J. Cell. Mol. Med. Vol 13, No 9B, 2009 pp. 3644-3654

AMPK-mediated autophagy inhibits apoptosis in cisplatin-treated tumour cells

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Received: August 27, 2008; Accepted: January 8, 2009

Abstract

The role of autophagy in cisplatin anticancer action was investigated using human U251 glioma, rat C6 glioma and mouse L929 fibrosarcoma cell lines. A dose- and time-dependent induction of autophagy was observed in tumour cells following cisplatin treatment, as demonstrated by up-regulation of autophagy-inducing protein beclin-1 and subsequent appearance of acridine orange-stained acidic autophagic vesicles. The presence of autophagosomes in cisplatin-treated cells was also confirmed by electron microscopy. Inhibition of autophagy with lysosomal inhibitors bafilomycin A1 and chloroquine, or a PI3 kinase inhibitor wortmannin, markedly augmented cisplatin-triggered oxidative stress and caspase activation, leading to an increase in DNA fragmentation and apoptotic cell death. The mechanisms underlying the protective effect of autophagy apparently involved the interference with cisplatin-induced modulation of Bcl-2 family proteins, as inhibition of autophagy potentiated cisplatin-mediated up-regulation of proapoptotic Bax and down-regulation of anti-apoptotic Bcl-2. Autophagy induction in cisplatin-treated cells was preceded by activation of adenosine monophosphate-activated protein kinase (AMPK) and concomitant down-regulation of mammalian target of rapamycin (mTOR)-mediated phosphorylation of p70S6 kinase. The ability of cisplatin to trigger autophagy was reduced by small interfering RNA (siRNA)-mediated AMPK silencing, while transfection with mTOR siRNA was sufficient to trigger autophagy in tumour cells. Finally, siRNA-mediated AMPK down-regulation and AMPK inhibitor compound C increased cisplatin-induced tumour cell death, while mTOR siRNA and AMPK activator metformin protected tumour cells from cisplatin. Taken together, these data suggest that cisplatin-triggered activation of AMPK and subsequent suppression of mTOR activity can induce an autophagic response that protects tumour cells from cisplatin-mediated apoptotic death.

Keywords: cisplatin • cancer • autophagy • apoptosis • AMPK • mTOR

Introduction

Cisplatin, one of the best-known anticancer agents, is a platinum-containing drug used as a first-line chemotherapy against epithelial malignancies such as lung, bladder, ovarian, testicular, head and neck, oesophageal, gastric, colon and pancreatic cancer, but also as a second- and third-line treatment against a number of metastatic malignancies, including breast and prostate cancer, melanoma, malignant gliomas and others [1]. The principal mode of its action is interaction with nucleophilic N7 sites of purine bases in DNA, leading to formation DNA–protein and DNA–DNA interstrand and intrastrand crosslinks [2]. Cisplatin-induced DNA damage results in inhibition of tumour cell division and induction of apoptosis (programmed cell death type I), which is characterized by activation of caspase enzyme family and consequent DNA fragmentation [2].

Macroautophagy (referred to hereafter as autophagy) is an alternative mode of cell demise, representing a self-cannibalization process that involves sequestration of cell structures in double-membraned organelles, called autophagosomes. This is followed by fusion of autophagosomes with lysosomes and formation of autophagolysosomes in which internal content is degraded by acidic lysosomal hydrolases [3]. The physiological role of autophagy is to remove long-lived proteins and damaged

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doi:10.1111/j.1582-4934.2009.00663.x

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 [4]. Induction of autophagy by some anticancer agents such as tamoxyfen, rapamycin, arsenic trioxide, temozolomide, brevinin-2R or fullerenes appears to be deleterious for tumour cells [5–11], thus indicating a possible use of autophagy as a 'magic bullet' in fighting apoptosis-resistant cancers [12]. On the other hand, a large body of evidence indicates that autophagy can also act as a survival mechanism that provides constituents necessary for sustaining essential cell metabolism in various stress conditions such as nutrient deprivation [13]. Moreover, inhibition of autophagy enhances the susceptibility of cancer cells to ionizing irradiation, hyperthermia, camptothecin, alkylating agents, hystone deacetylase inhibitors, sulindac sulphide and tumour necrosis factor-α [14–22], suggesting that tumour cells in some conditions might employ autophagy as a mechanism to evade therapy-induced death. It has recently been shown that cisplatin-triggered autophagy partially protects primary renal tubular epithelial cells from concomitant induction of apoptotic cell death by the drug [23–25]. The intracellular changes consistent with the induction of autophagy were also observed in non-tumorigenic NIH/3T3 mouse fibroblast cell line treated with cisplatin [26], but the role of autophagy in cell survival/apoptosis was not investigated in this study. Moreover, the ability of cisplatin to trigger autophagic response in tumour cells has not been evaluated thus far, although the understanding of the role that autophagy might play in cisplatin-induced apoptosis could provide novel approaches to cancer treatment.

Adenosine monophosphate-activated protein kinase (AMPK), which acts as a principal intracellular energy sensor, has recently been implicated in the induction of autophagy [27]. AMPK is activated in various cellular and environmental stress conditions when AMP/ATP ratio is elevated and it generally acts by switching off ATP-requiring processes, while switching on ATP-generating catabolic pathways [28]. The mechanism by which AMPK activates autophagic response presumably involves down-regulation of the kinase activity of mammalian target of rapamycin (mTOR), which has been known as an important negative regulator of autophagy [29]. AMPK can either promote or inhibit cell death in a context-dependent and/or cell type-dependent manner [30–32]. A recent study described that the induction of AMPK in tumour cells exposed to cisplatin was cytoprotective [33], but the possible involvement of AMPK in cisplatin-induced autophagy was not examined.

Development of tumour cell resistance to apoptosis induction is one of the major drawbacks in cisplatin therapy, and malignant gliomas are notorious for their ability to evade killing by conventional chemotherapeutics, including cisplatin [34]. In a search for novel approaches to increase cisplatin anticancer efficiency, we investigated the possible role of autophagy in cisplatin toxicity against U251 and C6 glioma cell lines, as well as the involvement of AMPK in cisplatin-induced autophagy in glioma cells.

Materials and methods

Chemicals, cells and cell cultures

All chemicals were from Sigma (St. Louis, MO, USA) except if stated otherwise. The mouse fibrosarcoma cell line L929 was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK), while the rat glioma cell line C6 and the human glioma cell line U251 were kindly donated by Dr Pedro Tranque (Universidad de Castilla-La Mancha, Albacete, Spain). The tumour cell lines were maintained at 37°C in a humidified atmosphere with 5% CO_2, in a HEPES (20 mM-buffered RPMI 1640 cell culture medium supplemented with 5% foetal calf serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 10 mM sodium pyruvate and penicillin/streptomycin. The cells were prepared for experiments using the conventional trypsinization procedure with trypsin/ethylenediaminetetraacetic acid and incubated in 96-well flat-bottom plates (1×10^4 cells/well) for the cell viability assessment and cell-based ELISA, 24-well plates (5×10^4 cells/well) for the flow cytometric analysis and RNA isolation, or in 90 mM Petri dishes (2×10^6 cells/dish) for Western blotting. Cells were rested for 24 hrs and then treated with cisplatin (4–50 μM), in the absence or presence of autophagy inhibitors bafilomycin A1 (100 ng/ml), wortmannin (100 nm) and chloroquine (20 μM), or AMPK modulators compound C (1 μM) and metformin (1 mM), as described in the 'Results' and figure legends.

Assessment of cell death and apoptosis

The release of intracellular enzyme lactate dehydrogenase (LDH), as a marker of cell membrane damage, and MTT assay for mitochondrial dehydrogenase activity were used to measure cell viability exactly as previously described [35]. The results were presented as a fold increase in comparison with control LDH release of untreated cells (arbitrarily set to 1) or as percentage of the mitochondrial dehydrogenase activity obtained in untreated cells (100%). DNA fragmentation, a marker of apoptosis, was assessed by flow cytometric analysis of cell stained with DNA-binding dye propidium iodide (PI). Briefly, cells were fixed in 70% ethanol and then incubated with RNase (50 μg/ml) and PI (40 μg/ml) for 30 min. at 37°C in the dark. Red fluorescence was analysed on a FACSCalibur flow cytometer (BD, Heidelberg, Germany), using FL2-W *versus* FL2-A dot plot to exclude cell aggregates and particle size-based gating to exclude cellular debris and necrotic cells. Cell distribution among cell cycle phases was determined by Cell Quest Pro software (BD) and hypodiploid cells in sub-G_0/G_1 compartment were considered apoptotic.

Determination of caspase activation and reactive oxygen species (ROS) production

Activation of caspases was measured by flow cytometry after labelling the cells with a cell-permeable, FITC-conjugated pan-caspase inhibitor (ApoStat; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The increase in green fluorescence (FL1) was considered as a measure of caspase activity within the individual cells of the treated population. Intracellular production of ROS was determined by measuring the intensity of green fluorescence emitted by redox-sensitive dye dihydrorhodamine 123 (DHR; Invitrogen, Paisley, UK), which was added to cell cultures (1 μM)

at the beginning of treatment. At the end of incubation, cells were detached by trypsinization, washed in PBS, and the green fluorescence (FL1) of DHR-stained cells was analysed using a FACSCalibur flow cytometer.

Autophagy detection by acridine orange staining and transmission electron microscopy (TEM)

The acidic autophagolysosomes were visualized by supravital acridine orange staining. After incubation, cells were washed with PBS and stained with acridine orange (1 μM; Sigma) for 15 min. at 37°C. Subsequently, cells were washed and analysed 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 analysed on a FACSCalibur flow cytometer using Cell Quest Pro software. The intensity of autophagy was quantified as red/green fluorescence ratio (FL3/FL1). For TEM, trypsinized cells were fixed with 2.5% glutaraldehyde in PBS, 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, Hillsboro, OR, USA).

Western blot analysis

Cells grown in a sub-confluent culture were lysed in lysis buffer (30 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonylfluoride and protease inhibitor cocktail) on ice for 30 min., centrifuged at 18,000 × *g* for 15 min. at 4°C, and the supernatants were collected as the cell lysates. Equal amounts of protein from each sample were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Marnes-la-Coquette, France). Following incubation with anti-beclin-1, anti-phospho-AMPK or anti-actin antibodies (Abcam, Cambridge, UK) as primary antibodies and peroxidase-conjugated goat anti-rabbit IgG (Jackson IP Laboratories, West Grove, PA, USA) as a secondary antibody, specific bands corresponding to beclin-1, phospho-AMPK and actin were visualized using enhanced chemiluminescence reagents for Western blot analysis (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

ELISA

The cell-based ELISA was performed as previously described [36], using rabbit polyclonal antibodies specific for beclin-1, phospho-AMPK and phospho-P70S6 kinase (S6K) (all from Abcam) as primary antibodies, while peroxidase-conjugated goat anti-rabbit IgG (Amersham Pharmacia Biotech) was used as secondary, detecting antibody. The absorbance at 450 nm was measured in an automated microplate reader after incubation with peroxydase substrate TMB and the obtained absorbances were normalized for cell number by crystal violet staining, as described in the original protocol [36]. ELISA for total human Bax-α and Bcl-2 in cell lysates was performed with DuoSet ELISA development Kit (R&D Systems) according to the manufacturer's instructions. The results were presented relative to the control value, which was arbitrarily set to 1.

Real-time RT-PCR analysis

Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Approximately 1 μg of RNA was used in the reverse transcription reaction using M-MuLV RT with random hexamers (Fermentas, Vilnus, Lithuania) according to the manufacturer's instructions. Real-time RT-PCR was performed on ABI Prism 7500 thermocycler (Applied Biosystems, Foster City, CA) in a 96-well reaction plate (MicroAmp Optical, ABI Foster City, CA, USA). The reactions were prepared according to the standard protocol for one-step QuantiTect SYBR Green RT-PCR (Applied Biosystems, Cheshire, UK), using commercially available primers for Bax-α or Bcl-2 (R&D Systems), and human GAPDH (Applied Biosystems) or rat β-actin (Sigma). Primers for β-actin were: 5′-CCCTGGCTCCTAGCACCAT-3′ (forward primer) 5′-GAGCCACCAATCCACACAGA-3′ (reverse primer), while GAPDH primers were 5′-CATCCATGACAACTTTGGTATCG-3′ (forward) and 5′-CCATCACGCCACAGTTTCC-3′ (reverse). Both GAPDH and β-actin primers were designed using Primer Express® software v2.0 (Applied Biosystems, Foster City, CA). Initial steps of RT-PCR were 2 min. at 50°C, followed by a 10 min. hold at 95°C. Cycles ($n = 40$) consisted of a 15-sec. melt at 95°C, followed by a 1-min. annealing/extension at 60°C. The final step was a 60°C incubation for 1 min. All reactions were performed in triplicates. The cycle of threshold (Ct) analysis was set at 0.1 relative fluorescence units. Averaged Ct values of control triplicates (GAPDH or actin) were subtracted from Ct values of target genes to obtain ΔCt, and then relative gene expression was determined as $2^{-\Delta Ct}$. The results were presented relative to the control value, which was arbitrarily set to 1. The S.D. values for the Bax/Bcl-2 ratio were calculated as follows: $C \times \sqrt{(a^2/A^2 + b^2/B^2)}$, where C = Bax/Bcl-2 ratio, while a and b represent S.D. of Bax (A) and Bcl-2 values (B), respectively.

Transfection with small interfering RNA

Small interfering RNA (siRNA) targeting human AMPKα1/2 and mTOR, as well as scrambled control siRNA, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). U251 cells were seeded in 96-well plates (1×10^4 cells/well) for cell-based ELISA and LDH release, 24-well plates (5×10^4 cells/well) for flow cytometric analysis and real-time RT-PCR, or 90 mM Petri dishes (2×10^6 cells/dish) for Western blotting. Transfection with siRNA was performed according to the manufacturer's protocol. Cells were allowed to grow 36 hrs following transfection and then treated with cisplatin. Subsequently, autophagy, LDH release, Bax/Bcl-2 mRNA levels and expression of beclin-1, phospho-AMPK or phospho-S6K were determined as outlined above.

Statistical analysis

The statistical significance of the differences between treatments was assessed using t-test or ANOVA followed by Student–Neuman–Keuls test for multiple comparisons. The value of $P < 0.05$ was considered significant.

Results

Cisplatin induces autophagy in tumour cells

Treatment with cisplatin for 16 hrs led to autophagy, as demonstrated by accumulation of acridine orange-stained red acidified

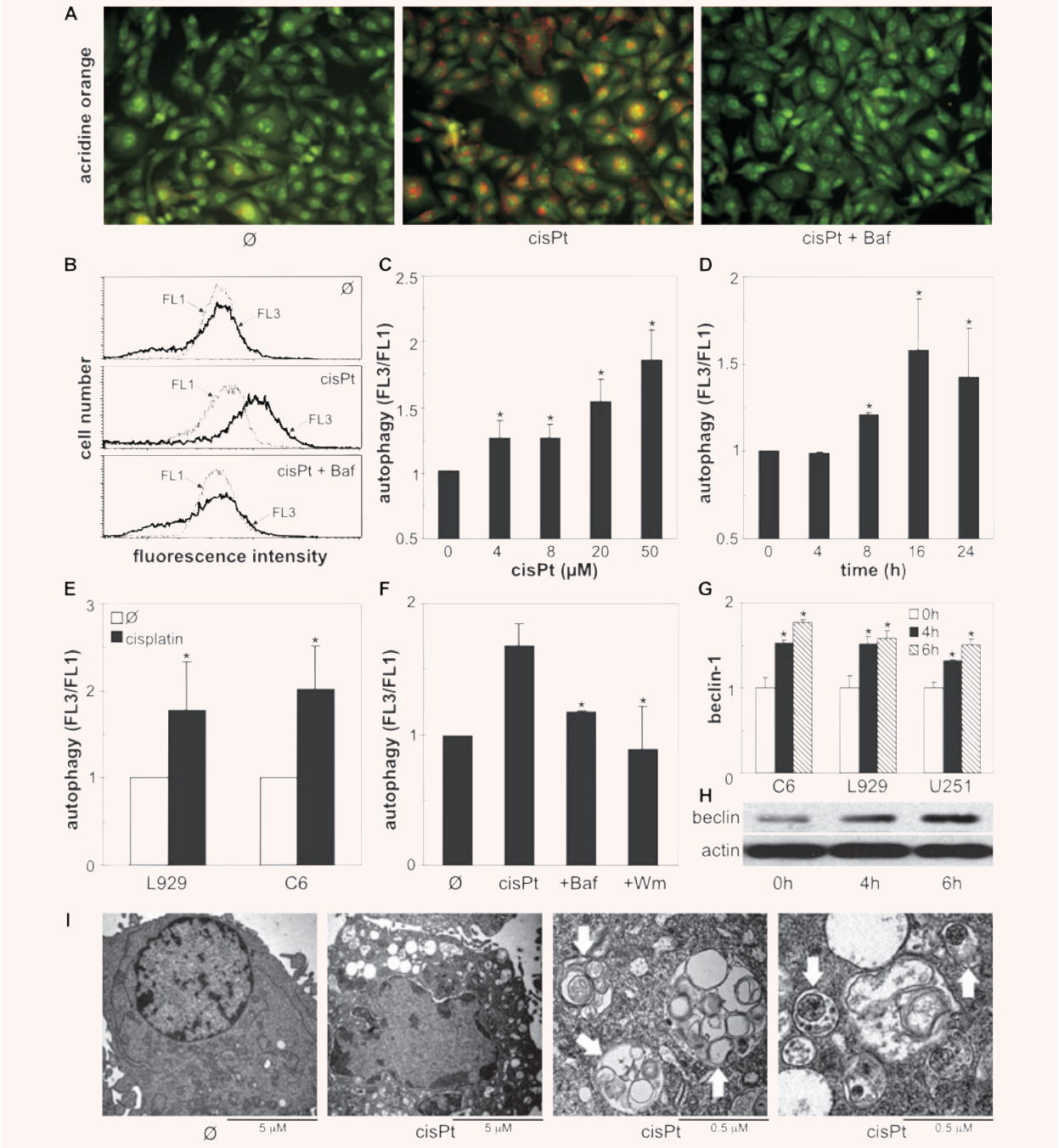

Fig. 1 Cisplatin-mediated induction of autophagy in tumour cells. (**A**, **B**) U251 glioma cells were incubated with cisplatin (cisPt 50 μM, 16 hrs) with or without bafilomycin A1 (Baf 100 ng/ml) and stained with acridine orange. The presence of autophagic vesicles was assessed by fluorescent microscopy (**A**) or flow cytometry (**B**). (**C**–**H**) U251 (**C**, **D**, **F**, **G**), L929 (**E**, **G**, **H**) and C6 cells (**E**, **G**) were incubated with different concentrations (**C**) or 50 μM (**D**–**H**) of cisplatin (cisPt) in the absence (**C**–**H**) or presence (**F**) of bafilomycin (Baf; 100 ng/ml) or wortmannin (Wm; 100 nm). Autophagy was analysed by flow cytometry after 16 hrs (**C**, **E**, **F**) or at the indicated time-points (**D**). Beclin-1 expression was determined at the indicated time-points by cell-based ELISA (**G**) or Western blotting (**H**). (**I**) TEM micrographs of control and cisplatin-treated (50 μM, 16 hrs) C6 cells. The arrows show typical autophagosomes (left), some of which contained cytoplasmic organelles (right). Data are mean + S.D. values from three independent experiments (**C**–**F**) or mean + S.D. values of triplicate measurements from a representative of three experiments (**G**).

vesicles in the cytoplasm of U251 human glioma cells (Fig. 1A). The accrual of acidic autophagolysosomes in cisplatin-treated cells was completely prevented by bafilomycin A1 (Fig. 1A), the vacuolar ATPase inhibitor that prevents fusion of autophagosomes with lysosomes. These findings were confirmed by flow cytometric analysis, showing that cisplatin-treated cells stained with acridine-orange displayed a bafilomycin-sensitive increase in red *versus* green (FL3/FL1) fluorescence ratio (Fig. 1B). Cisplatin-induced autophagy was both dose and time dependent, with the maximum observed after 16 hrs treatment with 50 μM of the drug (Fig. 1C and D). These conditions (16 hrs, 50 μM cisplatin) were therefore employed for the analysis of autophagy in subsequent experiments. Cisplatin also triggered the autophagic response in C6 rat glioma cells and L929 mouse fibrosarcoma cells (Fig. 1E), which was efficiently prevented by autophagy inhibitors bafilomycin and wortmannin, a PI3 kinase blocker (Fig. 1F). The increase in levels of beclin-1, a protein with a key role in autophagy, preceded the appearance of autophagic vesicles in all three cell lines, as demonstrated by cell-based ELISA analysis (Fig. 1G). The up-regulation of beclin-1 was confirmed by Western blot in L929 cells (Fig. 1H), while similar results were also obtained in C6 and U251 cells (not shown). Ultrastructural TEM analysis was consistent with the induction of autophagy by cisplatin (Fig. 1I). In addition to apoptotic changes in nuclear morphology, cisplatin-treated C6 cells displayed extensive cytoplasmic vacuolization (5 μM bar, right panel), with many vesicles showing multi-lamellar structure typical for autophagosomes (0.5 μM bar, left panel). The presence of vesicles engulfing cytoplasmic organelles (0.5 μM bar, right panel) confirmed initiation of autophagic digestion by cisplatin treatment.

Autophagy protects tumour cells from cisplatin-induced oxidative stress, caspase activation, DNA fragmentation and cell death

To assess the role of autophagy in cisplatin-induced death of tumour cells, we tested if cisplatin toxicity could be modulated with different autophagy-blocking agents – lysosomal inhibitors bafilomycin and chloroquine, and the PI3 class III inhibitor wortmannin. Each autophagy blocker significantly increased LDH release when administered simultaneously with cisplatin (Fig. 2A), without affecting LDH release in untreated cells (data not shown). These results were confirmed using MTT assay, which demonstrated that cisplatin-mediated decrease in mitochondrial dehydrogenase activity of U251 cells was potentiated by autophagy inhibitors (Fig. 2B). None of the autophagy inhibitors exerted a significant effect on tumour cell mitochondrial respiration in the absence of cisplatin (Fig. 2B; similar results were obtained with C6 and L929 cells – not shown). To get some insight into the mechanisms underlying the protective effect of autophagy, we assessed the effects of autophagy inhibitors on oxidative stress, caspase activation and DNA fragmentation, the hallmarks of apoptotic cell death. Expectedly, cisplatin treatment caused an increase in ROS production (Fig. 2C), caspase activation (Fig. 2D) and DNA fragmentation (Fig. 2E) in U251 glioma cells, as demonstrated by flow cytometric analysis of the cells stained with the appropriate reporter fluorochromes that react with ROS (DHR), activated caspases (ApoStat) or DNA (PI). Each of the three autophagy inhibitors markedly

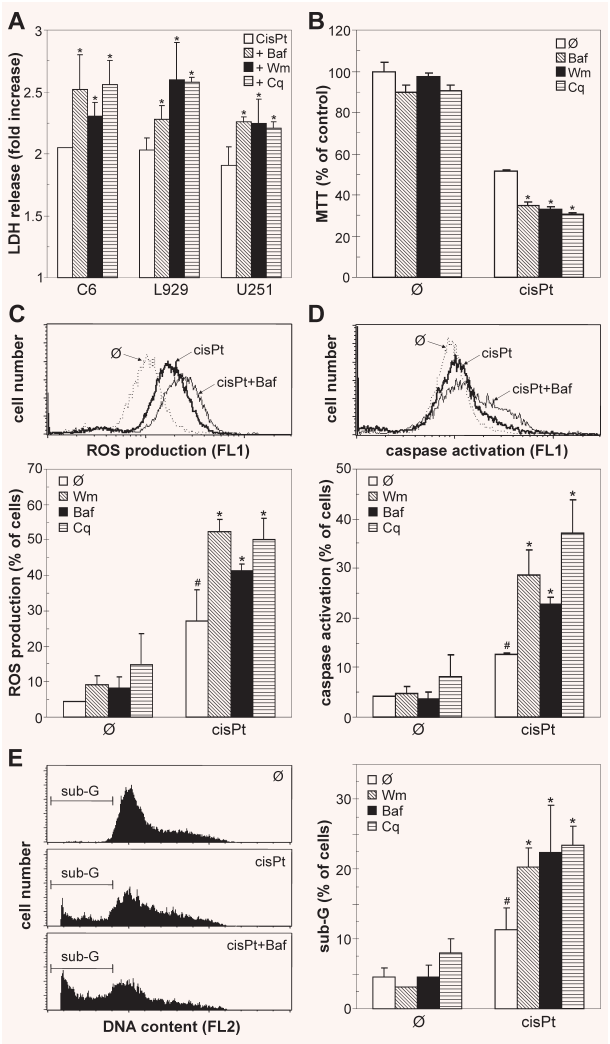

Fig. 2 The effects of autophagy inhibitors on cisplatin-induced apoptotic cell death. U251 cells (**A**–**E**), C6 or L929 cells (**A**) were incubated with cisplatin (cisPt; 50 μM) in the absence or presence of bafilomycin (Baf; 100 ng/ml), wortmannin (Wm; 100 nm) or chloroquine (Cq; 20 μM). LDH release (**A**), MTT reduction (**B**) and DNA fragmentation (**E**) were determined after 24 hrs, while ROS production (**C**) and caspase activation (**D**) were assessed after 16 hrs. (**A**, **B**) Data are mean + S.D. values of triplicates from a representative of three experiments. (**C**, **D**, **E**) Representative flow cytometry histograms are presented, while the accompanying graphs contain mean + S.D. values from three different experiments ($^{\#}P < 0.05$ and $^{*}P < 0.05$ refer to untreated and cisplatin-treated cells, respectively).

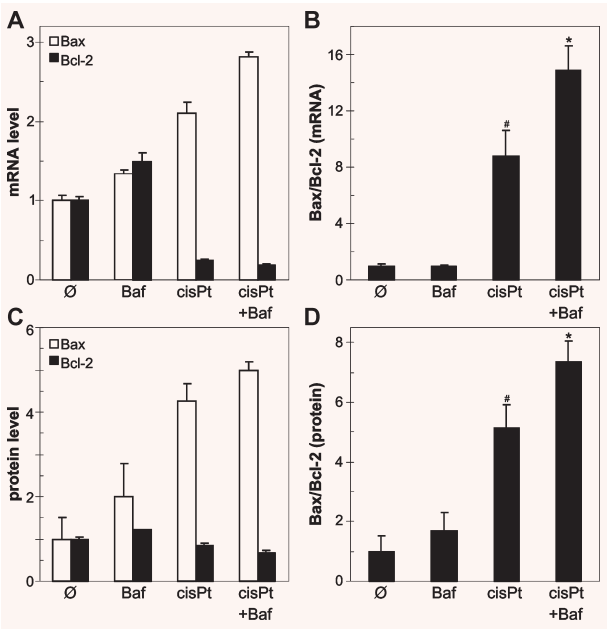

Fig. 3 The effect of bafilomycin A1 on Bax and Bcl-2 mRNA and protein levels. (**A**–**D**) U251 glioma cells were incubated with cisplatin (cisPt; 50 μM) and/or bafilomycin (Baf; 100 ng/ml). The intracellular levels of Bax and Bcl-2 mRNA (**A**) or protein (**C**) were determined by real time RT-PCR (**A**) or ELISA (**C**) after 8 or 16 hrs, respectively, and Bax/Bcl-2 ratio was calculated (**B**, **D**). Data are mean + S.D. values of triplicate measurements from a representative of three experiments ($^{\#}P < 0.05$ and $^{*}P < 0.05$ refer to untreated and cisplatin-treated cells, respectively).

increased cisplatin-induced oxidative stress, caspase activation and DNA fragmentation (Fig. 3C–E), thus further corroborating that autophagy was involved in tumour cell protection from cisplatin-induced apoptosis. Similar results were obtained with C6 glioma and L929 fibrosarcoma cells (data not shown).

Autophagy inhibits Bax/Bcl-2 ratio increase in cisplatin-treated tumour cells

Since dysregulation of the balance between proapoptotic (Bax) and anti-apoptotic (Bcl-2) members of the Bcl-2 family of intracellular proteins can lead to caspase activation and apoptosis, we investigated the role of autophagy in modulating Bax/Bcl-2 ratio in cisplatin-exposed tumour cells. Consistent with its pro-apoptotic action, cisplatin increased expression of Bax while reducing that of Bcl-2 in U251 glioma cells (Fig. 3A and C), which resulted in the increase in Bax/Bcl-2 ratio at both mRNA (Fig. 3B) and protein levels (Fig. 3D). The autophagy inhibitor bafilomycin alone was apparently able to induce the expression of both Bax and Bcl-2 mRNA (Fig. 3A), but the effect was somewhat less pronounced at the protein level (Fig. 3C) and did not result in a significant disturbance of Bax/Bcl-2 ratio (Fig. 3B, D). Nevertheless, bafilomycin further augmented cisplatin-induced alterations in Bax and Bcl-2 mRNA (Fig. 3A) and protein levels (Fig. 3C) in U251 glioma cells, which was accordingly reflected in a significant increase in Bax/Bcl-2 ratio (Fig. 3B, D). Similar results were obtained with C6 glioma cells (data not shown). It therefore appears that autophagy might protect tumour cells from cisplatin-mediated apoptosis by modulating the expression of Bcl-2 family members and preventing the pro-apoptotic increase in Bax/Bcl-2 ratio.

AMPK/mTOR pathway mediates cisplatin-induced autophagy

We next assessed the involvement of AMPK and mTOR signalling pathways in autophagy induction by cisplatin. Both cell-based ELISA and Western blot demonstrated an increase in AMPK phosphorylation in tumour cells after 2–4 hrs of cisplatin treatment (Fig. 4A and B; similar Western blot data were obtained in other cell lines – not shown). Cisplatin-triggered activation of AMPK was accompanied by a reduced phosphorylation/activation of S6K (Fig. 4C), an important mTOR target and its down-stream mediator. To get some further insight into the role of AMPK activation and mTOR down-regulation in cisplatin-triggered autophagy, a siRNA-mediated gene silencing was used. The ability of AMPK and mTOR siRNA to block the expression of AMPK and mTOR mRNA in U251 cells was confirmed by real-time RT-PCR analysis (data not shown). Accordingly, transfection of U251 cells with AMPK siRNA led to a significant reduction in both basal and cisplatin-induced AMPK activation, compared to control siRNA-transfected cells (Fig. 4D; insert shows Western blot confirmation of reduced phospho-AMPK levels in AMPK siRNA-transfected cells). Consistent with the role of AMPK in down-regulation of mTOR activity, cells transfected with AMPK siRNA displayed an increase in S6K activation, and cisplatin-mediated inhibition of S6K phosphorylation was markedly less pronounced (Fig. 4E). Cisplatin-triggered induction of beclin-1 expression (Fig. 4F) and autophagy (Fig. 4G) were also impaired in AMPK siRNA-transfected cells, thus confirming that AMPK activation was a principal mechanism underlying these events. In U251 cells transfected with siRNA targeting mTOR, compared with control siRNA, no difference was observed in basal or cisplatin-induced AMPK activation (Fig. 4D). On the other hand, basal S6K phosphorylation was markedly lower in mTOR siRNA-transfected cells and only marginally reduced by cisplatin treatment (Fig. 4E). Silencing of mTOR was sufficient to trigger beclin-1 up-regulation (Fig. 4F) and autophagy in U251 cells (Fig. 4G), thus indicating that mTOR down-regulation was involved in autophagy induction by cisplatin. However, cisplatin further increased both beclin-1 expression (Fig. 4F) and autophagy (Fig. 4G) in mTOR siRNA-transfected cells, indicating that these effects of the drug were partly independent of mTOR suppression. These data suggest that cisplatin-mediated induction of beclin-1 expression and autophagy were at least partly mediated by AMPK activation and subsequent down-regulation of mTOR.

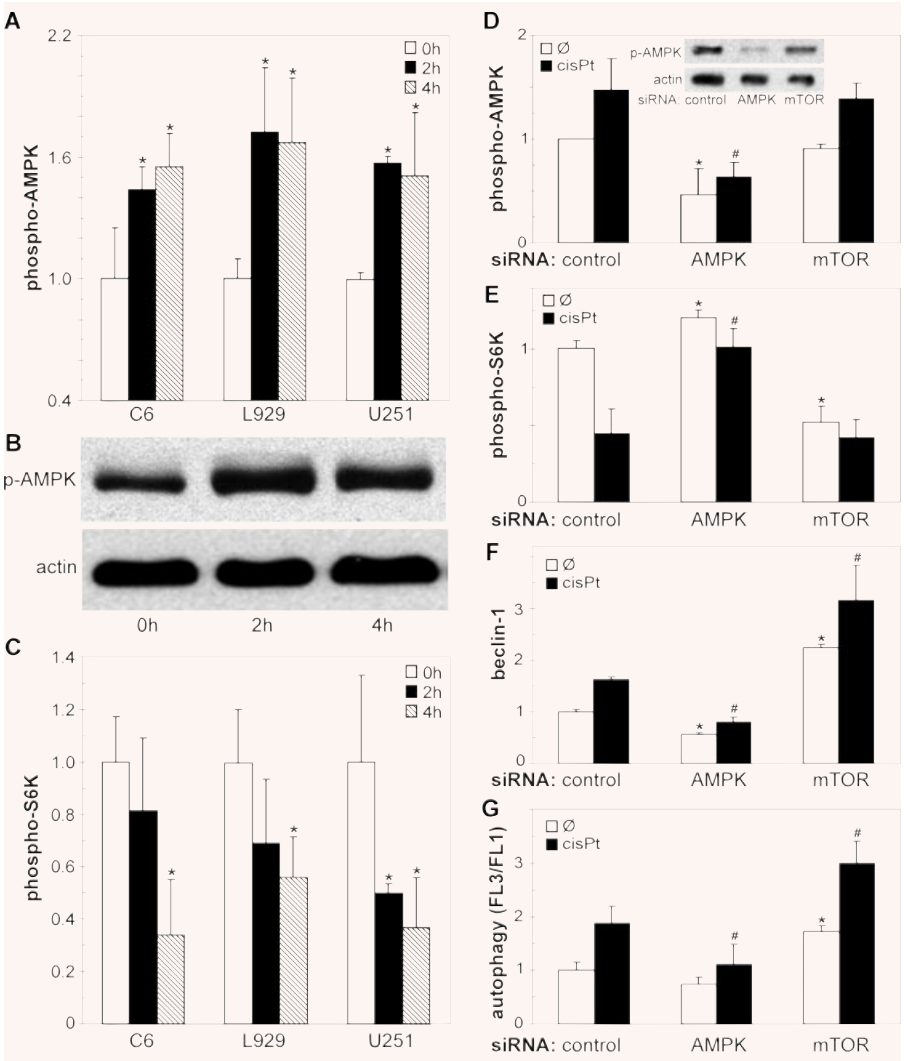

Fig. 4 The involvement of AMPK and mTOR in autophagy induction by cisplatin. (**A**, **C**) U251, C6 and L929 cells (**A**–**C**) were incubated with cisplatin (50 μM) and the levels of phosphorylated AMPK (**A**, **B**) and phosphorylated S6K (**C**) were determined by cell-based ELISA (**A**, **C**) or Western blotting (**B**) after 2 and 4 hrs. (**D**–**G**) U251 cells transfected with control, AMPK or mTOR siRNA were incubated in the absence or presence of cisplatin (cisPt; 50 μM). The levels of phospho-AMPK, phospho-S6K and beclin-1 were assessed by cell-based ELISA after 4 hrs, while the autophagy was measured by flow cytometry after 16 hrs. Insert in (**D**) shows Western blot analysis of phospho-AMPK level in untreated cells. (**A**, **C**, **D**–**G**) Data are mean + S.D. values of triplicate observations from a representative of three experiments ($^{*}P < 0.05$ and $^{\#}P < 0.05$ refer to control siRNA-transfected untreated and cisplatin-treated cells, respectively).

AMPK and mTOR modulate survival of cisplatin-treated tumour cells

To further confirm the involvement of AMPK in cisplatin-induced autophagy, we tested whether siRNA-mediated AMPK silencing could mimic the stimulatory effect of autophagy inhibition on cisplatin toxicity. Indeed, siRNA-mediated down-regulation of AMPK activity in U251 cells augmented the cisplatin toxicity, as demonstrated by an increase in cisplatin-induced LDH release in AMPK siRNA-transfected, compared to control cell cultures (Fig. 5A). Accordingly, AMPK silencing also mimicked bafilomycin-mediated increase in Bax/Bcl-2 mRNA ratio in cisplatin-treated U251 cells (Fig. 5B and C). On the other hand, transfection with mTOR siRNA inhibited cisplatin-mediated tumour cell killing (Fig. 5A), thus indicating that mTOR down-regulation might be involved in the cell protection by AMPK. The protective role of AMPK in cisplatin-mediated tumour cell killing was confirmed by using compound C, an AMPK inhibitor, and metformin, an AMPK activator, which respectively enhanced and reduced cisplatin-triggered LDH release in C6, U251 and L929 cell cultures (Fig. 5D–F).

Discussion

The presented data demonstrate the ability of cisplatin to initiate an autophagic response in different tumour cell lines, probably through AMPK/mTOR signalling pathway. Importantly, cisplatin-induced autophagy protected tumour cells from the concomitant induction of apoptotic death. It should be noted that pharmacological

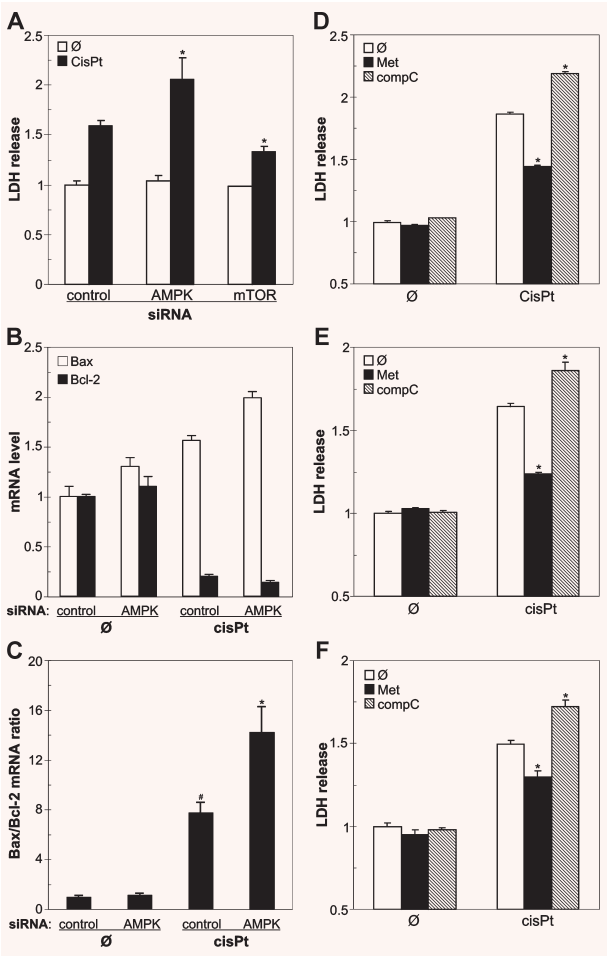

Fig. 5 Modulation of cisplatin-induced tumour cell death by AMPK and mTOR. (**A**) U251 cells transfected with control, AMPK or mTOR siRNA were incubated in the absence or presence of cisplatin (cisPt; 50 μM) and the LDH release was measured after 24 hrs. (**B**, **C**) The levels of Bax and Bcl-2 mRNA were determined by real-time RT-PCR in untreated and cisplatin (50 μM)-treated cells (8 hrs) transfected with control or AMPK siRNA (**B**), and Bax/Bcl-2 mRNA ratio was calculated (**C**). (**D**–**F**) U251 (**D**), C6 (**E**) and L929 cells (**F**) were incubated with cisplatin (50 μM) in the absence or presence of metformin (Met; 1 mM) or compound C (compC; 1 μM) and the LDH release was determined after 24 hrs. (**A**–**D**, **F**) Data are mean + S.D. values of triplicate observations from a representative of three (**A**) or two (**B**–**F**) experiments ($^{\#}P < 0.05$ and $^{*}P < 0.05$ refer to untreated and cisplatin-treated cells, respectively).

inhibition of autophagy at different stages may exert different effects on cell death [8, 15]. However, both early stage inhibitors (wortmannin) and late stage autophagy blockers (bafilomycin and chloroquine) augmented cisplatin toxicity in our study, thus supporting a role for autophagy in suppression of cisplatin-triggered apoptotic death of tumour cells. Apparently, the observed effects were not tumour cell type- or species specific, as similar results were observed in human U251 and rat C6 glioma cell lines, as well as in mouse L929 fibrosarcoma cells.

An increasing body of evidence supports the existence of a cross-talk between apoptosis and autophagy, including both positive and negative interactions [4]. However, only two studies so far examined this possibility in cisplatin-treated cells [23, 24]. Both studies were performed on renal tubular epithelial cells, demonstrating that the inhibition of autophagy was accompanied by an increase in cisplatin-induced caspase activation and subsequent nuclear fragmentation [23, 24], which are principal events in apoptotic cell death [37]. Our data extend these findings to cancer cells by showing that autophagy apparently restricts cisplatin-triggered activation of caspases and DNA fragmentation in U251 and C6 glioma and L929 fibrosarcoma cells. The mechanisms underlying these observations might include the interference of autophagy with the cisplatin-mediated dysregulation of the balance between proapoptotic and anti-apoptotic members of Bcl-2 family, as suggested by our data that pharmacological blockade of autophagy increases Bax/Bcl-2 ratio in cisplatin-treated tumour cells. The up-regulation of proapoptotic Bax and/or down-regulation of its inhibitor Bcl-2, followed by Bax incorporation into mitochondrial membrane and release of caspase-activators such as cytochrome c, is one of the crucial events in tumour cell apoptosis induced by cisplatin [38–42]. The autophagy-mediated decrease in Bax/Bcl-2 ratio in our study is consistent with the finding that arsenic trioxide-induced autophagy in U118 human glioma cells, which occurred in the absence of apoptosis, was accompanied by down-regulation of Bax and up-regulation of Bcl-2 protein levels [43]. Moreover, inhibition of autophagy during ischemia/reperfusion injury increased Bax mitochondrial translocation in cardiac myocytes [44] thus further supporting the possible role of autophagy in regulation of Bax/Bcl-2 balance. While this could be achieved through selective autophagic proteolysis of different Bcl-2 family proteins or interaction of beclin-1 with Bcl-2 [45], our RT-PCR data also indicate that autophagy could somehow modulate the expression of Bax and Bcl-2 genes.

In addition to increasing Bax/Bcl-2 ratio, caspase activation and DNA fragmentation as the key apoptotic parameters, inhibition of autophagic response markedly increased ROS production in cisplatin-treated tumour cells in our experiments. These results indicate a protective role of autophagy against oxidative stress, which has been known to promote cisplatin-triggered apoptosis through activation of redox-sensitive apoptotic signalling pathways, as well as *via* direct mitochondrial damage and subsequent release of apoptogenic factors [46–48]. Such a hypothesis is consistent with data obtained in different experimental systems, showing that autophagy attenuates oxidative stress through lysosome-mediated removal of oxidized proteins, thus preventing their involvement in perpetuation of oxidative damage [49, 50]. An overproduction of ROS is also involved in transcriptional and post-transcriptional perturbation of Bax/Bcl-2 balance [51, 52], which then leads to mitochondrial dysfunction and further increase in cell-damaging ROS production by mitochondria [53, 54]. Thus, our data indicate that autophagy might interfere with this proapoptotic positive feedback between ROS and Bcl-2 family proteins in cisplatin-treated tumour cells.

In a recent study, Kim *et al.* have described that cisplatin-mediated activation of AMPK protects human gastric carcinoma and colon carcinoma cells from the concomitant induction of apoptotic

cell death, through mechanisms involving down-regulation of p53/p21 and Bax expression [33]. Using siRNA-mediated AMPK silencing, we have confirmed the ability of AMPK to interfere with cisplatin-induced increase in Bax/Bcl-2 mRNA ratio in glioma cells. Moreover, our results seem to link the cytoprotective action of AMPK to the expression of beclin-1 and subsequent activation of autophagic response in cisplatin-treated glioma and fibrosarcoma cells. This observation is consistent with the previously reported autophagy-inducing role of AMPK in nutrient-starved hepatocytes [55], ischemic cardiac myocytes [56] and avicin D-treated cancer cells [57]. In some of these studies, AMPK-dependent induction of autophagy was mediated by downstream inactivation of mTOR [56, 57], a serine/threonine kinase that inhibits autophagy by blocking the expression of autophagy-inducing Atg proteins and/or through direct interference with their function in autophagosome formation [58]. Pharmacological suppression of mTOR by rapamycin, as well as transfection with dominant-negative mTOR or mTOR siRNA, are sufficient for the induction of autophagy in cancer cells [7, 59], as confirmed in our experiments using siRNA-mediated mTOR silencing. Since cisplatin-mediated AMPK activation in our study was clearly accompanied by a decrease in phosphorylation of the main mTOR target S6K, it seems conceivable to assume that cisplatin-triggered autophagy in cancer cells was at least partly mediated by mTOR down-regulation. The involvement of AMPK in cisplatin-mediated mTOR inactivation is supported by the finding that cisplatin could not significantly reduce S6K activation in glioma cells transfected with AMPK-targeting siRNA. Interestingly, although cisplatin did not cause additional mTOR suppression in mTOR siRNA-transfected cells, it was still able to increase both beclin-1 expression and autophagy. While this clearly indicates the existence of some mTOR-independent pathway(s) for the autophagy induction by cisplatin, the mechanisms underlying this effect remain to be explored.

In conclusion, the present study indicates that the cytoprotective role of AMPK in cisplatin-treated tumour cells is mediated through mTOR inhibition-dependent autophagy, which prevented cisplatin-triggered Bax/Bcl-2 increase and subsequent caspase activation. It should be noted, however, that beclin-1 has been shown to increase cisplatin-induced caspase activation and apoptosis in MKN28 human gastric cancer cells [60], which is consistent with our preliminary findings that autophagy inhibitors can protect B16 mouse melanoma cells from cisplatin-mediated cell death. In addition, inactivation of mTOR target S6K increased cisplatin sensitivity of human small cell lung cancer H69 cells [61], in contrast to cell-protective role of AMPK-mediated mTOR down-regulation in our study. It therefore appears that, depending on the tumour cell type, autophagy and/or AMPK/mTOR pathway might play distinct roles in cisplatin anticancer action. While this hypothesis remains to be further examined, our results nevertheless support the exploration of AMPK- and/or autophagy-modulating agents as adjuncts to cisplatin-based therapy of some cancers.

Acknowledgement

This work was supported by the Ministry of Science of the Republic of Serbia (Grants No. 145073, 143029 and 145058).

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