YD, Berg C, Nordheim A. Human WIPI-1 puncta-formation: a novel assay to assess mammalian autophagy. FEBS Lett 2007; 581:3396-404.
53. Takeuchi H, Kondo Y, Fujiwara K, Kanzawa T, Aoki H, Mills GB, Kondo S. Synergistic augmentation of rapamycin-induced autophagy in malignant glioma cells by phosphatidylinositol 3-kinase/protein kinase B inhibitors. Cancer Res 2005; 65:3336-46.
54. Coward J, Ambrosini G, Musi E, Truman JP, Haimovitz-Friedman A, Allegood JC, et al. Safingol (L-threo-sphinganine) induces autophagy in solid tumor cells through inhibition of PKC and the PI3-kinase pathway. Autophagy 2009; 5:184-93.
55. Wu YT, Tan HL, Shui G, Bauvy C, Huang Q, Wenk MR, et al. Dual role of 3-methyladenine in modulation of autophagy via different temporal patterns of inhibition on class I and III phosphoinositide 3-kinase. J Biol Chem 2010; 285:10850-61.

56. Youn JY, Wang T, Cai H. An ezrin/calpain/PI3K/AMPK/eNOSs1179 signaling cascade mediating VEGF-dependent endothelial nitric oxide production. Circ Res 2009; 104:50-9.
57. Li HB, Ge YK, Zheng XX, Zhang L. Salidroside stimulated glucose uptake in skeletal muscle cells by activating AMP-activated protein kinase. Eur J Pharmacol 2008; 588:165-9.
58. Kassenbrock CK, Hunter S, Garl P, Johnson GL, Anderson SM. Inhibition of Src family kinases blocks epidermal growth factor (EGF)-induced activation of Akt, phosphorylation of c-Cbl and ubiquitination of the EGF receptor. J Biol Chem 2002; 277:24967-75.
59. Sarbassov DD, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. Curr Opin Cell Biol 2005; 17:596-603.
60. Kim KY, Baek A, Hwang JE, Choi YA, Jeong J, Lee MS, et al. Adiponectin-activated AMPK stimulates dephosphorylation of AKT through protein phosphatase 2A activation. Cancer Res 2009; 69:4018-26.
61. Levine YC, Li GK, Michel T. Agonist-modulated regulation of AMP-activated protein kinase (AMPK) in endothelial cells. Evidence for an AMPK → Rac1 → Akt → endothelial nitric-oxide synthase pathway. J Biol Chem 2007; 282:20351-64.
62. Yu PB, Hong CC, Sachidanandan C, Babitt JL, Deng DY, Hoyng SA, et al. Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. Nat Chem Biol 2008; 4:33-41.
63. Emerling BM, Viollet B, Tormos KV, Chandel NS. Compound C inhibits hypoxic activation of HIF-1 independent of AMPK. FEBS Lett 2007; 581:5727-31.
64. Nam M, Lee WH, Bae EJ, Kim SG. Compound C inhibits clonal expansion of preadipocytes by increasing p21 level irrespectively of AMPK inhibition. Arch Biochem Biophys 2008; 479:74-81.
65. Paglin S, Hollister T, Delohery T, Hackett N, McMahill M, Sphicas E, et al. A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. Cancer Res 2001; 61:439-44.
66. Komata T, Kanzawa T, Nashimoto T, Aoki H, Endo S, Nameta M, et al. Mild heat shock induces autophagic growth arrest, but not apoptosis in U251-MG and U87-MG human malignant glioma cells. J Neurooncol 2004; 68:101-11.
67. Abedin MJ, Wang D, McDonnell MA, Lehmann U, Kelekar A. Autophagy delays apoptotic death in breast cancer cells following DNA damage. Cell Death Differ 2007; 14:500-10.
68. Amaravadi RK, Yu D, Lum JJ, Bui T, Christophorou MA, Evan GI, et al. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. J Clin Invest 2007; 117:326-36.
69. Carew JS, Nawrocki ST, Kahue CN, Zhang H, Yang C, Chung L, et al. Targeting autophagy augments the anticancer activity of the histone deacetylase inhibitor SAHA to overcome Bcr-Abl-mediated drug resistance. Blood 2007; 110:313-22.
70. Harhaji L, Mijatovic S, Maksimovic-Ivanic D, Popadic D, Isakovic A, Todorovic-Markovic B, Trajkovic V. Aloe emodin inhibits the cytotoxic action of tumor necrosis factor. Eur J Pharmacol 2007; 568:248-59.
71. Han J, Hou W, Goldstein LA, Lu C, Stolz DB, Yin XM, Rabinowich H. Involvement of protective autophagy in TRAIL resistance of apoptosis-defective tumor cells. J Biol Chem 2008; 283:19665-77.
72. Shingu T, Fujiwara K, Bögler O, Akiyama Y, Moritake K, Shinojima N, et al. Stage-specific effect of inhibition of autophagy on chemotherapy-induced cytotoxicity. Autophagy 2009; 5:537-9.
73. de Medina P, Silvente-Poirot S, Poirot M. Tamoxifen and AEBS ligands induced apoptosis and autophagy in breast cancer cells through the stimulation of sterol accumulation. Autophagy 2009; 5:1066-7.
74. Raicevic N, Mladenovic A, Perovic M, Harhaji L, Miljkovic D, Trajkovic V. Iron protects astrocytes from 6-hydroxydopamine toxicity. Neuropharmacology 2005; 48:720-31.