

Raptor mediates the antiproliferation of cardamonin by mTORC1 inhibition in SKOV3 cells

Daohua Shi*
Yanting Zhu*
Peiguang Niu
Jintuo Zhou
Huajiao Chen

Department of Pharmacy, Fujian Provincial Maternity and Children Hospital, Affiliated Hospital of Fujian Medical University, Fuzhou, Fujian, China

*These authors contributed equally to this work

Purpose: Cardamonin inhibits the proliferation of SKOV3 cells by suppressing the mammalian target of rapamycin complex 1 (mTORC1). However, the mechanism of cardamonin on mTORC1 inhibition has not been well demonstrated. The regulatory-associated protein of TOR (Raptor) is an essential component of mTORC1. Here, we investigated the role of Raptor in the mTORC1 inhibition effect of cardamonin in SKOV3 cells.

Methods: The expression of Raptor was knockdown by small interfering RNA (siRNA). The expressions of specific binding proteins of mTORC1 were analyzed by Western blotting, and the cell proliferation was detected by methyl thiazolyl tetrazolium (MTT) assay.

Results: Rapamycin, AZD8055, and cardamonin inhibited the activity of mammalian target of rapamycin (mTOR). Different from rapamycin and AZD8055, cardamonin suppressed the phosphorylation and protein expression of Raptor. Transfected with Raptor siRNA, the mTOR activation and proliferation of SKOV3 cells were decreased, and these effects were strengthened by cardamonin in Raptor siRNA SKOV3 cells. Cardamonin interfered with the lysosomal colocalization of mTOR with lysosomal associated membrane protein 2 (LAMP2), which was also hindered by Raptor siRNA. Furthermore, cardamonin strengthened the inhibitory effect on the lysosomal localization of mTOR in Raptor siRNA cells.

Conclusion: Our results suggested that Raptor mainly mediated the inhibition of cardamonin on mTORC1 in SKOV3 cells.

Keywords: cardamonin, Raptor, mTORC1, drug target, ovarian cancer

Introduction

The mammalian target of rapamycin (mTOR) is closely associated with the tumorigenesis and development of ovarian cancer.¹ Activated mTOR phosphorylates ribosomal protein S6 kinase 1 (S6K1) and 4E-binding protein 1 (4E-BP1), which participates in the regulation of protein translation, and mTOR downregulation inhibits the proliferation of cancer cells and tumor metastasis.²⁻⁵ Recently, mTOR inhibitors have achieved a great success in the cancer treatment.⁶⁻⁸ Therefore, mTOR is considered as an important target for cancer therapy.

mTOR exists as two distinct protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 senses nutrition or growth factors and regulates cell growth, metabolism, and autophagy; while mTORC2 directly activates Akt by phosphorylating its hydrophobic motif (Ser473) and regulates cell survival and cytoskeletal organization.⁹ Both mTOR complexes consisted of different components. The regulatory-associated protein of TOR (Raptor) and proline-rich Akt/PKB substrate 40 kDa (PRAS40) are the specific components of mTORC1. Raptor binds with mTOR to form a nutrient-sensitive complex and mediates the mTOR-catalyzed phosphorylation of S6K1 and 4E-BP1. In addition, Raptor mediates the translocation of mTOR to

Correspondence: Daohua Shi
Department of Pharmacy, Fujian Provincial Maternity and Children Hospital, Affiliated Hospital of Fujian Medical University, 18 Daoshan Road, 350001 Fuzhou, Fujian, China
Tel/fax +86 591 8831 2079
Email shidh@yeah.net

lysosomes, which is an essential procedure for the activation of mTOR.¹⁰ PRAS40 interacts with Raptor and participates in the regulation of mTOR activation and cell proliferation.^{11,12}

mTOR contains a number of distinct functional domains, including HEAT repeat domain, FK506-binding protein 12 kDa (FKBP12)-rapamycin-binding (FRB) domain, and the kinase domain. There exist two generations of mTOR inhibitors. Rapamycin, the first-generation mTOR inhibitor, connects with FKBP12 and binds to the FRB domain of mTOR. The second-generation is the adenosine triphosphate-competitive mTOR inhibitor, which binds to the kinase domain of mTOR and inhibits both mTORC1 and mTORC2.¹³ However, investigation on novel mTOR inhibitors is continually developing. Several studies revealed that flavonoids compounds could inhibit mTORC1 signaling pathway through disrupting the connection of mTOR and Raptor.^{14,15}

Cardamonin is the main flavonoid extracted from *Alpinia katsumadi* Hayata. It exhibits a wide range of pharmacological activity, including anti-inflammation, vasorelaxation,¹⁶ and enhancement on the therapeutic index of cisplatin.¹⁷ Cardamonin has been considered as a chemo-preventive agent in a variety of cancers, including breast, hematological, prostate, and colorectal cancers.^{18–21} Our previous studies have demonstrated that cardamonin decreases the proliferation of non-small-cell lung cancer A549 cells and ovarian cancer SKOV3 cells through blocking the cell cycle and inducing autophagy;^{22,23} moreover, it inhibits the metastasis of Lewis lung cancer in vivo.²⁴ These studies demonstrate that the antitumor effect of cardamonin is related to inhibition on mTORC1 signaling pathway.^{25–27} However, different to rapamycin, cardamonin inhibits mTOR without the assistance of FKBP12.^{22,25} Therefore, the mechanism of cardamonin on mTORC1 inhibition needs to be clarified. In the present study, we intend to investigate the effect of cardamonin on the specific binding proteins of mTORC1 and the lysosomal localization of mTOR in SKOV3 cells to reveal the underlying mechanism.

Materials and methods

Reagents

Cardamonin (no 110763, purity >99%; National Institutes for Food and Drug Control, Beijing, China), rapamycin (Sigma-Aldrich Co., St Louis, MO, USA), and AZD8055 (Axonmedchem, Groningen, the Netherlands) were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich Co.) to prepare the stock solution (2 mM, 10 μ M, and 10 μ M, respectively) and these solutions were stored at 4°C. Methyl thiazolyl tetrazolium (MTT) and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich Co. Lipofectamine 2000 was purchased from Thermo Fisher

Scientific (Waltham, MA, USA), and Raptor small interfering RNA (siRNA) was produced by GenePharma Co., Ltd (Shanghai, China). Antibodies against mTOR, p-mTOR (Ser2481), Raptor, p-Raptor (Ser792), PRAS40, p-PRAS40 (Ser183 and Thr246), S6K1, p-S6K1 (Thr389), and β -actin (Cell Signaling Technology, Beverly, MA, USA) were used at a 1:1,000 dilution. The secondary antibodies (anti-rabbit immunoglobulin G, horseradish peroxidase [HRP]-linked antibody) used for detection in all cases were from Cell Signaling Technology. The Alexa 488- and Alexa 647-conjugated secondary antibodies (goat anti-rabbit immunoglobulin G H&L) were from Abcam (Cambridge, MA, USA).

Cell culture

SKOV3 cells were obtained from the Boster Biological Technology Co., Ltd (Wuhan, Hubei, China) and cultured in Mccoy's 5A medium (M&C Gene Technology, Ltd., Beijing, China) with 10% fetal bovine serum (Hyclone), 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37°C in a humidified atmosphere with 5% CO₂.

Raptor small interfering RNA (siRNA) transfection

The siRNA sequences for Raptor and nonspecific siRNA (negative control) were sense 5'-GUG UCA CAC UGG AUU UGA UTT-3' and antisense 5'-AUC AAA UCC AGU GUG ACG CTT-3' and sense 5'-UUC UCC GAA CGU GUC ACG UTT-3' and antisense 5'-ACG UGA CAC GUU CGG AGA ATT-3', respectively. The cells were transfected with siRNA using Lipofectamine 2000 according to the manufacturer's protocol. The transfection efficiency was measured by Western blotting.

Cell proliferation analysis

Cell proliferation was determined by MTT assay. Cells were seeded in a 96-well plate (5 \times 10³ cells per well) and then treated with different drugs for 24 h. A total of 20 μ L MTT solution (5 mg/mL) was added to each well and incubated for another 4 h. The supernatant was discarded carefully and 150 μ L DMSO was added to each well and then shaken for 10 min. The absorbance was determined at 490 nm by a microplate reader (Model 1680; Bio-Rad Laboratories Inc., Hercules, CA, USA).

Western blotting analysis

After treating with indicated drugs for 2, 16, or 24 h, cells were washed twice with ice-cold phosphate buffered solution (PBS) and resuspended in lysis buffer comprising 50% glycerol, 100 mM NaF₂, 10 mM sodium pyrophosphate, 1% Triton X-100, 1 mM Na₃VO₄, 1 mM phenylmethanesulfonyl

fluoride, 10 µg/mL antipain, 10 µg/mL leu-peptin, and 10 µg/mL aprotinin for 30 min. Lysates were centrifuged at 14,000× *g* at 4°C for 20 min, and the supernatant was collected for experiments. Protein content was measured by the bicinchoninic acid method. Proteins (50 µg per lane) were separated on 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for mTOR and p-mTOR and on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for Raptor, p-Raptor, PRAS40, p-PRAS40, S6K1, and p-S6K1 and then transferred to polyvinylidene fluoride membranes. The membranes were blocked in 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 at room temperature for 1 h and then incubated with antibodies against mTOR, p-mTOR, Raptor, p-Raptor, PRAS40, p-PRAS40, S6K1, p-S6K1, and β-actin that were diluted in block buffer (1:1,000) at 4°C overnight. Then, the membranes were incubated with the appropriate HRP-linked secondary antibodies. Finally, HRP-enhanced chemiluminescence reagents were added to react with the secondary antibodies for 1–3 min, and the bands of specific proteins on the membranes were developed by autoradiography (KODAK Film, Shanghai, China). Protein bands were quantified by BioImaging Systems as a ratio to actin expression in each sample.

Immunofluorescence assays

The immunofluorescence assay was carried out as previously described.²⁸ A total of 5×10⁵ SKOV3 cells were plated on laser confocal petri dish. Twenty-four hours later, the slides were rinsed with PBS once and fixed with 4% paraformaldehyde in PBS at room temperature for 30 min. The slides were rinsed twice with PBS, and cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min, followed by rinsing with PBS for three times. The slides were incubated with primary antibody in 5% normal goat serum overnight at 4°C and then rinsed four times with PBS, and the slides were then incubated with secondary antibodies (diluted 1:1,000 in PBS) at room temperature in the dark for 1 h and rinsed with PBS for another four times. Finally, the slides were incubated with 4 ng/µL DAPI for 20 min in the dark. After rinsing four times with PBS, images were captured with a confocal microscope (TCS SP8; Leica Microsystems, Wetzlar, Germany). An average of 50 cells was imaged per condition, and the individual channel images were merged and analyzed with ImageJ (National Institutes of Health, Bethesda, MD, USA) to estimate the extent of colocalization.

Statistical analysis

Statistical analysis was performed using the SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). All data were

expressed as mean ± SD. Differences between groups were evaluated by the Student's *t*-test or one-way analysis of variance followed by Dunnett's post hoc test. *P*<0.05 was considered statistically significant.

Results

Cardamonin decreased the expression and phosphorylation of Raptor

After incubating with cardamonin, rapamycin, and AZD8055 for 2, 16, and 24 h, the phosphorylation of mTOR was decreased in the SKOV3 cells (Figure 1). The phosphorylation of Raptor was reduced by all these drugs, while the protein expression of Raptor was specifically decreased by cardamonin in a time-dependent manner (Figure 2). Cardamonin, rapamycin, and AZD8055 marginally inhibited the phosphorylation of PRAS40 (S183); however, the expression of PRAS40 and PRAS40-T246 was not affected (Figure 3). We speculated that Raptor participated in the mTOR inhibition by cardamonin.

Cardamonin inhibited the activity of mTORC1 signaling

As shown in Figure 4, the phosphorylation of S6K1 was significantly decreased by cardamonin, rapamycin, and AZD8055. However, the expression of S6K1 was not changed. Since S6K1 is a sensitive sensor of mTORC1, the result indicated that cardamonin inhibited the mTORC1 signaling.

Cardamonin decreased the phosphorylation of mTORC1-specific binding proteins in Raptor knockdown cells

After transfecting with Raptor siRNA, the protein expression of Raptor was decreased as expected. However, cardamonin had an additional inhibitory effect on the expression of Raptor in the Raptor siRNA cells (Figure 5). Raptor siRNA also decreased the phosphorylation of mTOR, Raptor, PRAS40-S183 (marginally), and S6K1, which were further inhibited by cardamonin in the Raptor siRNA SKOV3 cells (Figures 6 and 7), whereas rapamycin and AZD8055 had no additional inhibitory effect on these proteins.

Cardamonin inhibited the lysosomal localization of mTOR

Cardamonin suppressed the lysosomal colocalization of mTOR with LAMP2, an established lysosomal membrane protein. As expected, Raptor siRNA decreased the localization of mTOR to the lysosomal surface. Furthermore,

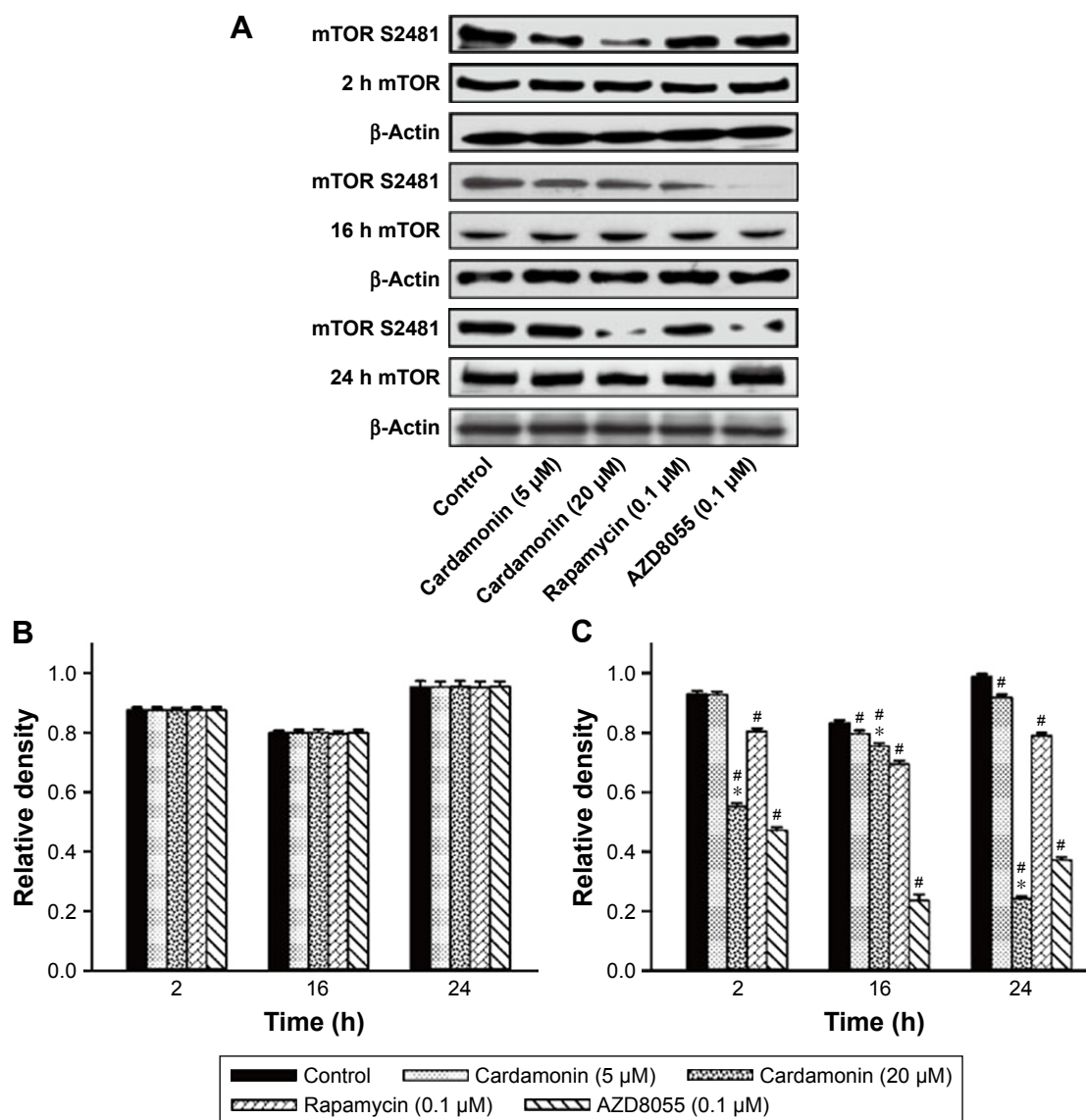


Figure 1 Cardamonin downregulated the expression of mTOR S2481.

Notes: SKOV3 cells were incubated with cardamonin (5 and 20 μM), rapamycin (0.1 μM), and AZD8055 (0.1 μM) for 2, 16, and 24 h. Western blotting was used to detect the expression of mTOR S2481 and total mTOR. (A) Autoradiograph of expression of mTOR S2481 and total mTOR. The density ratios of each band by mTOR/β-actin (B) and mTOR S2481/β-actin (C). n=3; *P<0.05 vs control group; #P<0.05 vs cardamonin (5 μM) group.

Abbreviation: mTOR, mammalian target of rapamycin.

cardamonin strengthened the inhibitory effect on lysosomal localization of mTOR in Raptor siRNA cells (Figure 8).

Cardamonin decreased the proliferation of Raptor siRNA SKOV3 cells

Cell viability was gradually reduced by cardamonin in both normal and Raptor siRNA cells in a dose-dependent manner. In addition, the antiproliferative effect of cardamonin was stronger on Raptor siRNA cells than that on normal cells (Figure 9). This inhibitory effect of cardamonin on cell proliferation was consistent with that on the expression of Raptor, whereas the inhibitory effect of cardamonin or

AZD8055 in normal cells and Raptor siRNA cells were similar. These results suggested that Raptor mainly mediated the antiproliferation effect of cardamonin.

Discussion

Ovarian cancer is the main cause of death among the gynecologic malignancies. Platinum-based adjuvant chemotherapy following cytoreductive surgery is the common treatment for ovarian cancer. However, the clinical outcome is unsatisfactory due to the severe adverse reaction and drug resistance of cancer cells. Thus, it will gain great clinical benefits to explore targeted drugs with better curative effects for ovarian cancer.

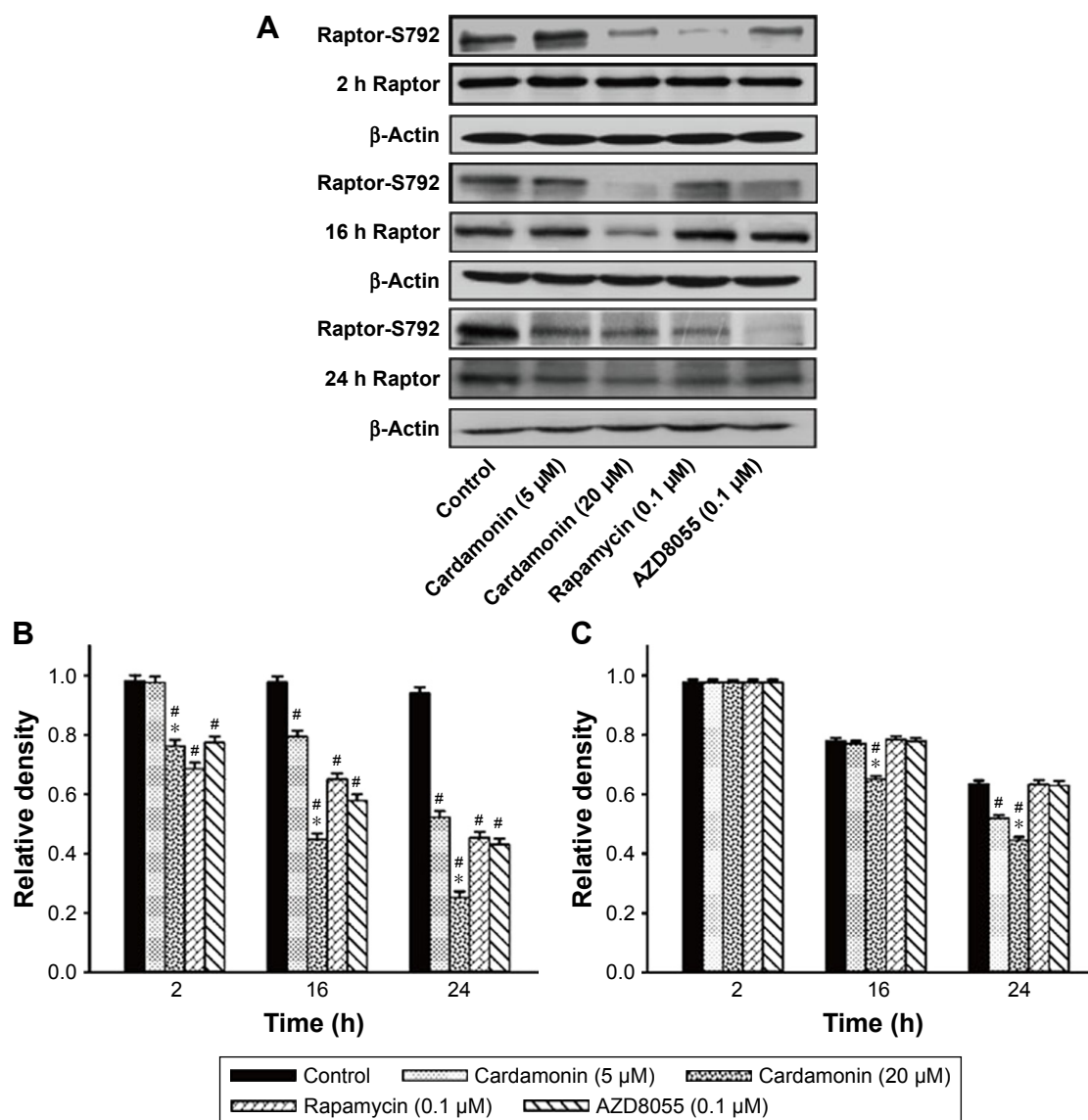


Figure 2 Cardamonin downregulated the expression of Raptor-S792 and total Raptor.

Notes: SKOV3 cells were incubated with cardamonin (5 and 20 μM), rapamycin (0.1 μM), and AZD8055 (0.1 μM) for 2, 16, and 24 h. Western blotting was used to detect the expression of Raptor-S792 and total Raptor. (A) Autoradiograph of expression of Raptor-S792 and total Raptor. The density ratios of each band by Raptor-S792/β-actin (B) and Raptor/β-actin (C). n=3; #P<0.05 vs control group; *P<0.05 vs cardamonin (5 μM) group.

mTOR is a promising drug target for cancer. It accelerates the tumorigenesis, development, and platinum resistance of ovarian cancer.¹ Our previous studies have demonstrated that cardamonin inhibits the proliferation and angiogenesis in SKOV3 cells through suppressing mTOR;²⁹ in addition, it has no effect on the specific component of mTORC2 and its downstream substrate Akt (Shi et al, unpublished data, 2018). It speculates that cardamonin is an mTORC1 inhibitor.^{23,29} As Raptor and PRAS40 are important in the activation of mTORC1 and S6K1, we investigated the effect of cardamonin on the phosphorylation and expression of these proteins. In the present study, the activity of mTOR and S6K1 was significantly decreased by cardamonin. After treating with cardamonin for 2, 16, and 24 h,

the expression of Raptor, Raptor-S792, and PRAS40-S183 was reduced in SKOV3 cells, whereas the expression of PRAS40-T246 was not affected. Since the phosphorylation of PRAS40 at Ser183 rather than Thr246 was regulated by mTORC1,³⁰ we further confirmed that cardamonin inhibited mTORC1. Interestingly, both rapamycin and AZD8055 have no effect on the expression of Raptor. It suggests that Raptor plays a critical role in mTORC1 inhibition by cardamonin, which is different from rapamycin and AZD8055.

Since cardamonin decreases the indispensable binding protein of mTORC1, it is worthwhile investigating the effect of cardamonin in Raptor knockdown cells. In accordance with other studies, we successfully knocked

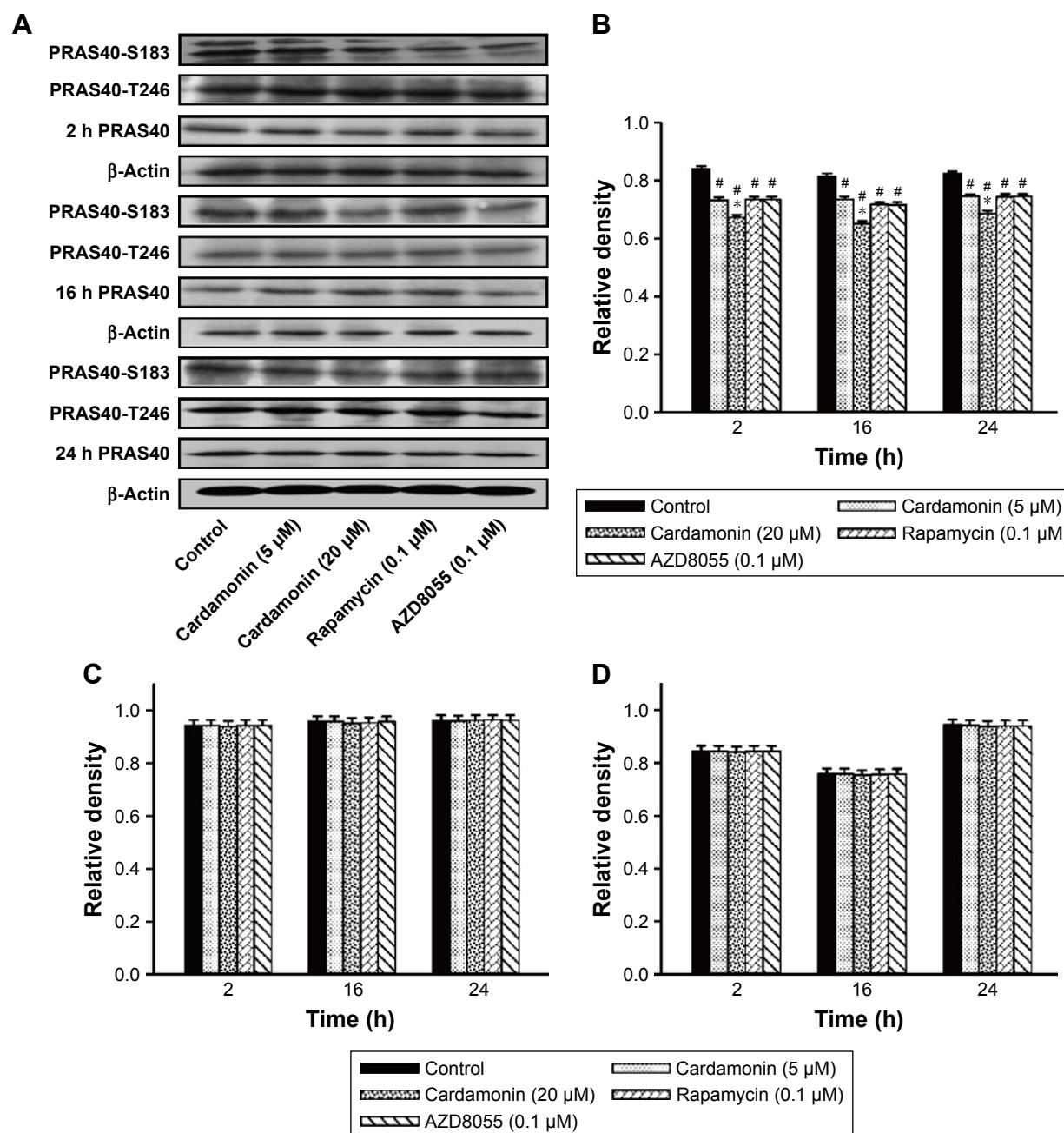


Figure 3 Cardamonin downregulated the expression of PRAS40-S183.

Notes: SKOV3 cells were incubated with cardamonin (5 and 20 μM), rapamycin (0.1 μM), and AZD8055 (0.1 μM) for 2, 16, and 24 h. Western blotting was used to detect the expression of PRAS40-S183, PRAS40-T246, and total PRAS40. (A) Autoradiograph of expression of PRAS40-S183, PRAS40-T246, and total PRAS40. The density ratios of each band by PRAS40-S183/β-actin (B), PRAS40-T246/β-actin (C), and PRAS40/β-actin (D). n=3, #P<0.05 vs control group; *P<0.05 vs cardamonin (5 μM) group.

down the expression of Raptor by siRNA in SKOV3 cells.^{31,32} Moreover, the phosphorylation of mTOR and S6K1 was decreased. However, the expression of Raptor was partially downregulated by siRNA. After treating with cardamonin, the protein expression of Raptor and the phosphorylation of mTOR and S6K1 were further reduced. We speculated that the further inhibitory effect of cardamonin on mTORC1 signal pathway in Raptor siRNA SKOV3 cells was due to the

incomplete knockdown of Raptor. It was worth noting that both rapamycin and AZD8055 had no additional inhibitory effect on the expression of Raptor.

Mechanism of mTORC1 activation remains mysterious. It is expected that proteins that signal the availability of nutrition to mTORC1 are also likely to interact with it, but so far, no good candidates have been identified. Recent studies have demonstrated that Raptor-mediated translocation of mTOR to

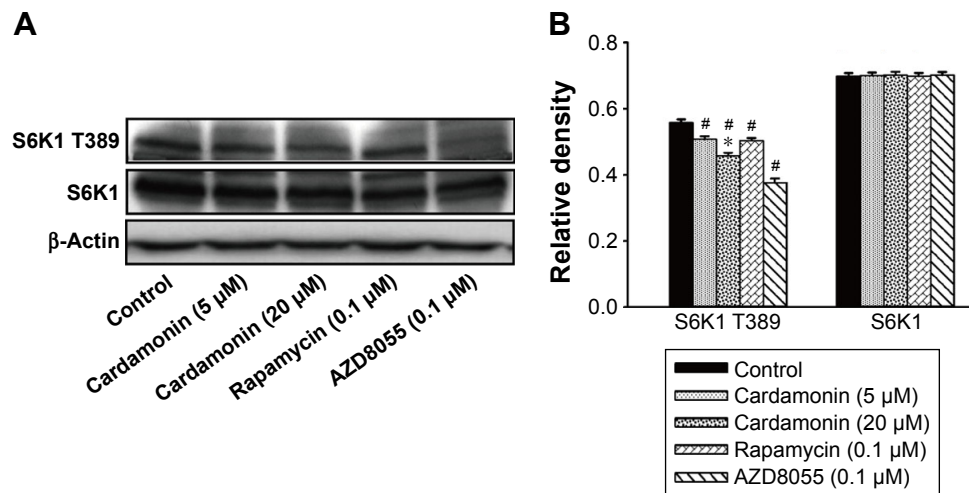


Figure 4 Cardamonin downregulated the expression of p-S6K1.

Notes: SKOV3 cells were incubated with cardamonin (5 and 20 μM), rapamycin (0.1 μM), and AZD8055 (0.1 μM) for 24 h. Western blotting was used to detect the expression of S6K1 T389 and total S6K1. **(A)** Autoradiograph of expression of S6K1 T389 and total S6K1. **(B)** The density ratios of each band by S6K1 T389/β-actin and S6K1/β-actin. *n*=3; **P*<0.05 vs control group; **P*<0.05 vs cardamonin (5 μM) group.

lysosomes is an essential procedure for the activation of mTOR.^{33–35} Cardamonin and Raptor siRNA decreased the localization of mTOR to the lysosomal surface. Furthermore, the inhibitory effect of cardamonin on mTOR lysosomal localization was strengthened in Raptor siRNA cells. It suggested

that Raptor was the potential target of cardamonin on mTORC1 inhibition. Nevertheless, more studies are needed to confirm whether cardamonin has a direct effect on mTOR.

Previous studies have shown that the proliferation is suppressed by cardamonin and mTOR inhibitors in various cancer cells.^{36,37} To illustrate whether the antiproliferative effect of cardamonin was associated with Raptor downregulation, we performed MTT assay in both normal cells and Raptor siRNA cells. The results showed that the cell viability was decreased by cardamonin. Similar to Raptor inhibition, the antiproliferation effect of cardamonin in Raptor siRNA-transfected cells was stronger than that in normal SKOV3 cells. However, the effective dose of cardamonin is much higher than that of rapamycin and AZD8055. It indicated that Raptor was involved in the antiproliferative effect of cardamonin in SKOV3 cells. Several studies have demonstrated that cardamonin downregulates the proliferation of cancer cells through inhibiting nuclear factor-κB, signal transducers and activators of transcription 3, and c-Jun N-terminal kinase.^{38–40} The effective concentration of cardamonin (10–20 μM) in these studies is higher than that of rapamycin, which is consistent with our results in the present and previous studies.^{23,29} Although cardamonin interferes with other signaling pathways, our results in this study suggest that Raptor mainly mediates the antiproliferative effect of cardamonin in SKOV3 cells.

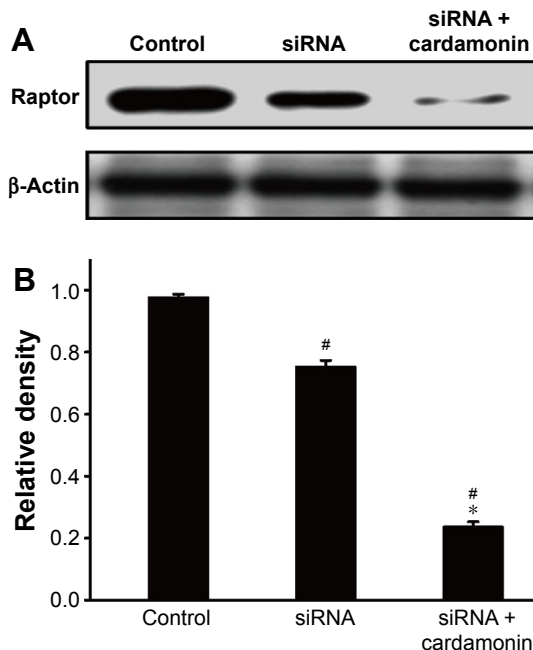


Figure 5 Analysis of Raptor interference.

Notes: SKOV3 cells were transfected with Raptor siRNA for 24 h and then treated with cardamonin (20 μM) for 24 h. Cell extracts were analyzed by Western blotting for the expression of total Raptor. **(A)** Expression of total Raptor in Raptor RNAi SKOV3 cells. **(B)** The density ratios of each protein expression band by Raptor/β-actin in Raptor RNAi SKOV3 cells. *n*=5; **P*<0.05 vs control group; **P*<0.05 vs siRNA group.

Abbreviation: siRNA, small interfering RNA.

Conclusion

Our results reveal that Raptor is the potential target of cardamonin on antiproliferation by suppressing the mTORC1 activity in ovarian cancer SKOV3 cells.

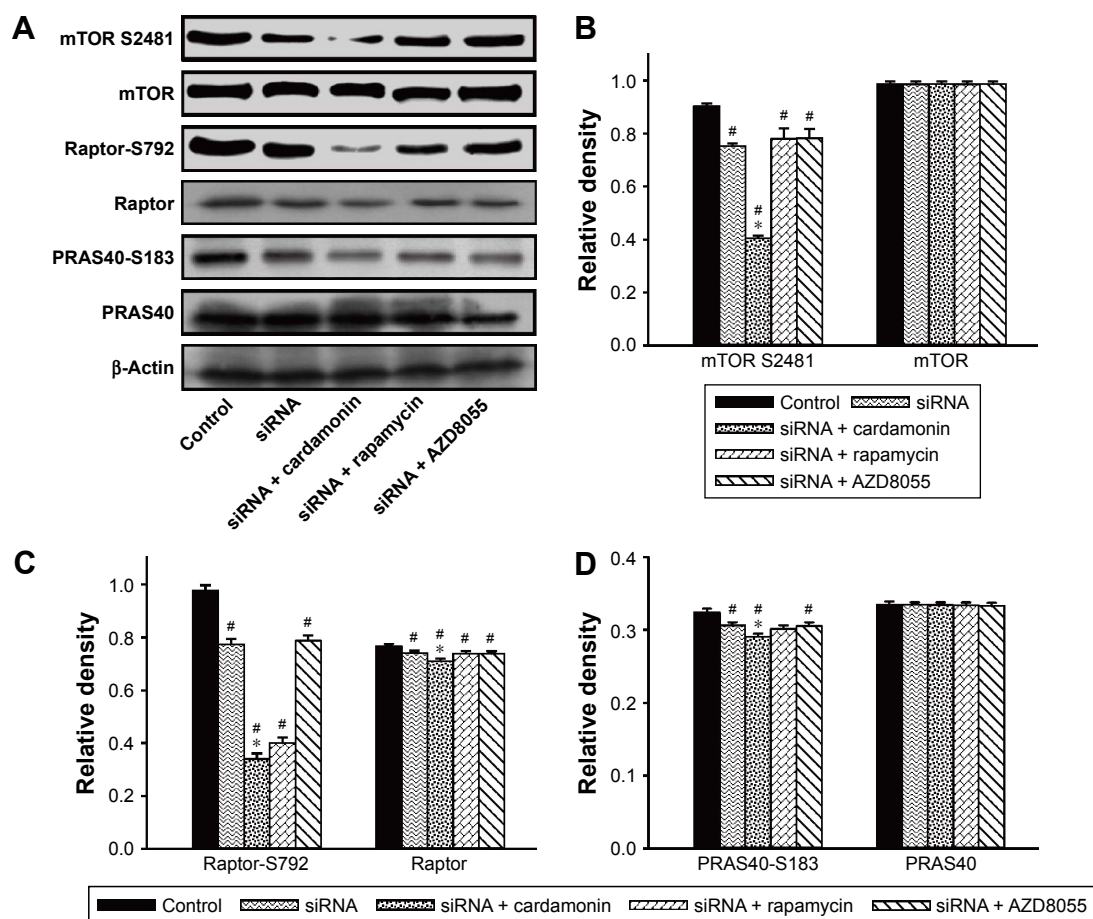


Figure 6 Cardamonin downregulated the phosphorylation of mTORC1-specific binding proteins.

Notes: SKOV3 cells were transfected with Raptor siRNA for 24 h and then treated with cardamonin (20 μ M), rapamycin (0.1 μ M), and AZD8055 (0.1 μ M) for 24 h. Expression of protein extracted from total cells was assayed by Western blotting. **(A)** The level of phosphorylation and total protein of mTORC1-specific binding proteins in Raptor RNAi SKOV3 cells. **(B)** The density ratios of each band by mTOR S2481/ β -actin and mTOR/ β -actin in Raptor RNAi SKOV3 cells. **(C)** The density ratios of each band by Raptor-S792/ β -actin and Raptor/ β -actin in Raptor RNAi SKOV3 cells. **(D)** The density ratios of each band by PRAS40-S183/ β -actin and PRAS40/ β -actin in Raptor RNAi SKOV3 cells. n=3; * P <0.05 vs control group; # P <0.05 vs siRNA group.

Abbreviations: mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; siRNA, small interfering RNA.

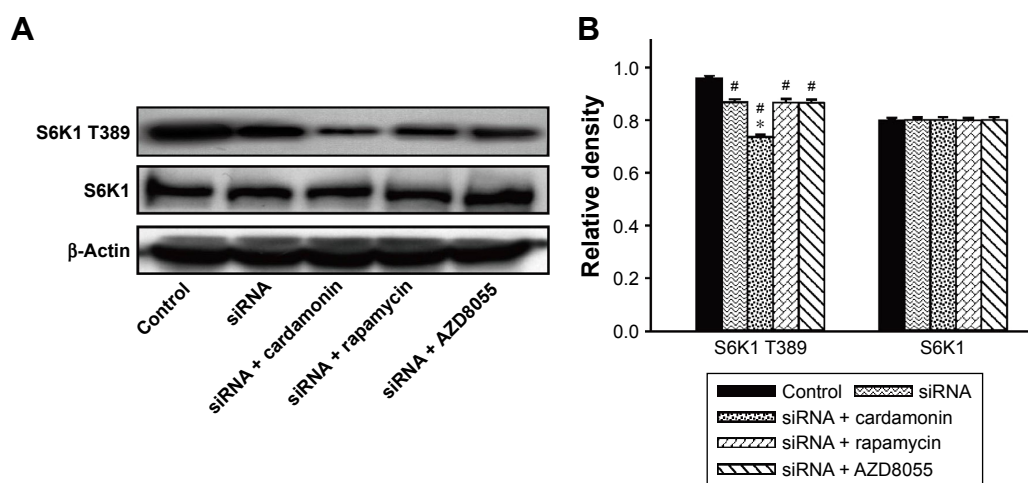


Figure 7 Cardamonin downregulated the phosphorylation of S6K1.

Notes: SKOV3 cells were transfected with Raptor siRNA for 24 h and then treated with cardamonin (20 μ M), rapamycin (0.1 μ M), and AZD8055 (0.1 μ M) for 24 h. Protein expression of phosphorylation and total S6K1 extracted from the total cell extracts were assayed by Western blotting. **(A)** The level of phosphorylation and total S6K1 in Raptor RNAi SKOV3 cells. **(B)** The density ratios of each band by S6K1 T389/ β -actin and S6K1/ β -actin in Raptor knock-down SKOV3 cells. n=3; * P <0.05 vs control group; # P <0.05 vs siRNA group.

Abbreviation: siRNA, small interfering RNA.

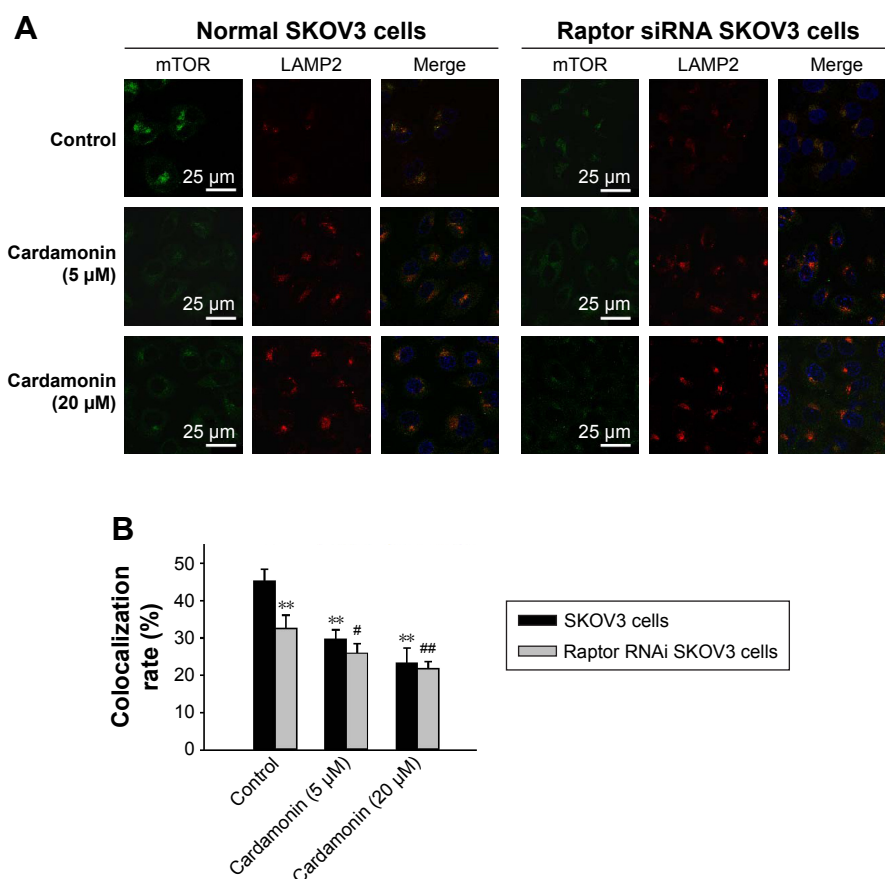


Figure 8 Raptor interference enhanced the inhibitory effect of cardamonin on the lysosomal localization of mTOR.

Notes: Both normal and Raptor siRNA SKOV3 cells were treated with cardamonin (5 and 20 μ M) for 150 min. The lysosomal localization of mTOR was determined by immunofluorescence assays. **(A)** Images of SKOV3 cells coimmunostained for mTOR (green), LAMP2 (red), and nucleus (blue). **(B)** Quantitation of the colocalization. $n=3$; $^{**}P<0.01$ vs SKOV3 control group; $^{*}P<0.05$, $^{###}P<0.05$ vs Raptor siRNA SKOV3 control group.

Abbreviations: mTOR, mammalian target of rapamycin; siRNA, small interfering RNA.

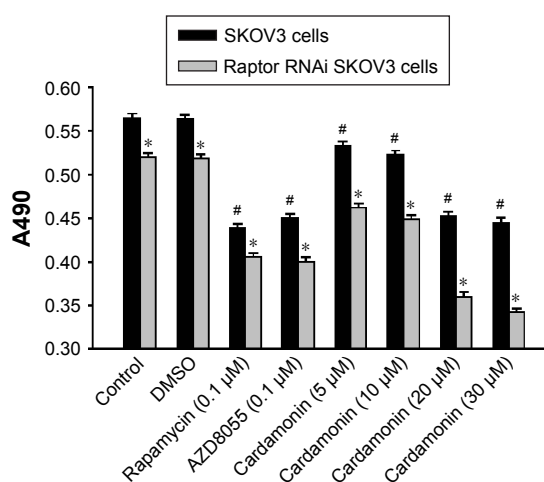


Figure 9 Inhibitory effect of cardamonin on the proliferation of SKOV3 cell-transfected Raptor-siRNA.

Notes: SKOV3 cells were seeded in a 96-well plate and then treated with DMSO (0.1%), cardamonin (5, 10, 20, and 30 μ M), rapamycin (0.1 μ M), and AZD8055 (0.1 μ M) for 24 h. A total of 20 μ L MTT solution (0.5 mg/mL) was added to each well, incubated at 37°C for 4 h, then the supernatant was discarded, 150 μ L DMSO added into each well, and shaken for 15 min. Finally, the absorbance was determined at 490 nm by a microplate reader. $n=6$; $^{*}P<0.05$ vs control group; $^{*}P<0.05$ vs nontransfection groups.

Abbreviations: DMSO, dimethyl sulfoxide; MTT, methyl thiazolyl tetrazolium; siRNA, small interfering RNA.

Acknowledgments

The authors gratefully thank Dr Peng Huang (University of Calgary, Calgary, AB, Canada) for helpful comments. This study was supported by the Natural Science Foundation of Fujian Province (2015J01368 and 2016J01492) and Innovative Medical Foundation of Fujian Provincial Health and Family Planning Commission (2016-CX-13), China.

Disclosure

The authors report no conflicts of interest in this work.

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