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Original article

Autophagy induced by cardamonin is associated with mTORC1 inhibition in SKOV3 cells

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ABSTRACT

Background: The mammalian target of rapamycin (mTOR) integrates energy level to modulate cell proliferation and autophagy. Cardamonin exhibits anti-proliferative activity through inhibiting mTOR. In this study, the effect of cardamonin on autophagy and its mechanism on mTOR inhibition were investigated.

Methods: Cell viability and proliferation were measured by MTT assay and BrdU incorporation, respectively. Cell apoptosis was assayed by flow cytometry and cell autophagy was detected by electron microscopy and GFP-LC3 fluorescence. The mechanism of cardamonin on mTORC1 inhibition was investigated by Raptor siRNA and Raptor over-expression.

Results: The cell viability and proliferation were inhibited by cardamonin. The autophagosomes and the protein level of LC3-II were increased by cardamonin. Cell apoptosis and the levels of cleaved PARP and Caspase-3 were increased by cardamonin. Cardamonin inhibited the phosphorylation of mTOR and ribosome S6 protein kinase 1 (S6K1) as well as the protein level of regulatory associated protein of mTOR (Raptor). However, cardamonin had no effect on the component of mTORC2 and its downstream substrate Akt. The inhibitory effect of cardamonin on the phosphorylation of mTOR and S6K1 was eliminated by Raptor knockdown with siRNA, whereas this effect of cardamonin was stronger than that of rapamycin and AZD8055 in Raptor over-expression cells. Cell viability was inhibited by cardamonin in both Raptor knockdown and Raptor over-expression cells, which was consistent with the inhibitory effect of cardamonin on mTOR.

Conclusion: These findings demonstrated that the autophagy induced by cardamonin was associated with mTORC1 inhibition through decreasing the protein level of Raptor in SKOV3 cells.

Introduction

Autophagy is a catabolic membrane-trafficking process involved in the degradation of intracellular material within lysosomes, which leading to the organelle turnover and providing energy and macromolecular precursors. It supports the growth and survival of cancer cells. However, autophagy has opposing, context-dependent roles in cancer [1]. The convincing evidence proves that under certain conditions autophagy can also foster cell death. The autophagic cell death induced by anticancer agents underlines the potential of autophagy as a novel modality for cancer therapy [2,3].

Proteins that constitute the basic machinery of autophagy have been well characterized. The mammalian target of rapamycin (mTOR) integrates energy level and plays an important role in the autophagic process [4]. Due to the dual role of autophagy, mTOR inhibition might lead to cell death or survival, depending on whether this protein works as a cell death inducer or a survival mechanism [5]. There is considerable rationale that mTOR inhibition contributes to the initiation of autophagy even in the presence of sufficient nutrients and growth hormones [6,7]. Amplified mTOR activation has been found in most ovarian cancer, which resulting in autophagy suppression and excessive cell proliferation [8]. It speculates that induction of autophagic cell death by mTOR inhibition is a therapeutic strategy for ovarian cancer.

mTOR exerts its biological activity in two different complexes called mTOR complex 1 (mTORC1) and mTORC2. The regulatory-

Abbreviations: FKBP12, FK506 binding protein 12 kDa; FRB domain, FKBP12-rapamycin binding domain; mTOR, mammalian target of rapamycin; mTORC1/2, mTOR complex 1/2; NF-κB, nuclear factor-κB; Raptor, regulatory associated protein of mTOR; Rictor, rapamycin-insensitive companion of mTOR; S6K1, ribosome S6 protein kinase 1.

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associated protein of mTOR (Raptor) is an indispensable component of mTORC1. It interacts with mTOR and regulates the mTOR-catalyzed phosphorylation of ribosome S6 protein kinase 1 (S6K1) [9]. The rapamycin-insensitive companion of mTOR (Rictor) is essential for mTORC2, which serves as the upstream of Akt [10]. Recent studies find that the components of mTOR complex are critical in activating mTOR and its downstream target. Flavonoid compounds could decrease the association of mTOR and its components, and subsequently reduce the phosphorylation of S6K1 [11,12]. It provides novel ways for the development of mTOR inhibitors.

Numerous Chinese herbs have been applied to cancer treatment as complementary and alternative medicines to enhance the efficacy or dampen the side effect of chemotherapy drugs. Cardamonin is a natural chalcone compound that exhibits antiproliferation activity in various cancer cells [13]. Several studies have demonstrated that cardamonin inhibits nuclear factor-κB (NF-κB) and mitogen-activated protein kinase [14]; however, the anti-tumor mechanism of cardamonin has not been completely clarified. Some investigations confirm that cardamonin inhibits the proliferation of A549 cells and metastasis of Lewis lung cancer through suppressing mTOR. Furthermore, different with the classical mTOR inhibitor (rapamycin), cardamonin inhibits mTOR without the assistance of FK506 binding protein 12 kDa (FKBP12) [15,16]. Therefore, the exact mechanism of cardamonin on mTOR inhibition needs to be illustrated. In addition, cardamonin triggers autophagy in HCT116 colon cancer cells [17]. Nevertheless, it is necessary to clarify the autophagic effect of cardamonin in ovarian cancer cells. In the present study, the effect of cardamonin on proliferation, autophagy and apoptosis was examined in SKOV3 cells. To further investigate the underlying mechanism of cardamonin on mTOR inhibition, we detected the protein levels of the components of mTOR complexes that were associated with the activation of mTOR signal pathway.

Materials and methods

Chemical reagents

Solutions and supplements for cell culture were purchased from Gibcol (Grand Island, NY, USA). Cardamonin (Fig. 1A), rapamycin, AZD8055, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and bafilomycin A1 were purchased from Sigma (St. Louis, MO, USA). Antibodies against mTOR, phospho-mTOR (Ser 2448), S6K1, phospho-S6K1 (Thr 389), Akt, phospho-Akt (Ser 473), Raptor, Rictor, LC3, Atg 5, Beclin-1, PARP, Caspase-3, β-actin and the secondary antibody (anti-rabbit IgG, mouse radish peroxidase-linked antibody) were purchased from Cell Signaling Technologies (Beverly, MA, USA). Annexin V-FITC/PI apoptosis detection kit and bromodeoxyuridine (BrdU) for cell proliferation assay kit were from BD Biosciences (San Jose, CA, USA).

Cell culture

SKOV3 cells were obtained from the Boster Biological Technology Co., Ltd (Wuhan, Hubei, China), and cultured in McCoy's 5A medium that supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL).

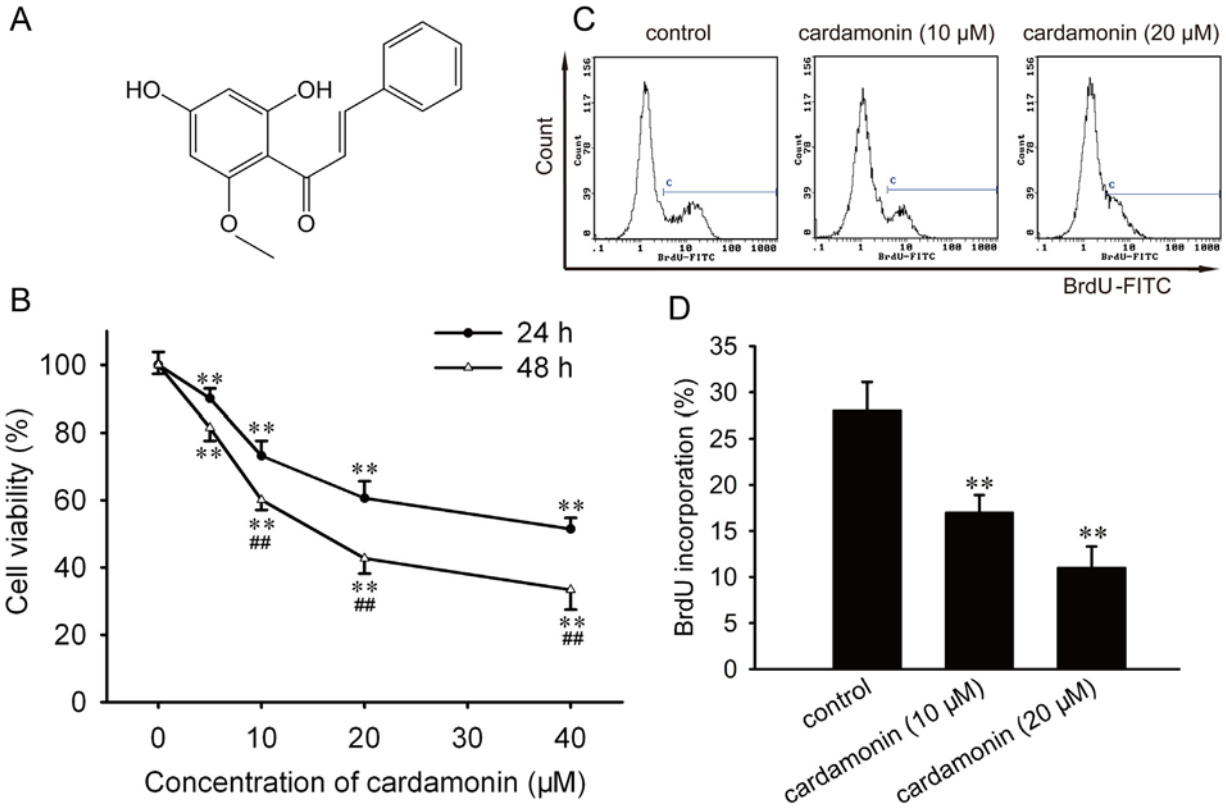

Fig. 1. Cardamonin inhibited the cell viability of SKOV3 cells. (A) Chemical structure of cardamonin. (B) Cells were treated with cardamonin at various concentrations (5, 10, 20 and 40 μM) for 24 and 48 h. Viable cells were detected by MTT assay ($n = 5$) and viability was determined by the ratio between drugs exposed cells and untreated control cells. Cells were treated with cardamonin for 48 h. Then the cells were incubated with BrdU for 4 h. BrdU incorporation was measured and analyzed by Flow cytometry ($n = 3$). (C) Representative flow cytometry scatter plots depict BrdU positive cells. (D) The percentage of BrdU incorporation ratio. Data were described with means ± SD. $^{**}p < 0.01$ *vs.* control; $^{\#\#}p < 0.01$ *vs.* 24 h.

Cell viability assay

MTT assay was used to analyze the effect of cardamonin on cell viability as previously described with modifications [18,19]. Cells with 180 μL suspension were seeded and cultured overnight in 96-well plates (5×10^3 cells per well). Cell viability was assessed after addition 20 μL of cardamonin to the indicated concentrations for 24 or 48 h in the presence of 10% FBS. The number of surviving cells were assessed by a microplate reader (Bio-Rad, USA) for the determination of the A_{570} nm of the dissolved formazan product after addition of 10 μL MTT (5 mg/mL) in the medium for 4 h.

BrdU incorporation assay

Cell proliferation assay was performed by a BrdU kit. After treatment with indicated drugs for 48 h, the SKOV3 cells were incubated with BrdU for 4 h. Then the cells were stained according to the manufacturer's instructions and analyzed by a flow cytometry (BD Biosciences).

Electron microscopy

After treatment with drugs for 48 h, SKOV3 cells were prefixed in 2% glutaraldehyde at 4 °C, followed by incubating with 1% OsO_4 for 3 h at 4 °C. And then the cells were dehydrated in a graded series of ethanol and flat embedded in epon. Ultrathin sections were doubly stained with uranyl acetate and observed by a JEM-2000EX electron microscopy (JEOL, Japan).

Quantitation of autophagy with GFP-LC3

SKOV3 cells were transfected with 5 μg GFP-LC3 (GenePharma, Shanghai, China) with Lipofectamine 2000 (Invitrogen) for 48 h. After treatment with cardamonin for 48 h, the GFP-LC3 cells were visualized by the Leica fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). Cell numbers were counted to normalize the measurement. 200 GFP-positive cells were selected for observation and the percentage of cells with GFP-LC3 punctate dots was calculated.

Apoptosis analysis

Cell apoptosis was detected by Annexin V-FITC/propidium iodide (PI) kit according to the manufacturer's instructions. After treatment with indicated drugs for 48 h, cells were collected and washed with cold PBS twice, and then resuspended with binding buffer at a concentration of 1×10^6 cells/mL. Annexin V/FITC and PI staining solution (5 μL) were added to 500 μL forementioned cell suspension, and incubated at room temperature for 15 min in dark and then analyzed by the flow cytometer (BD Biosciences).

Western blot

After treatment with indicated drugs for 48 h, cells were washed with ice-cold PBS twice and lysed in RIPA lysis buffer (addition of 1 mM phenylmethylsulfonyl fluoride) for 20 min at 4 °C. Lysates were centrifuged at 15,000*g* at 4 °C for 20 min, and protein concentrations of the supernatants were determined using BCA protein assay reagent. 40 μg proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride filters. The membranes were blocked with 5% BSA in 1 × TBST for 1 h and then incubated overnight with primary antibody. Immunoblots were visualized with horseradish peroxidase-coupled goat anti-rabbit immunoglobulin by using the enhanced chemiluminescence and exposure to X-ray film to produce bands within the linear range.

Small interfering RNA (siRNA) transfection

siRNAs were purchased from GenePharma (Shanghai, China). The target sequences for Raptor were 5′-CGA GAU UGG ACG ACC AAA UTT-3′. A non-specific siRNA sequence (5′-GUA UGA CAA CAG CCU CAA GTT-3′) was used as control. The cells were transfected with siRNA using Lipofectamine 2000 for 24 h according to the manufacturer's protocol. The media were replaced with fresh one with indicated drugs and cultured for another 48 h. Then the protein was isolated for Western Blot assay.

Raptor over-expression plasmids construction and stable transfection

The eukaryotic expressing vector of Raptor (GV208-Raptor) and the lentivirus encoding GV208-Raptor were generated by Genechem, Inc. (Shanghai, China). After lentivirus transfection for 16 h, the medium was replaced with normal medium and the cells were cultured for 72 h. Then the culture medium was replaced with selection medium containing puromycin (2.5 μg/mL). The selection medium was changed every 3 or 4 days until the puromycin resistant colonies appeared. The colonies were then individually picked out and subcultured in the puromycin-containing medium.

Statistical analysis

All data were expressed as mean ± SD. Statistical analysis was performed by one-way ANOVA, and Tukey-Kramer multiple comparison tests with SPSS 19.0 software. A value of $p < 0.05$ was considered as statistically significant.

Results

Cardamonin inhibited the cell viability and proliferation of SKOV3 cells

Cardamonin significantly inhibited the viability of SKOV3 cells in a concentration- and time-dependent manner (Fig. 1B). Cell proliferation was significantly inhibited by cardamonin in the BrdU incorporation assay (Fig. 1C and D).

Cardamonin induced autophagy in SKOV3 cells

After treatment with different drugs for 48 h, the ultrastructure of cells was analyzed by electron microscopy. Numerous autophagosomes were observed in cells that cultured with cardamonin, rapamycin and AZD8055, respectively, but few in the control cells (Fig. 2A and B). Furthermore, the fluorescence imaging analysis showed that cardamonin significantly increased GFP-LC3 punctation (Fig. 2C and D). The protein level of LC3-II was significantly increased by cardamonin. Rapamycin and AZD8055 increased the levels of Atg 5 and Beclin-1; however, cardamonin had no effect on these proteins (Fig. 2E–H). The autophagic flux was analyzed using bafilomycin A1. While bafilomycin A1 expectedly increased the protein level of LC3-II, treatment with cardamonin additionally increased the LC3-II (Fig. 2I and J).

Cardamonin increased apoptotic cell death in SKOV3 cells

The population of dead cell was increased to 12.8% (10 μM) and 21.7% (20 μM) by cardamonin, while the percentage of early apoptotic cells was slightly increased. Similar results were observed in the rapamycin and AZD8055 exposure group (Fig. 3A and B). On the other hand, we have found that the levels of cleaved PARP and Caspase-3 were increased after exposure to cardamonin (Fig. 3C–E).

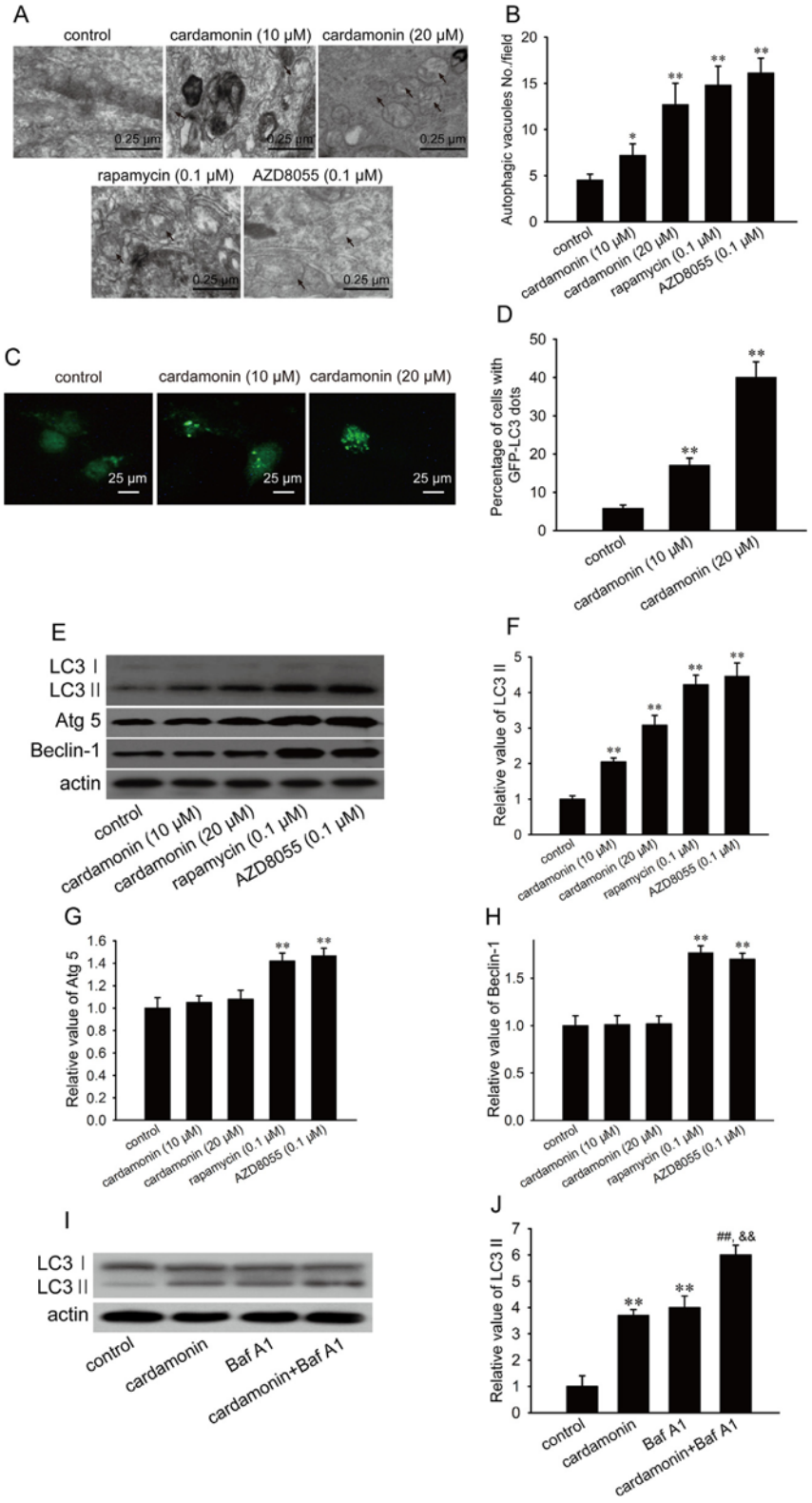

Fig. 2. Cardamonin induced autophagy in SKOV3 cells. SKOV3 cells were treated with different drugs as indicated for 48 h. (A) The ultrastructural changes were observed by transmission electron microscopy ($n = 3$). The arrow indicates an autophagosome including residual digested material in cells. (B) The quantification of autophagic vacuoles per area (100 μm^2). After GFP-LC3 transfection, SKOV3 cells were treated with cardamonin for 48 h. (C) The cells were examined under a fluorescence microscopy ($n = 3$). (D) The percentage of cells with GFP-LC3 punctate dots. (E) Protein levels of LC3, Atg 5 and Beclin-1 were detected by Western blot analysis. Actin was used as an equal loading control ($n = 3$). (F-H) The protein band intensities were quantified and normalized to actin using ImageJ. Data were presented by mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* control. SKOV3 cells were treated with cardamonin (20 μM), bafilomycin A1 (Baf A1, 10 nM) alone respectively or with cardamonin in the presence of Baf A1 for 4 h. (I) Protein level of LC3 was detected by Western blot analysis. Actin was used as an equal loading control ($n = 3$). (J) The protein band intensities were quantified and normalized to actin using ImageJ. Data were presented by mean ± SD. ** $p < 0.01$ *vs.* control. $^{\#\#}$ $p < 0.01$ *vs.* cardamonin, $^{\&\&}$ $p < 0.01$ *vs.* Baf A1.

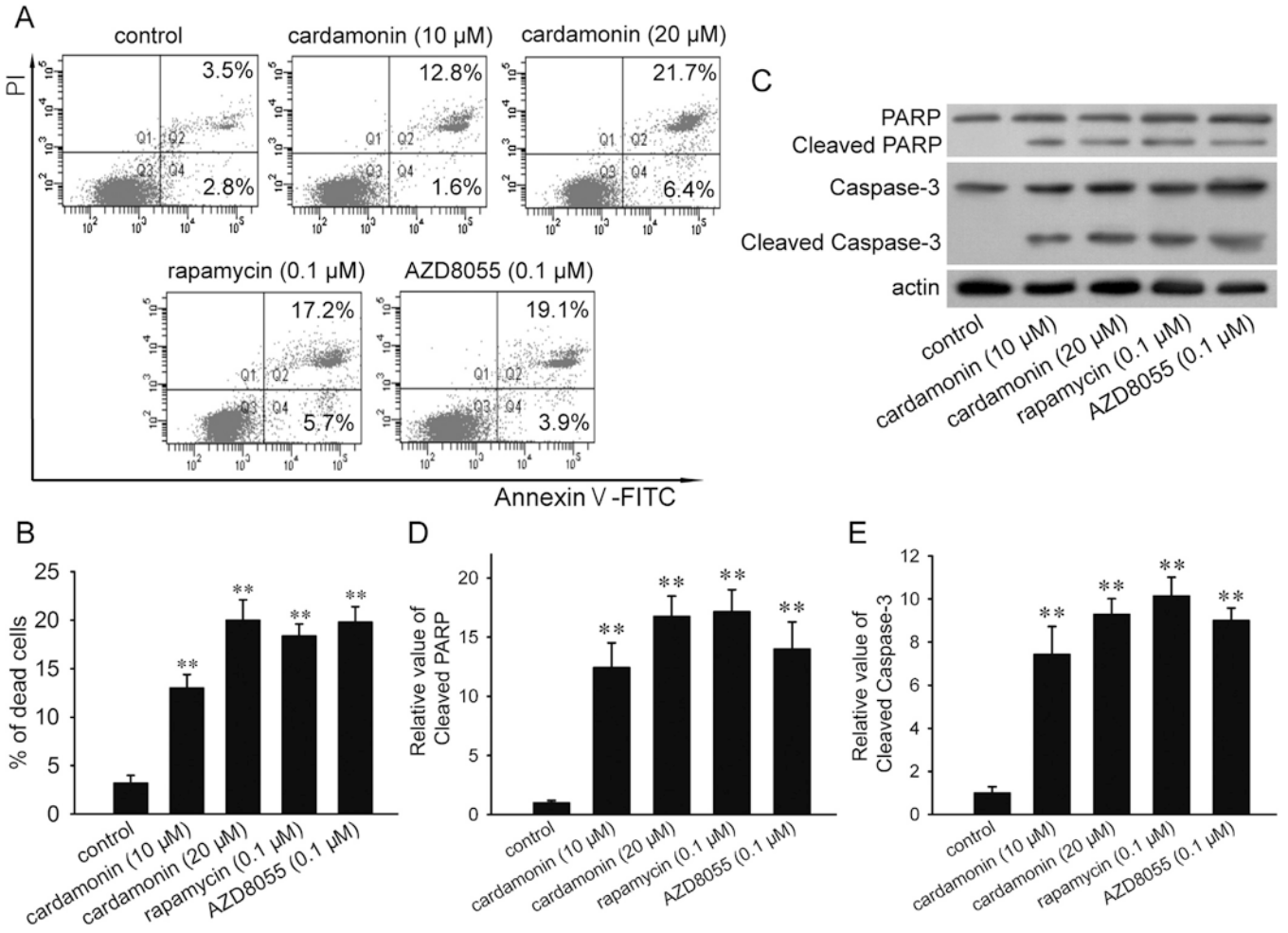

Fig. 3. Cardamonin increased apoptosis of SKOV3 cells. Effect of cardamonin on the apoptosis of SKOV3 cells was examined by Annexin V-PI stain assay. Cells were treated with drugs as indicated for 48 h. Then the cells were stained with Annexin V-FITC and PI ($n = 3$). (A) Representative flow cytometry scatter plots depict percentage of Annexin V and PI staining. (B) The percentage of dead cells. (C) The levels of cleaved PARP and Caspase-3 were detected by Western blot analysis. Actin was used as an equal loading control ($n = 3$). (D-E) The cleaved protein band intensities were quantified and normalized to actin using ImageJ. Data were presented by mean ± SD. ** $p < 0.01$ *vs.* control.

Raptor mediated the inhibitory effect of cardamonin on mTORC1

As shown in Fig. 4, the protein level of Raptor was inhibited by cardamonin, whereas that of Rictor was not affected. In accordance with rapamycin, the phosphorylation of mTOR and S6K1 was inhibited by cardamonin. However, cardamonin had no effect on the phosphorylation of Akt, which was decreased by AZD8055, a dual inhibitor of mTORC1 and mTORC2 (Fig. 5). After Raptor siRNA transfection, the phosphorylation of mTOR and S6K1 was reduced. In contrast, phosphorylation of Akt was not decreased. The inhibitory effects of cardamonin, rapamycin and AZD8055 on the phosphorylation of mTOR and S6K1 were eliminated in Raptor siRNA knockdown cells; while AZD8055 retained the inhibition on the phosphorylation of Akt (Fig. 6). The phosphorylation of mTOR and S6K1 were increased in the Raptor over-expression cells. The inhibitory effect of cardamonin on the phosphorylation of mTOR and S6K1 was stronger than that of rapamycin and AZD8055 in the Raptor over-expression cells. Neither Raptor over-expression nor cardamonin decreased the phosphorylation of Akt (Fig. 7).

The effect of cardamonin on the cell viability in the Raptor knockdown and Raptor over-expression cells

The cell viability was decreased by Raptor siRNA transfection. Cardamonin had no obvious effect on the viability in Raptor knockdown cells; however, both rapamycin and AZD8055 further inhibited cell viability. Conversely, the cell viability was increased in Raptor over-expression condition. The inhibitory effect of cardamonin on the cell viability was stronger than that of rapamycin and AZD8055 on Raptor over-expression cells (Fig. 8).

Discussion

Autophagy is a tightly-regulated catabolic process that the cellular components are targeted to lysosomes for degradation. It results in adaptive cell survival or death depending on the circumstances [20]. Excessive autophagy leads to progressive consumption of cellular components, and ultimately induces cell death [21]. The autophagic cell death refers to a mode of cell death that is regulated by specific proteins in the autophagic pathway [5]. Numerous flavonoid compounds, including vitexin, quercetin and fisetin, induce autophagic cell death through inhibiting mTOR [22–24]. Similar with these compounds, we found that cardamonin induced autophagy in SKOV3 cells. It increased the protein level of LC3-II and autophagosomes formation. To confirm that the increased LC3-II reflected true induction of autophagy but not the inhibition of lysosomal degradation, we investigated the influence of cardamonin on LC3-II in the presence of bafilomycin A1. The result suggested

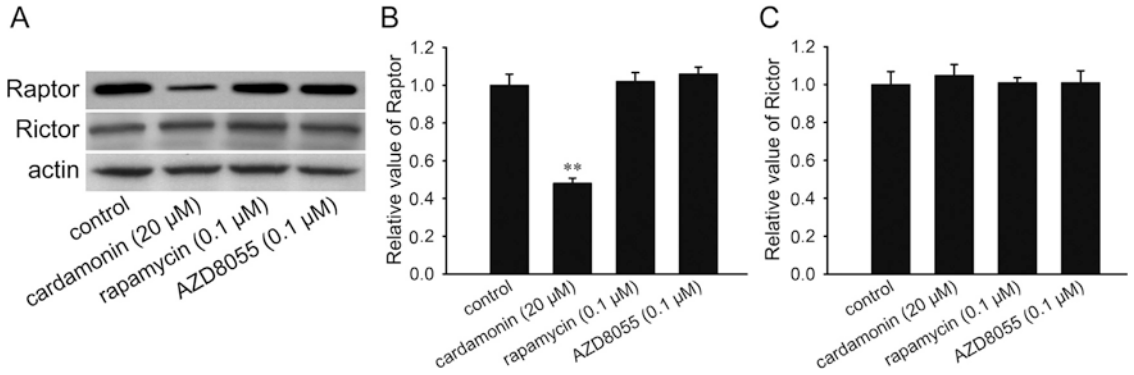

Fig. 4. Cardamonin inhibited the protein level Raptor. (A) The protein levels of Raptor and Rictor were detected by Western blot analysis. Actin was used as an equal loading control ($n = 3$). (B, C) The protein band intensities were quantified and normalized to actin using ImageJ. Data were presented by mean ± SD. ** $p < 0.01$ *vs.* control.

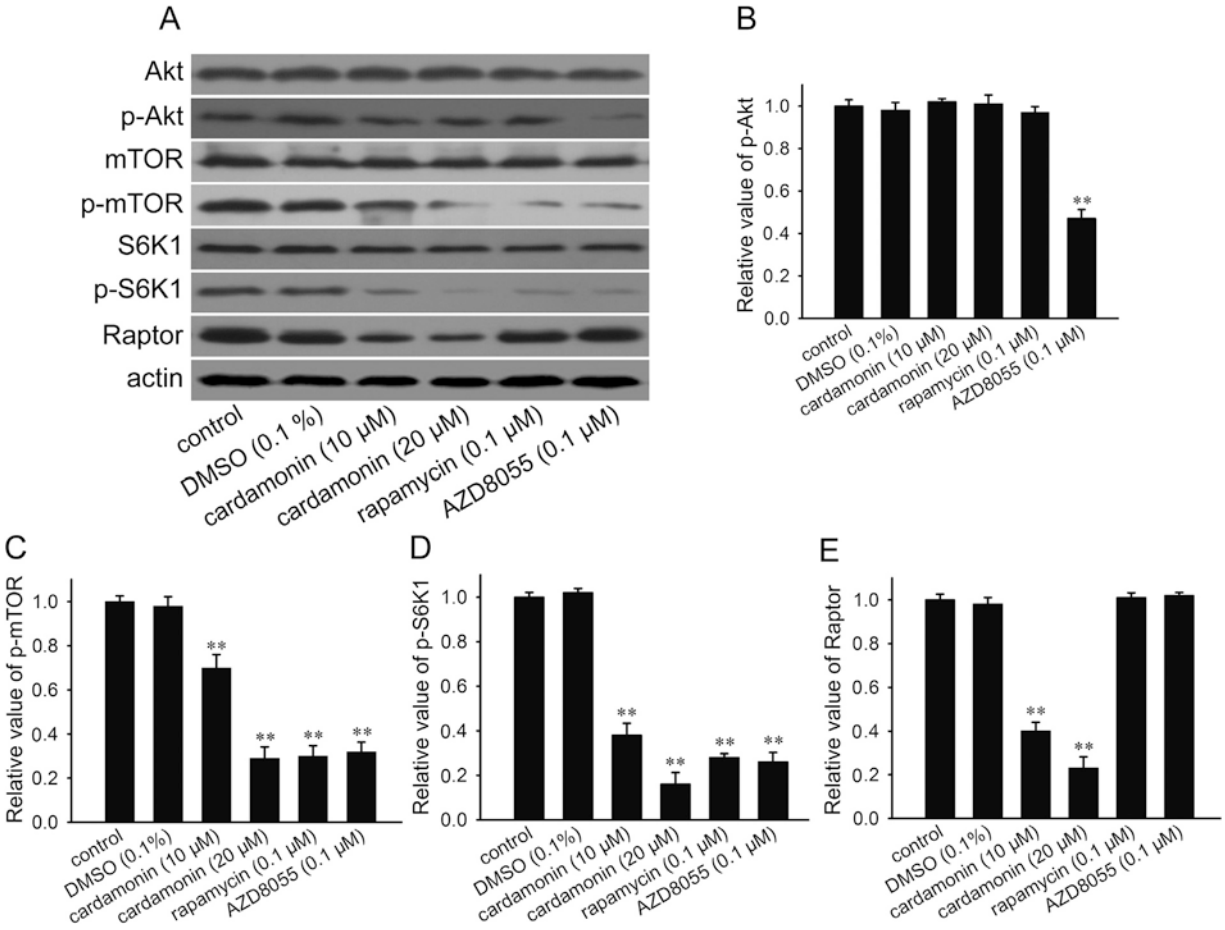

Fig. 5. Cardamonin inhibited the mTORC1 signaling pathway. (A) The protein levels of Akt, p-Akt, mTOR, p-mTOR, S6K1, p-S6K1 and Raptor were determined by Western Blot. Actin was used as an equal loading control ($n = 3$). (B-E) The protein band intensities were quantified and normalized to actin using ImageJ. Data were presented by mean ± SD. ** $p < 0.01$ *vs.* control.

that cardamonin increased the protein level of LC3-II, rather than prevented autophagic proteolysis. However, cardamonin had no effect on the protein levels of Atg 5 and Beclin-1, which was consistent with dimethyl cardamonin [25].

The activation of mTOR stimulates excessive cell proliferation and it considered as a promising therapeutic target for cancer treatment. Despite the exact mechanism was not clearly demonstrated, cardamonin significantly decreased the viability of the ovarian cancer SKOV3 cells through inhibiting the mTOR signaling pathway [26]. Previous studies have demonstrated that mTOR acts as the upstream effector of apoptosis [27,28]. And apoptosis can be induced by mTOR inhibition in many cancer cells [29,30]. In the present study, we found that cell apoptosis and the levels of cleaved PARP and Caspase-3 were promoted by cardamonin, which were in agreement with the apoptosis induction effect of cardamonin in nasopharyngeal carcinoma cells, myeloma cells, colon cancer cells and triple negative breast cancer cells [31–33].

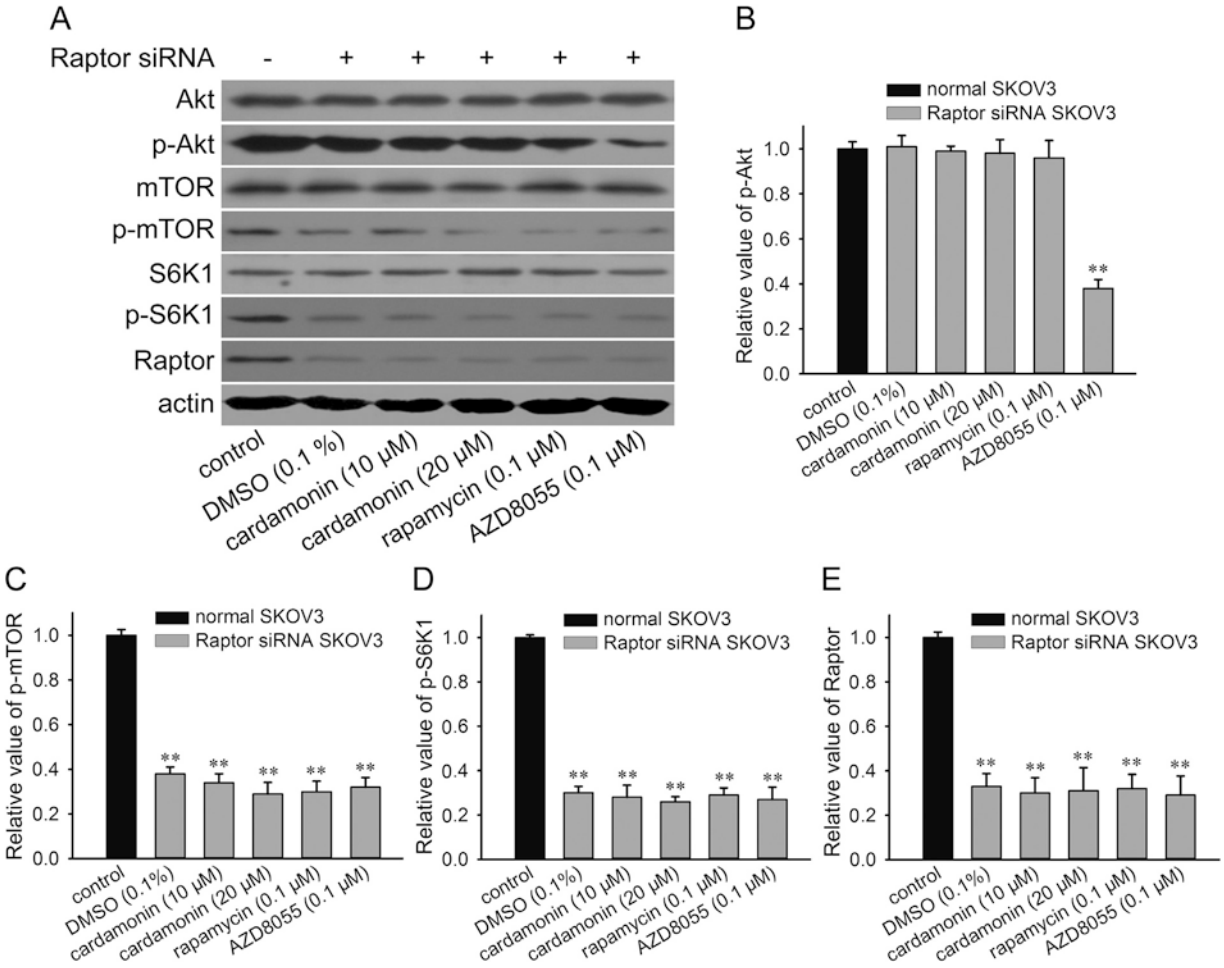

Fig. 6. Raptor siRNA abrogates the inhibitory effect of cardamonin on mTORC1. (A) The protein levels of Akt, p-Akt, mTOR, p-mTOR, S6K1, p-S6K1 and Raptor were determined by Western Blot. Actin was used as an equal loading control ($n = 3$). (B-E) The protein band intensities were quantified and normalized to actin using ImageJ. Data were presented by mean ± SD. ** $p < 0.01$ *vs.* control.

mTOR consists of several distinct functional domains. The FKBP12-rapamycin binding (FRB) domain and kinase domain are responsible for the binding site of mTOR inhibitors. The rapamycin-FKBP12 complex binds with the FRB domain and inhibits the activity of mTORC1, rather than mTORC2 [34]. AZD8055 targets to the kinase domain and suppresses the activity of mTORC1 and mTORC2 [35]. Previous results demonstrated that cardamonin inhibited the activation of mTOR and S6K1 without the assistance of FKBP12; furthermore, the mRNA expression of Raptor was decreased by cardamonin [16,36]. In the present study, we found that the protein level of Raptor was decreased by cardamonin. The result suggested that cardamonin inhibited the mTORC1 signaling pathway through reducing the protein level of Raptor, which was different with the existing mTOR inhibitors. Since Raptor regulates the activation of mTOR and its signaling transduction [9,37], we further investigate the inhibitory effect of cardamonin on mTOR and S6K1 in the Raptor knockdown and Raptor over-expression SKOV3 cells. As expected, the phosphorylation of mTOR and S6K1 was significantly decreased by Raptor siRNA and increased by Raptor over-expression, respectively. Raptor siRNA abrogated the inhibitory effect of cardamonin on the phosphorylation of mTOR and S6K1. However, the inhibitory effect of cardamonin on the phosphorylation of mTOR and S6K1 was stronger than that of rapamycin and AZD8055 in Raptor over-expression cells. The phosphorylation of Akt was not affected by cardamonin, but it was obviously decreased by the dual inhibitor of mTORC1 and mTORC2 (AZD8055). It suggested that Raptor was the potential target of cardamonin and it was a novel mTORC1 inhibitor.

Cisplatin resistance is one of the major limitations for the chemotherapy of ovarian cancer. mTOR is frequently hyper-activated in the cisplatin resistant ovarian cancer cells [19]. The analog of rapamycin (rapalogues), including temsirolimus and everolimus, significantly enhanced the therapeutic efficacy of platinum in ovarian cancer [38,39]. Similar with the rapalogues, cardamonin enhanced the anti-proliferation effect of cisplatin by suppressing the activity of mTOR in SKOV3 cells [40]. As a potential mTOR inhibitor, cardamonin has clinical advantages in cancer therapy. The inhibitory effect of cardamonin on the cell viability in SKOV3 cancer cells is stronger than that in normal human umbilical artery smooth muscle cells [36]. In addition to fewer hepatotoxicity and nephrotoxicity [41], cardamonin had neph-roprotective effect against cisplatin induced renal injury through suppressing oxidative stress and inflammation in rats [42]. Therefore, we speculate that cardamonin has little toxicity on human normal cells.

In conclusion, the results demonstrated that the autophagy induced by cardamonin was associated with mTORC1 inhibition in SKOV3 cells. Different with the existing mTOR inhibitors, Raptor downregulation was involved in the mTORC1 inhibition by cardamonin. This study provides important evidences for further development of cardamonin as a novel mTORC1 inhibitor.

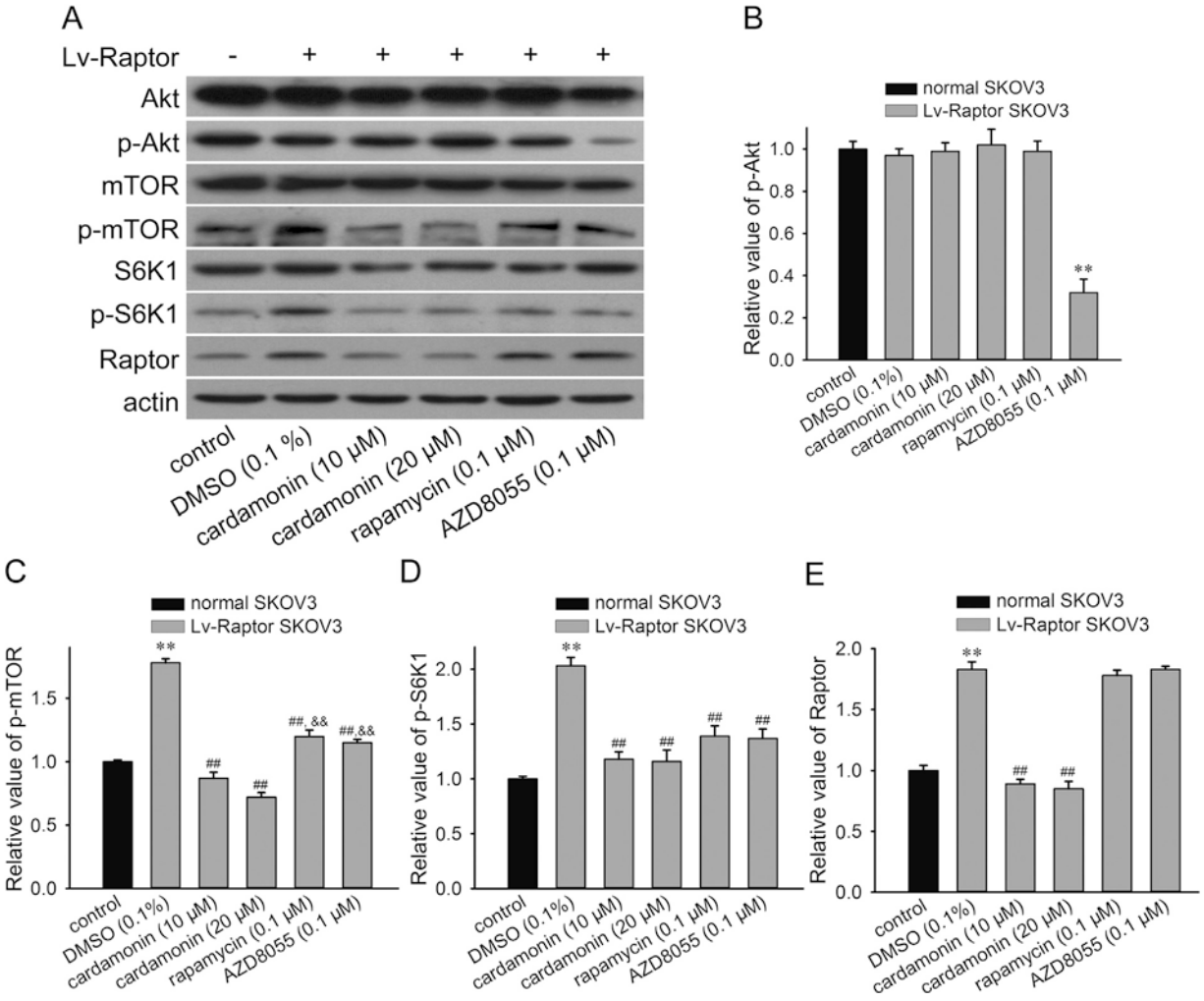

Fig. 7. Cardamonin inhibited the mTORC1 signaling pathway. (A) The protein levels of Akt, p-Akt, mTOR, p-mTOR, S6K1, p-S6K1 and Raptor were determined by Western Blot. Actin was used as an equal loading control ($n = 3$). (B-E) The protein band intensities were quantified and normalized to actin using ImageJ. Data were presented by mean ± SD. ** $p < 0.01$ *vs.* control; $^{\#\#}$ $p < 0.01$ *vs.* Lv-Raptor SKOV3 DMSO (0.1%); $^{\&\&}$ $p < 0.01$ *vs.* Lv-Raptor SKOV3 cardamonin (20 μM).

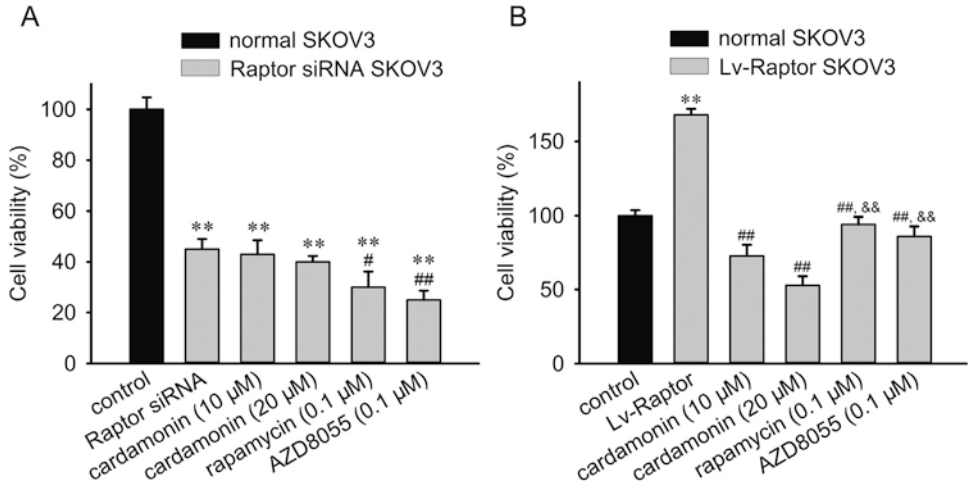

Fig. 8. The effect of cardamonin on the cell viability in Raptor knockdown and Raptor over-expression cells. (A) The Raptor knockdown cells and (B) Raptor over-expression cells were respectively treated with cardamonin (10 and 20 μM), rapamycin (0.1 μM) and AZD8055 (0.1 μM) for 48 h. Viable cells were detected by MTT assay ($n = 5$) and viability was determined by the ratio between drugs exposed cells and untreated control cells. Data were described with means ± SD. ** $p < 0.01$ *vs.* control; (A) $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ *vs.* Raptor siRNA; (B) $^{\#\#}$ $p < 0.01$ *vs.* Lv-Raptor; $^{\&\&}$ $p < 0.01$ *vs.* Lv-Raptor cardamonin (20 μM).

Conflicts of interest

None.

Acknowledgements

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