Autophagy blockade sensitizes the anticancer activity of CA-4 via JNK-Bcl-2 pathway

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ABSTRACT

Combretastatin A-4 (CA-4) has already entered clinical trials of solid tumors over ten years. However, the limited anticancer activity and dose-dependent toxicity restrict its clinical application. Here, we offered convincing evidence that CA-4 induced autophagy in various cancer cells, which was demonstrated by acidic orange staining of intracellular acidic vesicles, the degradation of p62, the conversion of LC3-I to LC3-II and GFP-LC3 punctate fluorescence. Interestingly, CA-4-mediated apoptotic cell death was further potentiated by pretreatment with autophagy inhibitors (3-methyladenine and bafilomycin A1) or small interfering RNAs against the autophagic genes (Atg5 and Beclin 1). The enhanced anticancer activity of CA-4 and 3-MA was further confirmed in the SGC-7901 xenograft tumor model. These findings suggested that CA-4-elicted autophagic response played a protective role that impeded the eventual cell death while autophagy inhibition was expected to improve chemotherapeutic efficacy of CA-4. Meanwhile, CA-4 treatment led to phosphorylation/activation of JNK and JNK-dependent phosphorylation of Bcl-2. Importantly, JNK inhibitor or JNK siRNA inhibited autophagy but promoted CA-4-induced apoptosis, indicating a key requirement of JNK-Bcl-2 pathway in the activation of autophagy by CA-4. We also identified that pretreatment of Bcl-2 inhibitor (ABT-737) could significantly enhance anticancer activity of CA-4 due to inhibition of autophagy. Taken together, our data suggested that the JNK-Bcl-2 pathway was considered as the critical regulator of CA-4-induced protective autophagy and a potential drug target for chemotherapeutic combination.

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Introduction

In the case of tumor vascular disrupting agents (VDAs), the tubulin binding natural product CA-4, originally isolated from the South African Combretum caffrum tree, possesses favorable anticancer activity. Its water-soluble phosphate prodrug (CA-4P) has already entered clinical trials of various types of solid tumor by decreasing tumor blood flow and inducing necrosis in the central core of tumors (Dowlati et al., 2002; Rustin et al., 2003). However, CA-4P has not showed consistent single-agent activity in clinical trials. It induces little tumor growth retardation with evident peripheral residue and recurrence, which may be due to the limited anticancer activity. Besides, the increment of systemic administration of CA-4P can cause dose-dependent toxicity such as tumor pain, myocardial ischemia and prolonged QT interval (Nagaiah and Remick, 2010). Therefore, numerous attempts have been made to improve antitumor activity and reduce toxicity, including a combination with chemotherapeutic agents, such as 5-fluorouracil, paclitaxel and TRAIL/Apo-2L (Zhang et al., 2011; Zweifel et al., 2011). However, those combination regimens still failed in the clinical applications. In this case, the mechanisms warrant investigation to enhance the anticancer activity of CA-4.

Autophagy is a lysosomal degradation pathway for the degradation of redundant or faulty cell components (Meijer and Codogno, 2004). It can be stimulated by various stress situations, including external stimuli (e.g. hypoxia, energy depletion, pharmacological agents), as well as internal stressors. Autophagy is regulated at the molecular level by a family of dedicated genes called Atg (AuTophaGy-related) genes and is executed at basal levels in virtually all cells as a homeostatic mechanism for maintaining cellular integrity. Apart from its normal role in cell physiology, extensive studies reveal that alterations in autophagy occur in various human diseases, including neurodegeneration, steatosis, infection, aging and cancer (Baehrecke, 2005; Mathew et al., 2007; White and DiPaola, 2009). Especially, the double-edged sword function of autophagy in cancer, acting either as both a tumor suppressor by preventing the accumulation of damaged proteins and organelles or as a mechanism of cell survival that can promote the growth of established tumors, has been widely recognized and remains of great interest (Pan et al., 2013; White and DiPaola, 2009). Especially, the double-edged sword function of autophagy in cancer, acting either as both a tumor suppressor by preventing the accumulation of damaged proteins and organelles or as a mechanism of cell survival that can promote the growth of established tumors, has been widely recognized and remains of great interest (Pan et al., 2013; White and DiPaola, 2009). Currently, there is an accumulation of evidence for autophagy’s involvement in mediating resistance of cancer cells to chemotherapy, suggesting that autophagy may also be induced by CA-4 as prosurvival response (Carew et al., 2007; Liu et al., 2011).
In the present study, we demonstrated for the first time that CA-4 could induce autophagic response in various cancer cell lines. Furthermore, inhibition of autophagy by specific chemical or genetic inhibitors aggravated the cell death in response to CA-4 stimulation. In addition, CA-4 plus autophagy inhibitor (3-MA) caused marked tumor growth inhibition compared with single agents alone on human gastric cancer xenografts in nude mice, suggesting that CA-4-induced autophagy might play a self-protective role against its own cytotoxic effect. Our data also showed that the autophagy induced by CA-4 was independent of mTOR and was mediated through activation of JNK and subsequent phosphorylation of Bcl-2, ultimately leading to autophagy-dependent interference with CA-4-induced apoptosis. Thus, targeting the autophagic pathway might provide a new strategy for the potential use of CA-4.

Materials and methods

Reagents. CA-4 (~99% purity) was bestowed as a gift by Dr. Jinshan Shen (Shanghai Institute of Materia Medica, Chinese Academy of Science, Shanghai, China), dissolved in dimethyl sulfoxide (DMSO) (10.0 mM stock solution) and stored at −20 °C. DMSO, propidium iodide (PI), sulforhodamine B (SRB), acridine orange (AO), SP600125, bafilomycin A1 (Baf A1) and 3-methyladenine (3-MA) were purchased from Sigma Chemical Co. (St. Louis, MO). ABT-737 was obtained from Selleck Chemicals LLC (Houston, TX). The primary antibodies against PARP, procaspase-3, β-actin, p-p70S6K, p-4EBP1, Beclin 1, Aktg, Bcl-2, JNK, and HRP-labeled secondary anti-goat, anti-mouse and anti-rabbit antibodies were from Santa Cruz Biotech (Santa Cruz, CA); LC3, PI3K III, p-Akt (ser473), p-mTOR (ser2448), cleaved caspase-3, JNK-P (Thr183/Tyr185), Bcl-2-P (Ser70) and p62 were from Cell Signaling Technology (Beverly, MA).

Cell lines and cultures. All the cell lines were purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Human gastric cancer cells SGC-7901 and hepatocellular carcinoma cells SMMC-7721 were maintained in RPMI 1640 (Invitrogen, USA), human breast tumor cells MDA-MB-231 was cultured in L15 medium (Invitrogen, USA). All media were supplemented with 10% fetal bovine serum (Invitrogen, USA) in a humidified atmosphere of 5% CO2 at 37 °C.

Detection of apoptosis by PI staining. Apoptosis was analyzed by measuring sub-G1 peaks (indication of DNA fragmentation) on a flow cytometer after cells were fixed with ethanol and stained with PI as described before (Liu et al., 2010).

Western blot analysis. Western blot analysis was performed as described before (Liu et al., 2010). The same amount of protein was loaded in each lane, separated by 8% SDS-PAGE and then transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in PBST for 1 h at room temperature, and then incubated with the appropriate primary antibodies (dilution range 1:500–1:1000) overnight at 4 °C in PBST. After three washes in PBST, the membrane was incubated for 1 h at room temperature with the appropriate horseradish peroxidase-labeled secondary antibody at 1:5000 dilution. Bound antibodies were detected using ECL.

Immunoprecipitation. Cell lysates (500 μg of total protein) were incubated with a slow rotation for 4 h with Bcl-2 antibodies (1:25) followed by Protein A/G-conjugated agarose for an additional 1 h. The beads were washed four times with lysis buffer containing protease inhibitor and phosphatase inhibitors and the supernatant was removed by centrifugation (3000 g, 1 min). The pellet was mixed with a loading buffer and a denaturing agent, and the sample was heated to 95 °C for 10 min followed by Western blot.

Acridine orange staining. AVOs were quantified by flow cytometry after cells were stained by AO as described before (Xi et al., 2011). Briefly, cells were treated with or without CA-4 for 8 h. Cells were then stained with 1 μg/mL AO in complete medium at 37 °C for 15 min, and examined by flow cytometry within 1 h.

Determination of autophagic activity. GFP-LC3 cells presented a diffuse distribution under control, while a punctate pattern of GFP-LC3 was increased in number and fluorescence intensity by autophagy (Zhao et al., 2010). SCC-7901, SMMC-7721 and MDA-MB-231 cells were plated in 24-well plates, and transfected with a green fluorescent protein (GFP)-tagged LC3 expressing vector (2 μg/well) using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 24 h transfection, cells were exposed to CA-4 for an additional 24 h. The punctate patterns of LC3 in transfected cells were visualized by fluorescence microscopy (Leica DMI 4000 B).

siRNA and plasmid transfection. The siRNA sequence was duplexes produced by GenePharma, Co (Shanghai, China). The optimal sense sequences of the Atg5, Beclin 1 and JNK-1 siRNA were and 5′-3′ GGAA UAUCCUCGAGAAAUU, 5′-3′ CAGTTGGCACATAAAT, and 5′-3′ UCACAGUCGAGAACAAU, respectively (Fung et al., 2008; Shen et al., 2008; Zhao et al., 2010). The transfection was performed using Opti-MEM, siRNA and oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations.

Cell proliferation assay. In vitro cell proliferation assays were performed using the SRB assay, as previously described (Zhang et al., 2013).

Animals and antitumor activity in vivo. Human gastric cancer SGC-7901 xenograft was established by injecting 5 × 106 cells subcutaneously into nude mice. When the tumors reached a volume of 50–150 mm³, the mice were randomized to control and treated groups, and received vehicle (0.9% sodium chloride, i.p. administration), CA-4 (20 mg/kg, 1% DMSO), 7% Cremophor/ethanol (3:1), and 92% sodium chloride, i.p. administration), 3-MA (20 mg/kg, i.p. administration) every 3 days for a total of 8 times (days 4, 7, 10, 13, 16, 19, 21 and 24). Mice weight and tumor volume were measured individually every 3 days with microlipers until animals were euthanized. Tumor volume was calculated using the formula: V = 0.5 × a × b², where a and b were the long and short diameters of the tumor, respectively. The tumor volume at day n was expressed as RTV according to the following formula: RTV = TVn / TV0, where TVn was the tumor volume at day n and TV0 and short diameters of the tumor, respectively. The tumor volume at day n was expressed as RTV according to the following formula:

\[
RTV = \frac{TV_n}{TV_0}
\]

where TVn was the tumor volume at day n and TV0 was the tumor volume at day 0. Therapeutic effects of treatment were expressed in terms of T/C % using the calculation formula T/C (%) = mean RTV of the treated group/mean RTV of the control group × 100%. Animal studies were preformed in accordance with protocols approved by the ZJU Animal Subjects Committee.

Assay of cardiac and hepatic marker enzymes. Activities of creatine kinase-MB (CK-MB), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the serum were measured using commercial kits (Biosino Bio-technology and Science Inc., Beijing, China).

Statistical analysis. Unless otherwise stated, results were expressed as mean ± SD of three independent experiments. The significance of differences between the experiment conditions was determined by the two tailed Student’s t-tests and indicated with p < 0.05*, p < 0.01** and p < 0.001***.

Results

Autophagy was induced in several cancer cell lines after CA-4 treatment

AO is a lysosomotropic agent and is able to stain the acidic vesicular organelles (AVOs), which offers a rapid and quantitative method to measure induction of autophagy (Xi et al., 2011). Firstly, to investigate whether CA-4 could induce autophagy in three human cancer cell lines
including human gastric cancer cells SGC-7901, breast tumor cells MDA-MB-231 and human hepatocellular carcinoma SMMC-7721 cells, CA-4-treated cancer cells were stained with AO to detect and quantify AVOs. FACS analysis showed that the percentage of cells with AVOs was higher in the CA-4-treated group compared with the control group (Figs. 1A and B). To confirm that the observed vesicles were indeed related to autophagy, we then examined LC3 conversion and p62 degradation, two selective markers of autophagy. As shown in Fig. 1C, we observed the conversion of the LC3 (LC3-I to LC3-II) and the degradation of p62 in these cell lines shortly after 24 h exposure to the indicated concentrations of CA-4. Consistent with Western blot results, CA-4 treatment showed increased percentage of cells displaying punctate GFP-LC3 after 24 h than control group (Fig. 1D), providing further evidence that the autophagic process was being activated by CA-4 treatment.

Fig. 1. CA-4 induced autophagy in human cancer cells. (A) The formation of AVOs was quantified by flow cytometry after AO staining in SGC-7901, SMMC-7721 and MDA-MB-231 cells treated with CA-4 for 8 h. The gated region (M1) in each histogram represented the percentage of cells with AVOs. (B) Percentage of AVOs development in total cells was calculated from 3 independent experiments. (C) SGC-7901, SMMC-7721 and MDA-MB-231 cells were treated with CA-4 for 24 h, and whole cell lysates were then subjected to immunoblotting with antibodies against p62, LC3 and β-actin. Numbers below each band indicated the relative ratio between LC3-II and LC3-I isoforms after normalized to β-actin. (D) Formation of GFP-LC3 vacuoles (dots) was detected after CA-4 treatment for additional 24 h. Scale bars, 10 μm. The percentage of cells with punctate GFP-LC3 fluorescence was calculated by randomly counting 200 cells under the fluorescent microscope.
Autophagy inhibition enhanced CA-4-induced apoptosis

Recent data suggest that autophagy inhibition may enhance chemosensitization in human cancer cells (Carew et al., 2007; Liu et al., 2011). Thus, we determined whether inhibition of autophagy, utilizing pharmacological or genetic means, could enhance CA-4-induced apoptosis. To inhibit autophagy, we firstly utilized the class III phosphatidylinositol 3-kinase (PI3K) inhibitor 3-MA that had been shown to sensitize cancer cells to chemotherapy-induced apoptosis. As depicted in Fig. 2A, 3-MA plus CA-4 treatment resulted in an increased percentage of apoptotic cancer cells than CA-4 alone using PI staining assay. Consistent with these results, treatment of SGC-7901 and SMMC-7721 cells with CA-4 had only a bottom line effect on apoptosis induction, as shown by minimal levels of cleavage of PARP and caspase-3, which serve as markers of apoptosis; however, cancer cells’ pre-exposure to 3-MA followed by CA-4 treatment resulted in the enhanced cleavage of these proteins (Fig. 2B). Baf A1, a specific inhibitor of vacuolar type H+ -ATPase, prevents autophagy at the late stage by inhibiting fusion between autophagosomes and lysosomes (Fujimoto et al., 2009). As shown in Figs. 2C and D, Baf A1 similarly enhanced CA-4-induced apoptotic signaling, as shown by caspase and PARP cleavage. In addition, we proceeded to confirm the effect of autophagy inhibition on CA-4 induced apoptosis more directly by using small interfering RNA (siRNA) targeting silencing the expression of autophagy related genes beclin 1 or Atg5 (Maiuri et al., 2007). Fig. 2F shows that Atg5 or Beclin 1 knockdown resulted in a decrease of LC3-II accumulation after CA-4 treatment for 48 h when compared with random control in SGC-7901 cells. Consistent with the results using pharmacological means, suppression of autophagy by silencing Atg5 or Beclin 1 aggravated CA-4-induced apoptosis in SGC-7901 cells as indicated by an increase in sub-G1 population and in the amount of cleaved caspase-3 and PARP (Figs. 2E and F). These results suggest that CA-4 induces a canonical autophagy, and induction of autophagy has a protective role in tumor cells subjected to the cytotoxicity of CA-4.

The enhanced antitumor effect of CA-4 in combination with 3-MA against SGC-7901 xenografts

In light of the enhanced apoptosis by combing CA-4 with 3-MA in vitro, we then explored whether the enhanced anticancer effect of 3-MA and CA-4 was actually applicable in vivo. As shown in Fig. 3A and Table 1, the administration of CA-4 produced little antitumor effect in mean RTV compared with that of the control group (mean RTV, CA-4 vs control: 20.94 vs 34.51; p < 0.05). 3-MA exerted a slight tumor growth inhibitory effect (mean RTV, 3-MA vs control: 19.05 vs 34.51; p > 0.05). CA-4 plus 3-MA caused marked tumor growth inhibition.
CA-4-induced autophagy was mediated through JNK-Bcl-2 pathway

It has been well established that the Akt-mTOR pathway negatively regulates autophagy, inhibition of mTOR increases the expression of autophagy-related genes, such as Atg8 (LC3), and the kinase activity of Atg1 (Jung et al., 2010). We therefore examined whether CA-4-induced autophagy was resulted from the inhibition of phosphorylation of mTOR and its downstream targets p70 ribosomal protein S6 kinase. Unexpectedly, CA-4 treatment for 24 h induced a sharp increase in the phosphorylation of mTOR, as well as its downstream targets p70 ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), indicating that CA-4 might induce autophagy through an Akt/mTOR-independent pathway (Fig. 4A).

Beclin 1, the mammalian ortholog of yeast Atg6, has a central role in autophagy. It interacts with its binding partner class III phosphoinositide 3-kinase (PI3K III), also named Vps34, and is required for the initiation of the formation of the autophagosome in autophagy (Yang et al., 2011). Thus, we assessed cellular levels of Beclin 1 and PI3K III in SGC-7901...
7901 cells treated with CA-4, but failed to determine significant changes as shown in Fig. 4B, suggesting that autophagy induced by CA-4 was unlikely via upregulating the expression of Beclin 1 and PI3K III. We thus hypothesized that some other factors might play key roles in the autophagy induced by CA-4. Several investigators have described that JNK activation leads to Bcl-2 phosphorylation, dissociation of Bcl-2 from Beclin 1, and stimulation of autophagy (Levine et al., 2008; Lorin et al., 2010). Thus, it is intriguing to know whether JNK-Bcl-2 contributes to CA-4 induced autophagy. Treatment of cells with CA-4 led to increased phosphorylation of JNK, which indicated the possibility of JNK involvement during autophagy. We also observed a concentration-dependent increase of the phosphorylated (activated) form of Bcl-2 (Ser70) following treatment with CA-4, while total protein levels of Bcl-2 was not significantly affected by CA-4 treatment (Fig. 4C). Next, we tested whether CA-4 could affect the interaction between Beclin 1 and Bcl-2 in SGC-7901 cells. Intriguingly, we observed that CA-4 could disrupt the interaction between Beclin 1 and Bcl-2, indicating that the interaction of Beclin 1 and Bcl-2 might have a key role in CA-4 induced autophagy (Fig. 4D). These findings define a mechanism that JNK-Bcl-2 pathway might regulate autophagic activity in response to CA-4.

Inhibition of JNK activity sensitized cancer cells to CA-4-mediated cell death

To further evaluate the relationship between CA-4-induced autophagy and the JNK signaling, cells were treated with CA-4 in the presence of SP600125 (a JNK activity-specific inhibitor). Our data showed that JNK inhibitor significantly inhibited CA-4-induced LC3-II production and decreased Bcl-2 phosphorylation (Fig. 5A). Inhibition of JNK activity by SP600125 stimulated CA-4-induced apoptosis (Fig. 5B). To further explore the importance of JNK in modulating autophagy induced by CA-4, we used siRNA to knockdown JNK expression. As shown in Fig. 5C, silencing JNK in SGC-7901 cells significantly blocked LC3-II induction and inhibited phosphorylation of Bcl-2. Besides, inhibition of autophagy by JNK knockdown increased CA-4-induced apoptosis as shown in Fig. 5D. Collectively, these results validated that JNK activation could be an essential step in the stimulation of autophagy, and inhibition of JNK activity might promote CA-4-induced cell death.

Inhibition of Bcl-2 phosphorylation sensitized cancer cell lines to CA-4-mediated cell death

Our previous observations exhibited that increased phosphorylation of Bcl-2 in CA-4–treated SGC-7901 cells was correlated with protective autophagy induced by CA-4. ABT-737, a BH3 mimetic small-molecule Bcl-2 antagonist, binds with high affinity to Bcl-2 and is currently under evaluation in clinical trials (Kang and Reynolds, 2009). Therefore, we postulated that ABT-737 might be used to modulate the autophagy capacity of Bcl-2 by competitive binding to the BH3 acceptor groove of Bcl-2 and then diminish the amount of Bcl-2 phosphorylation induced by CA-4, thus inhibit autophagy and push cell into death. In order to confirm the hypothesis, SGC-7901 cells were pretreated with 4 μM ABT-737 for 4 h and then incubated with 10 nM CA-4 for another 24 h. As indicated in Fig. 6A, in contrast to CA-4 monontreatment, pretreatment with ABT-737 inhibited Bcl-2 phosphorylation and the conversion of LC3-I into LC3-II. Furthermore, ABT-737 pretreatment significantly augmented CA-4-induced cytotoxicity and apoptosis in several cancer cells, respectively (Figs. 6A–C). Collectively, these data indicated that Bcl-2 was important in modulating CA-4–induced autophagy, and Bcl-2 might be a potential drug target for chemotherapeutic combination.

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Tumor volume (mm³)</th>
<th>Tumor weight (g)</th>
<th>Mean RTV</th>
<th>Inhibition rate (%)</th>
<th>T/C (100%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Start</td>
<td>End</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>16.4 ± 0.21</td>
<td>20.2 ± 0.32</td>
<td>108.88 ± 21.87</td>
<td>2824.35 ± 428.05</td>
<td>1.14 ± 0.63</td>
<td>34.51 -</td>
</tr>
<tr>
<td>3-MA</td>
<td>16.5 ± 0.32</td>
<td>18.6 ± 0.23</td>
<td>117.76 ± 24.74</td>
<td>1836.92 ± 180.24</td>
<td>0.77 ± 0.40</td>
<td>19.05 32.67</td>
</tr>
<tr>
<td>CA-4</td>
<td>16.9 ± 0.44</td>
<td>19.4 ± 0.51</td>
<td>115.14 ± 30.18</td>
<td>1733.27 ± 216.87</td>
<td>0.86 ± 0.57</td>
<td>20.94 23.89</td>
</tr>
<tr>
<td>Combination</td>
<td>16.9 ± 0.31</td>
<td>18.8 ± 0.33</td>
<td>110.73 ± 17.06</td>
<td>1208.25 ± 237.03</td>
<td>0.42 ± 0.25</td>
<td>11.23 62.72</td>
</tr>
</tbody>
</table>

* Relative to control, p < 0.05.
* Relative to 3-MA group, p < 0.05.
* Relative to CA-4 group, p < 0.05.
Autophagy is a degradative process in eukaryotic cells that results in the breakdown of intracellular material within lysosomes (Maiuri et al., 2007). It serves as an alternative route of programmed cell death called type-2 programmed cell death or autophagic cell death, whereas a lot of evidence showed that autophagy is not always prodeath but can be prosurvival under conditions of cellular stress induced by nutrient deprivation or chemotherapy, thus allowing cells to evade apoptosis. In tumor cells, the role of autophagy may depend on tumor types, the stage of tumorigenesis, and the nature and extent of the insult (Shingu et al., 2009). Recent findings have revealed that CA-4 induced autophagy in colon cancer cells (Greene et al., 2012). However, the underlying mechanisms remain unknown. Besides, the role of CA-4 induced autophagy might differ depending on the cell type and cell-specific condition. In this context, we investigated whether CA-4 could induce autophagy in other cancer cells. In our study, we showed that CA-4 also induced autophagy in human gastric cancer cells SGC-7901, breast tumor cells MDA-MB-231 and human hepatocellular carcinoma SMMC-7721 cells, as evidenced by autophagosomal marker LC3 conversion, accumulation of AO-labeled acidic vesicles consistent with autophagolysosomes, punctate formation of GFP-LC3 and decreased p62 protein levels. These findings showed that CA-4 could induce autophagy in several cancer cells, not strictly in a cell type-specific manner. In an effort to determine whether autophagy served a prosurvival or prodeath role in response to treatment with CA-4, we adopted pharmacological and genetic approaches to inhibit autophagy. We utilized 3-MA and Baf A1 as inhibitors of autophagy. Our data showed that 3-MA or Baf A1 treatment significantly enhanced CA-4 induced apoptosis in SGC-7901 and SMMC-7721 cells, indicating that CA-4-triggered autophagy was protective. Furthermore, inhibition of autophagy related genes (Atg5 or Beclin 1) resulted in a significant apoptotic cell death in SGC-7901 cells treated with CA-4. In addition, the enhanced anticancer effect of 3-MA and CA-4 was further demonstrated using the SGC-7901 xenograft tumor model in vivo. Meanwhile, autophagy inhibition with 3-MA could antagonize CA-4-induced cardiotoxicity in vivo. We thus concluded that the CA-4-triggered autophagy was protective in various cancer cells and inhibition of autophagy could be a promising strategy to enhance the antitumor efficiency and/or to overcome drug resistance of CA-4 without apparent toxic effects.

We next focused on the potential pathways involved in CA-4-induced induction of autophagy, which might help in seeking better solutions to enhance the efficacy of currently used antineoplastic agent CA-4 in cancer cells. A number of complicated signaling pathways are involved in the regulation of autophagy, Beclin 1 is essential for the double-membrane autophagosome formation, which is required during the initial steps of autophagy (Pattingre et al., 2005). The activation of JNK, also referred to as stress-activated kinases, mediated Bcl-2 phosphorylation (Wei et al., 2008). Bcl-2 phosphorylation disrupts the interactions between Beclin-1 and Bcl-2, releasing Beclin-1 to promote autophagy. Our data showed that CA-4 could enhance the phosphorylation of JNK and Bcl-2 in SGC-7901 cells and inhibiting JNK abrogated CA-4-induced Bcl-2 phosphorylation. Meanwhile, we showed that CA-4 could disrupt the interaction between Beclin 1 and Bcl-2 and released Beclin-1 could induce autophagy. Thus, it was reasonable to hypothesize that CA-4 treatment might lead to JNK activation, which resulted in Bcl-2 phosphorylation, thereby disrupting the interaction of Bcl-2 and Beclin 1, and stimulation of autophagy. In addition, the Akt/mTOR pathway is the classic pathway often involved in regulating autophagy. Inhibition of the Akt/mTOR pathway has consistently been associated with triggering autophagy in cancer cells (Jung et al., 2010).
Rapamycin is the mTORC1 inhibitor and induces autophagy in different tumor cells including glioma cells (Takeuchi et al., 2005). However, in our study, we found the expression of phospho-Akt (ser473), phospho-mTOR (ser2448) as well as the levels of phosphorylated p70S6 kinase and 4E-BP1 was increased in SGC-7901 cells after exposure to CA-4. These results suggested CA-4 induced autophagy via an mTOR-independent pathway.

In regard to the previous data showing that phosphorylation of Bcl-2 contributed to the CA-4 induced protective autophagy, we speculated that inhibiting autophagy by decreasing Bcl-2 phosphorylation might enhance the antitumor characteristic by CA-4. ABT-737 is a potent small-molecule inhibitor of antiapoptotic proteins Bcl-2 with markedly higher affinity (Kline et al., 2007). In the present study, we observed that CA-4 plus ABT-737 (pretreated) could reduce the phosphorylation of Bcl-2, in parallel with the conversion of LC3-I to LC3-II. Furthermore, CA-4 plus ABT-737 (pretreated) could significantly enhance the apoptosis of cancer cells. Inhibition of autophagy could indeed be one of the underlying mechanisms of the synergistic cytotoxicity induced by CA-4 and ABT-737. However, the involvement of degradation of Mcl-1 (data not shown) in synergistic effect of CA-4 and ABT-737 also cannot be excluded. Mechanisms of this combination regimen might need further investigation.

In conclusion, we demonstrated that autophagy triggered by CA-4 occurred in many types of cancer cells. In addition, our data showed that the activation of JNK signaling pathway was responsible for the induction of autophagy by CA-4 via increasing phosphorylation of Bcl-2 to promote its release from Beclin 1. Thus, our data suggested that the JNK-Bcl-2 pathway was considered to be the critical regulator of CA-4-induced autophagy and a potential drug target for chemotherapeutic combination. Meanwhile, we found that ABT-737 treatment could have promising therapeutic potential as CA-4-based adjuvant chemotherapy against cancer based on this knowledge.

Conflict of interest

The authors declare that there are no conflicts of interest.

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