

RCE-4, a potential anti-cervical cancer drug isolated from *Reineckia carnea*, induces autophagy via the dual blockade of PI3K and ERK pathways in cervical cancer CaSki cells

WEI XIANG¹, REN-JING ZHANG², GUI-LAN JIN³, LI TIAN¹, FAN CHENG¹, JUN-ZHI WANG¹,
XIANG-FEI XING³, WEI XI³, SHU-JUN TANG⁴ and JIAN-FENG CHEN¹

¹Hubei Key Laboratory of Natural Products Research and Development, College of Biological and Pharmaceutical Sciences, China Three Gorges University, Yichang, Hubei 443002; ²Department of Pathology, Affiliated Hospital of North Sichuan Medical College, Nanchong, Sichuan 637000; ³The First People's Hospital of Yichang and The People's Hospital of China Three Gorges University, Yichang, Hubei 443002, P.R. China; ⁴Department of Biomedical Engineering, Illinois Institute of Technology, Chicago, IL 60616, USA

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Abstract. The steroidal saponin RCE-4 (1 β , 3 β , 5 β , 25S)-spirostan-1, 3-diol 1-[α -L-rhamnopyranosyl-(1 \rightarrow 2)- β -D-xylopyranoside], isolated from *Reineckia carnea*, exerts significant anti-cervical cancer activity by inducing apoptosis. The potential effect of RCE-4 on proliferation inhibition and autophagy induction has rarely been studied. Therefore, the focus of the present study was to investigate the effects of RCE-4 on proliferation, and to elucidate the detailed mechanisms involved in autophagy induction in cervical cancer cells. CaSki cells were treated with RCE-4 or/and autophagy inhibitors, and the effect of RCE-4 on cellular proliferation was assessed by MTT assay. The pro-autophagic properties of RCE-4 were subsequently confirmed using monomeric red fluorescent protein-green fluorescent protein-microtubule-associated proteins 1A/1B light chain 3B (LC3) adenoviruses and CYTO-ID autophagy assays, and by assessing the accumulation of lipid-modified LC3 (LC3II). The mechanisms of RCE-4-induced autophagy were investigated by western blot analysis. The results demonstrated that inhibiting autophagy significantly promoted RCE-4-induced cell death, indicating that autophagy served a protective role following RCE-4 treatment. In addition, RCE-4-induced autophagy was reflected by increased expression levels of the serine/threonine-protein

kinase ULK1, phosphorylated (p)-ULK1, p-Beclin-1 and LC3II, the formation of autophagosomes and autolysosomes, and sequestosome 1 (p62) degradation. Subsequent analysis indicated that RCE-4 activated the AMP-activated protein kinase (AMPK) pathway by upregulating AMPK and p-AMPK, and also inhibited the PI3K and extracellular signal-regulated kinase (ERK) signaling pathways by down-regulating p-PI3K, p-Akt, p-mTOR, Ras, c-Raf, p-c-Raf, dual specificity mitogen-activated protein kinase kinase (MEK)1/2, p-MEK1/2 and p-Erk1/2. Additionally, with increased treatment times RCE-4 may impair lysosomal cathepsin activity and inhibit autophagy flux by suppressing the expression of AMPK, p-AMPK, ULK1, p-ULK1 and p-Beclin-1, and upregulating that of p62. These results indicated that the dual RCE-4-induced inhibition of the PI3K and ERK pathways may result in a more significant anti-tumor effect and prevent chemoresistance, compared with the inhibition of either single pathway; furthermore, dual blockade of PI3K and ERK, and the AMPK pathway may be involved in the regulation of autophagy caused by RCE-4. Taken together, RCE-4 induced autophagy to protect cancer cells against apoptosis, but AMPK-mediated autophagy was inhibited in the later stages of RCE-4 treatment. In addition, autophagy inhibition improved the therapeutic effect of RCE-4. These data highlight RCE-4 as a potential candidate for cervical cancer treatment.

Correspondence to: Professor Jian-Feng Chen, Hubei Key Laboratory of Natural Products Research and Development, College of Biological and Pharmaceutical Sciences, China Three Gorges University, 8 University Avenue, Yichang, Hubei 443002, P.R. China
E-mail: chenjianfeng2003@126.com

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Introduction

Natural products are valuable resources for the screening of anti-cervical cancer drugs, and include compounds such as artesunate (1), resveratrol (2), betulinic acid (3) and Timosaponin A-III (4). *Reineckia carnea* (Liliaceae), a traditional Chinese herb containing the saponin (1 β , 3 β , 5 β , 25S)-spirostan-1, 3-diol 1-[α -L-rhamnopyranosyl-(1 \rightarrow 2)- β -D-xylopyranoside] (RCE-4; Fig. 1A) as a biologically active component, has been used to treat diseases including rheumatism, coughs and hepatitis (5,6). To the best of our knowledge, the steroidal saponins primarily exert anti-inflammatory

and antitumor activity, with toxicity towards both normal and tumor cells (7). However, RCE-4 exhibited a significant anti-inflammatory (8) and notable cytotoxic effects on cancer CaSki, HeLa, HT-29 and CNE-2 cell lines, whereas it exerted relatively weak cytotoxic effects in normal Marc-145 and MDCK cells (9). Furthermore, CaSki cell xenograft experiments using nude mice demonstrated that RCE-4 inhibited tumor growth and had notably low levels of toxicity in normal tissues, including those of the liver and the uterus (10).

Drug-induced tumor-cell death is a complex process that includes various modes of programmed cell death, including apoptosis, autophagy and necrosis (11). Cross-talk has been observed between autophagy (type II programmed cell death), a process in which newly formed membrane-encapsulated vesicles phagocytose and consume cellular components including damaged organelles and misfolded protein aggregates, and apoptosis, which share various cell death mediators, such as Bcl-2 and Beclin-1 (12). Normally, autophagic self-degradation not only removes damaged organelles and misfolded proteins (13,14), but also recycles nutrients. However, deregulation of autophagy may induce tumor cell death, and may therefore serve as a cell survival pathway to inhibit apoptosis, either in combination with apoptosis or as a secondary mechanism when the former is ineffective (15-17). Mechanistically, numerous studies have shown that the PI3K/AKT and Ras/Raf/dual specificity mitogen-activated protein kinase (MEK)/ERK pathways serve an important role in autophagy by inhibiting the expression of mTOR, which is a key homeostatic regulator of cellular proliferation (18). In addition, the AMP-activated protein kinase (AMPK) signaling pathway, which is closely associated with energy metabolism, has also been indicated to activate autophagy (19-21).

Conversely, cross-talk between the Ras/Raf/MEK/Erk and PI3K/Akt pathways in regulating tumor cell proliferation, differentiation, apoptosis and senescence has been identified. This cross-talk may provide the focus for further studies investigating drug therapies that inhibit both signaling networks (22-24). Specifically, activation of the PI3K/Akt pathway promotes cell survival by inhibiting cell cycle progression; additionally, high activity levels of Erk, which is regulated by the Ras/Raf/MEK/Erk pathway, promote cyclin D1 expression and cellular proliferation. Moreover, activation of the PI3K/Akt or Ras/Raf/MEK/Erk pathways inhibits apoptosis by phosphorylating and blocking apoptosis-associated targets including Bcl-2, Bad and caspase-9 (3,25). These studies indicate that the ERK and the PI3K pathways promote cell proliferation and survival. However, inhibitors of each single pathway have limited anti-tumor activity, as the inhibition of one pathway results in the compensatory activation of the other (18). This may also partially explain why cancer treatment often results in resistance to chemotherapeutic drugs. For example, the MEK inhibitor PD98059 blocks the ERK pathway, leading to an abnormal increase in AKT activation levels (26). In addition, in breast cancer cells, the activity of an PI3K inhibitor was demonstrated to be enhanced, resulting in a decreased level of proliferation and enhanced anti-tumor activity by co-treatment with an ERK pathway inhibitor (27). These examples suggest that simultaneous inhibition of the ERK and PI3K pathways in the treatment of certain tumor cells may contribute to the improved anti-tumor activity of chemotherapeutic drugs.

In previous studies, RCE-4, as a potential anti-cervical cancer chemotherapy drug, was demonstrated to induce mitochondria-mediated apoptosis, which was considered to be the mechanism for inhibiting the proliferation of CaSki and HeLa cells (5,9). The aim of the present study was to clarify whether RCE-4-induced autophagy is a survival or death mechanism during cervical cancer treatment. Subsequently, the levels of autophagy were assessed by treating cells with different concentrations of RCE-4, and to determine the degree of autophagy at different treatment exposure times. Given the key roles of the AMPK, PI3K and ERK pathways in cancer cell proliferation and autophagy, it was necessary to additionally identify whether RCE-4 treatment affected the expression and phosphorylation statuses of proteins involved in these pathways. Collectively, the aim of the present study was to improve the understanding of the anti-tumor mechanisms of RCE-4.

Materials and methods

Reagents and antibodies. The RCE-4 preparation used in the present study was isolated from *R. carnea* and its structure is demonstrated in Fig. 1A. A 50-mM stock solution of purified RCE-4 was prepared in DMSO, and diluted to the desired concentration with RPMI-1640 medium prior to use. Rabbit monoclonal antibodies against β -actin (cat. no. 4970), AMPK α (cat. no. 5831), phosphorylated (p)-AMPK α (cat. no. 2535), the serine/threonine-protein kinase ULK1 (ULK1; cat. no. 8054), p-ULK1 (cat. no. 14202), Beclin-1 (cat. no. 3495), p-Beclin-1 (cat. no. 14717), microtubule-associated proteins 1A/1B light chain 3B (LC3; cat. no. 12741), sequestosome 1 (p62; cat. no. 8052), mTOR (cat. no. 2983), p-mTOR (cat. no. 5536), PI3K (cat. no. 4257), p-PI3K (cat. no. 4228), Akt (cat. no. 4691), p-Akt (cat. no. 4060), Ras (cat. no. 3339), c-Raf (cat. no. 9422), p-c-Raf (cat. no. 9431), MEK1/2 (cat. no. 8727), p-MEK1/2 (cat. no. 9154), p-Erk1/2 (cat. no. 4370) and Erk1/2 (cat. no. 4695), all purchased from Cell Signaling Technology, Inc., were used at a dilution of 1:1,000. The horseradish peroxidase-labeled secondary antibody (anti-rabbit IgG), which was purchased from Cell Signaling Technology, Inc. (cat. no. 7074V), was used at a dilution of 1:5,000. The CYTO-ID Autophagy Detection kit (cat. no. ENZ-51031) was purchased from Enzo Life Sciences Inc., and the autophagy inhibitors bafilomycin A1 (Baf A1), 3-methyladenine (3-MA) and chloroquine (CQ) were obtained from MedChemExpress. The monomeric red fluorescent protein (mRFP)-green fluorescent protein (GFP)-LC3 tandem fluorescent protein kit was purchased from Han heng Biotechnology Co., Ltd.

Cell culture. The human cervical cancer CaSki cell line was obtained from the Cell Bank of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 0.2% HEPES at 37°C and 5% CO₂.

Growth inhibitory assays. CaSki cells (2x10⁴ cells/well) were seeded into 96-well plates, incubated at 37°C for 12 h and subsequently treated with 1, 2, 4, 6 and 8 μ M RCE-4 for an additional 48 h. For the autophagy inhibition assay,

the CaSki cells were pretreated with the selective autophagy inhibitors 3-MA (8 mM), CQ (30 μ M) and Baf A1 (0.4 μ M) for 6 h, and then treated with RCE-4 (0, 1, 2, 4, 6 and 8 μ M) for an additional 48 h. Subsequently, 20 μ l 5 mg/ml MTT (Sigma-Aldrich; Merck KGaA) reagent was added to each well, and the cells were cultured for another 4 h; the medium was gently removed, and the adherent cells were lysed in 150 μ l DMSO solution for 10 min with shaking. The absorbance at 490 nm was measured using a microplate reader (Tecan Group, Ltd.), and the inhibition rate was calculated according to the following formula: Inhibition rate = [1 - optical density (OD) value of the administration group/OD value of blank control group] x 100%.

CYTO-ID autophagy detection assay. The CYTO-ID Autophagy Detection kit was used to selectively label pre-autophagosomes, autophagosomes and autolysosomes; bright fluorescence is detectable in autophagic vacuoles, thereby providing a quantitative method for autophagy detection (28). The CYTO-ID autophagy detection assay was performed according to the manufacturer's kit protocol. CaSki cells were treated with RCE-4 at concentrations of 0, 8, 12 and 16 μ M for 6, 12 and 24 h each. Following the collection of the adherent cells and those in solution, the cells were resuspended in 1X assay buffer (provided by the CYTO-ID Autophagy Detection kit) and centrifuged (1,000 x g) at room temperature for 5 min to remove residual supernatant impurities, including cell growth medium and serum. After carefully removing the buffer, cells were suspended and stained with 500 μ l of 0.1% (v/v) CYTO-ID Green staining solution (1 μ l CYTO-ID Green Detection Reagent diluted with 1 ml 1X Assay buffer) for 30 min at room temperature in the dark. After treatment, stained cells were collected via centrifugation and washed with 1X Assay Buffer. The cell pellets were then resuspend in 500 μ l of fresh 1X Assay Buffer to analyze autophagy status. To detect autophagic vesicles, the green fluorescence emission (530 nm) of $\geq 1 \times 10^4$ cells was detected using flow cytometry (BD FACSVerser; BD Biosciences), and the data were analyzed using BD FACSuite™ software (version 1.0.0.1477; BD Biosciences).

Assessment of autophagic flux. To determine the autophagy levels in living cells, mRFP-GFP-LC3 adenoviruses or plasmids have been widely used to identify autophagosomes and autolysosomes (29,30). However, this method is only able to indicate a specific point within the process, rather than the autophagic status of the cells at different points following drug treatment. Following culture of the mRFP-GFP-LC3 adenovirus-transfected CaSki cells in cell culture dishes (Wuxi NEST Biotechnology Co., Ltd.) for 6 h at a multiplicity of infection of 200, 12 μ M RCE-4 was added for an additional 6, 12, 24 or 30 h. The fluorescence values of the green dots, indicating autophagosomes, and red dots, indicating autophagosomes and autolysosomes, were observed using laser confocal fluorescence microscopy (magificatin, x1,000; LCFM; Olympus FV1200; Olympus Corporation). The number of red and yellow dots (indicating autophagosomes) in the merged images was then recorded.

Western blot analysis. CaSki cells were exposed to RCE-4 at a concentration of 0, 8, 12 and 16 μ M for 6, 12 or 24 h. Following

incubation, the cells lysed in radioimmunoprecipitation assay buffer (containing 1% phosphorylated protease inhibitor and 1% PMSF) were centrifuged (12,000 x g) at 4°C for 10 min, and the total protein was quantified using a bicinchoninic (BCA) protein concentration acid assay kit (Beyotime Institute of Biotechnology). The protein samples were adjusted to a uniform concentration with 5X buffer and water, and denatured by boiling at 100°C for 10 min. The proteins (60 μ g/well) were separated using SDS-PAGE with a 6-15% polyacrylamide gel, and transferred onto a PVDF membrane (EMD Millipore). Following blocking at room temperature for 2 h with 5% skim milk, the membrane was incubated with the corresponding primary antibodies at 4°C overnight. The membrane was washed 5 times with TBS + 0.05% Tween-20 (TBST) and the secondary antibody was subsequently added prior to further incubation for 1.5 h at 37°C. An enhanced chemiluminescent kit (Beyotime Institute of Biotechnology) was used to visualize the protein bands, and the results were recorded with Kodak Film or a Luminescence Imaging System (Tanon 5200; Tanon Science and Technology Co., Ltd.). The grey values were analyzed with β -actin as a loading control. Densitometry was analyzed using Image J version 2.1 software (National Institutes of Health).

Statistical analysis. The data are expressed as the mean \pm standard deviation. GraphPad prism v.5.0 (GraphPad Software, Inc.) was used for all statistical analyses, and the differences between the treatment groups and control group were assessed by one-way analysis of variance followed by Dunnett's post hoc tests. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Autophagy inhibition increases the level of RCE-4-induced CaSki cell death. To investigate the effects of RCE-4 on the growth of human cervical cancer cells, an MTT assay was performed using CaSki cells. The results revealed that RCE-4 treatment significantly decreased the viability of CaSki cells after 48 h of treatment, which was compared with an RCE-4-untreated control group (Fig. 1B). In addition, the RCE-4-induced inhibition was enhanced following pretreatment with the autophagy inhibitors 3-MA, CQ or Baf A1 for 6 h. The half maximal inhibitory concentration values were 4.18, 3.36, 3.32 and 1.54 μ M for the RCE-4 control, the 3-MA+RCE-4, the CQ+RCE-4 and the Baf A1+RCE-4 groups, respectively. It was demonstrated that the inhibition of autophagy significantly sensitized CaSki cells to RCE-4-induced cell death, inferring that RCE-4 activated autophagy in anti-cervical cancer cells (Fig. 1B).

RCE-4 triggers autophagy in cervical cancer cells. Using the CYTO-ID autophagy assay, mRFP-GFP-LC3 tandem fluorescent protein kit and western blot analysis, RCE-4 was determined to induce autophagy in CaSki cells. The CYTO-ID autophagy assay kit was used to demonstrate the presence of autophagosomes in RCE-4 treated CaSki cells; the results indicated an increase in the formation of autophagosomes following treatment with 8, 12 and 16 μ M RCE-4 for 6, 12 and 24 h (Fig. 2A).

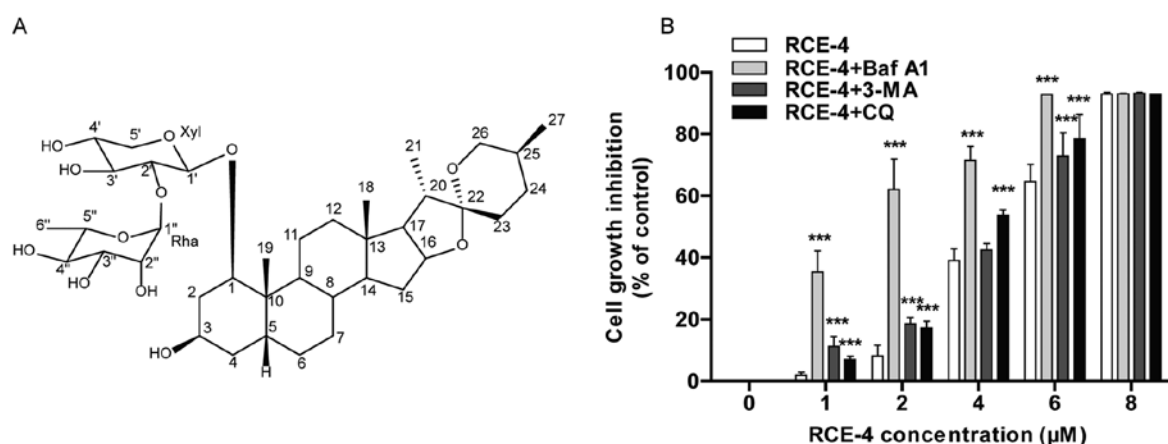


Figure 1. Autophagy inhibitors 3-MA, CQ and Baf A1 enhance the ability of RCE-4 to inhibit CaSki cell proliferation. (A) Chemical structure of RCE-4. (B) CaSki cells were treated with RCE-4 for 48 h, following pretreatment with autophagy inhibitors for 6 h, and proliferation inhibition was assessed by MTT assay. Values for each RCE-4 concentration were generated from 3 independent experiments, and are presented as the mean \pm standard deviation. *** P <0.001 vs. control group (the corresponding concentration of RCE-4 without autophagy inhibitor treatment). 3-MA, 3-methyladenine; CQ, chloroquine; Baf A1, bafilomycin A1; RCE-4, (1 β , 3 β , 5 β , 25S)-spirostan-1, 3-diol 1-[α -L-rhamnopyranosyl-(1 \rightarrow 2)- β -D-xylopyranoside].

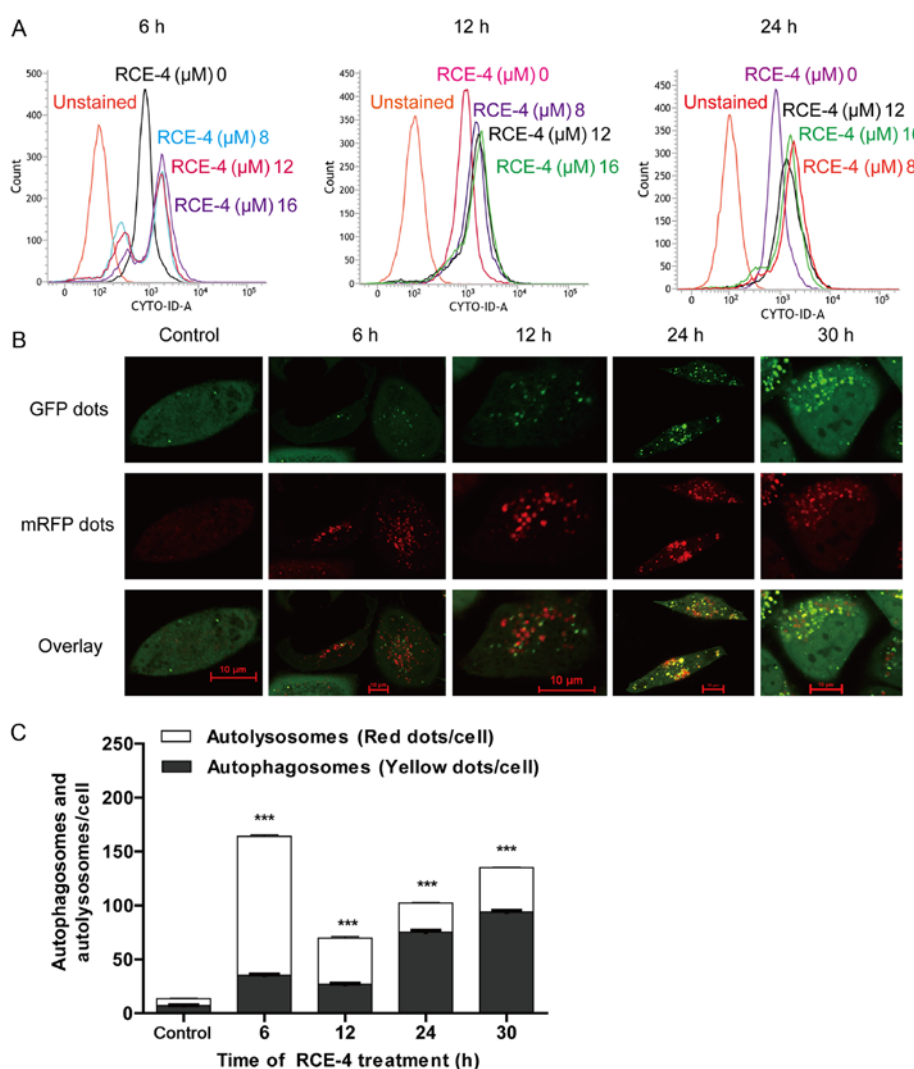


Figure 2. RCE-4 promotes autophagy in CaSki cells. (A) CYTO-ID histograms of RCE-4-treated cells indicated a shift to the right in comparison with the RCE-4-untreated group (control group) after an exposure period of 6, 12 or 24 h, indicating an increase in the mean fluorescence intensity of the RCE-4-treated group. (B) Cells were transfected with mRFP-GFP-LC3 adenoviruses for 6 h, and treated with RCE-4 for a further 6, 12, 24 or 30 h. The formation of autophagosomes (yellow dots) and autolysosomes (red dots) in each cell was observed and quantified by laser confocal fluorescence microscopy. A total of 50 cells for each condition were counted. Scale bars=10 μ m. (C) Changes in the number and percentage of autophagosomes and autolysosomes in the control and RCE-4-treated groups are presented at different RCE-4 treatment times. *** P <0.001 vs. RCE-4-untreated group. GFP, green fluorescent protein; mRFP, monomeric red fluorescent protein; RCE-4, (1 β , 3 β , 5 β , 25S)-spirostan-1, 3-diol 1-[α -L-rhamnopyranosyl-(1 \rightarrow 2)- β -D-xylopyranoside].

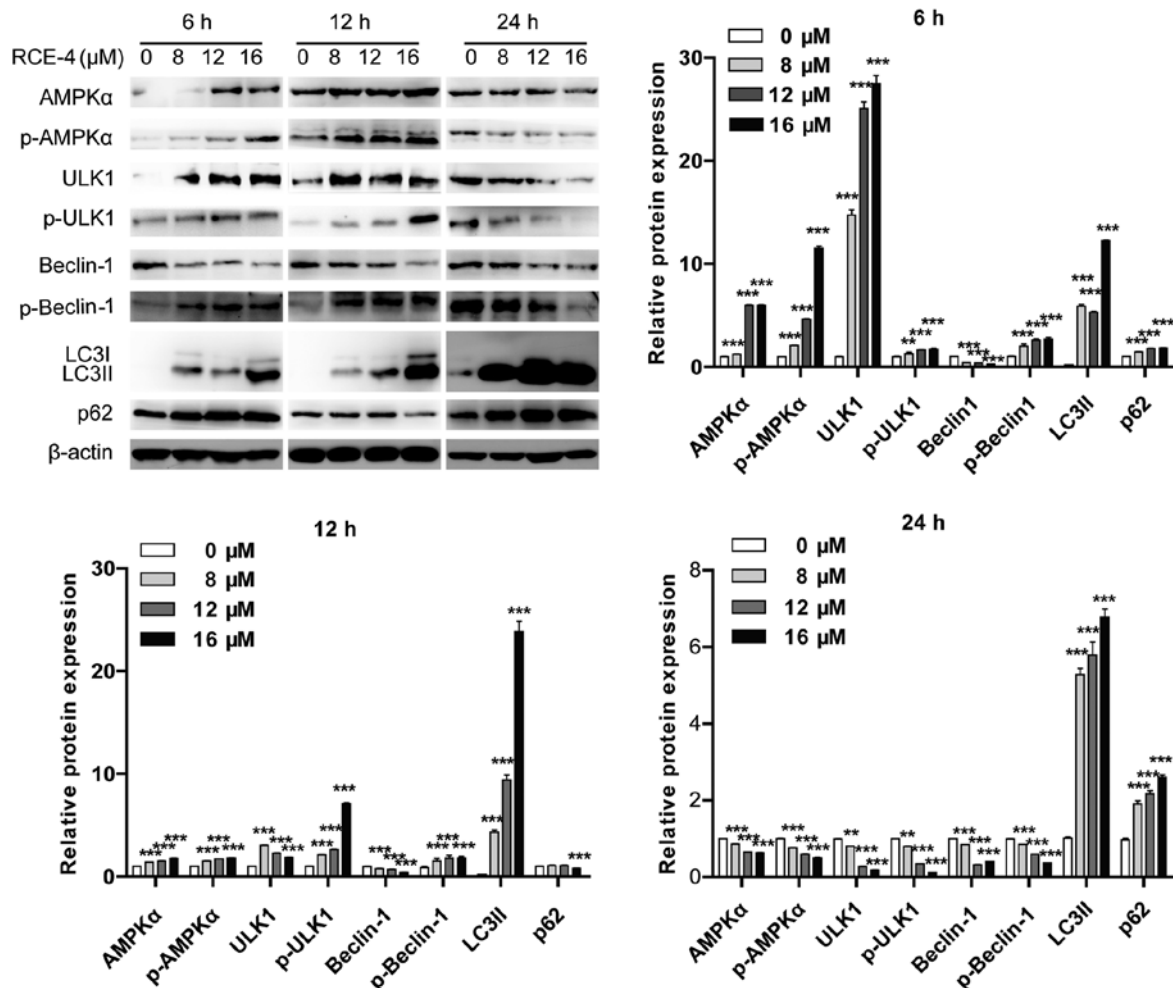


Figure 3. Expression levels of autophagy- (ULK1, p-ULK1, Beclin-1, p-Beclin-1, LC3II and p62) and AMPK pathway-associated proteins (AMPK and p-AMPK) in CaSki cells following treatment with different concentrations of RCE-4 for 6, 12 or 24 h. Bar graphs represent the western blot grey values, presented as the mean \pm standard deviation. ***P<0.01 and ****P<0.001 vs. 0 μ M RCE-4 group. ULK1, serine/threonine-protein kinase ULK1; p-, phosphorylated; LC3, microtubule-associated proteins 1A/1B light chain 3B; AMPK, AMP-activated protein kinase; p62, sequestosome 1; RCE-4, (1 β , 3 β , 5 β , 25S)-spirostan-1, 3-diol 1-[α -L-rhamnopyranosyl-(1 \rightarrow 2)- β -D-xylopyranoside].

The location and number of autophagosomes and autolysosomes in RCE-4-treated cells was visualized using LCFM after CaSki cell transfection with mRFP-GFP-LC3 adenoviruses. mRFP is stably present in autolysosomes in an acidic environment, while GFP fluorescence is easily quenched. The emission of red and green fluorescence from one cell was recorded and merged; consequently, yellow dots, indicating autophagosomes, and red dots, indicating autolysosomes, appear in the merged images. When CaSki cells were treated with RCE-4 at a concentration of 12 μ M for 6 or 12 h, an increased percentage of red fluorescent dots in the combined images was observed compared with the control group, representing autophagy activation. Conversely, when treated for 24 or 30 h, autophagy may have been inhibited, based on a decreased percentage of red fluorescent dots (Fig. 2B and C).

As indicated in Fig. 3, RCE-4 increased ULK1, p-ULK1, p-Beclin-1 and lipid-modified LC3 (LC3II) expression levels, and downregulated p62 protein expression levels when used for 12 h, compared with the control group. There were no significant alterations between the expression levels of autophagy-associated proteins, with the exception of p62, in CaSki cells treated with RCE-4 for 6 and 12 h. Upregulation

of the p62 expression level may be due to a compensatory increase in the number of autophagosomes at the initial stages of autophagy (31). Furthermore, following treatment with RCE-4 for 24 h, the expression levels of ULK1 and p-Beclin-1 were downregulated, and that of p62 was upregulated, which further supported the inhibition of autophagy after prolonged treatment. Notably, it was expected that RCE-4 would induce an upregulation in the expression levels of Beclin-1, as it promotes autophagy; however, Beclin-1 expression was not upregulated in the RCE-4-treated cells.

Conversely, the increase in the LC3II protein expression level may be due to increased autophagosome formation following autophagy activation, or incomplete autolysosome clearance. Following pretreatment with autophagy inhibitors 3-MA, Baf A1 or CQ for 6 h, the LC3II expression level in the 3-MA+RCE-4 group was decreased, compared with that of the RCE-4 group. However, the LC3II expression levels of the Baf A1+RCE-4 and the CQ+RCE-4 groups were increased following autophagy inhibition (Fig. 4A). Differences in LC3II relative protein expression were analyzed via densitometric analysis and presented in Fig. 4B. These results support the hypothesis that RCE-4 increases autophagic flux rather than inhibits the degradation

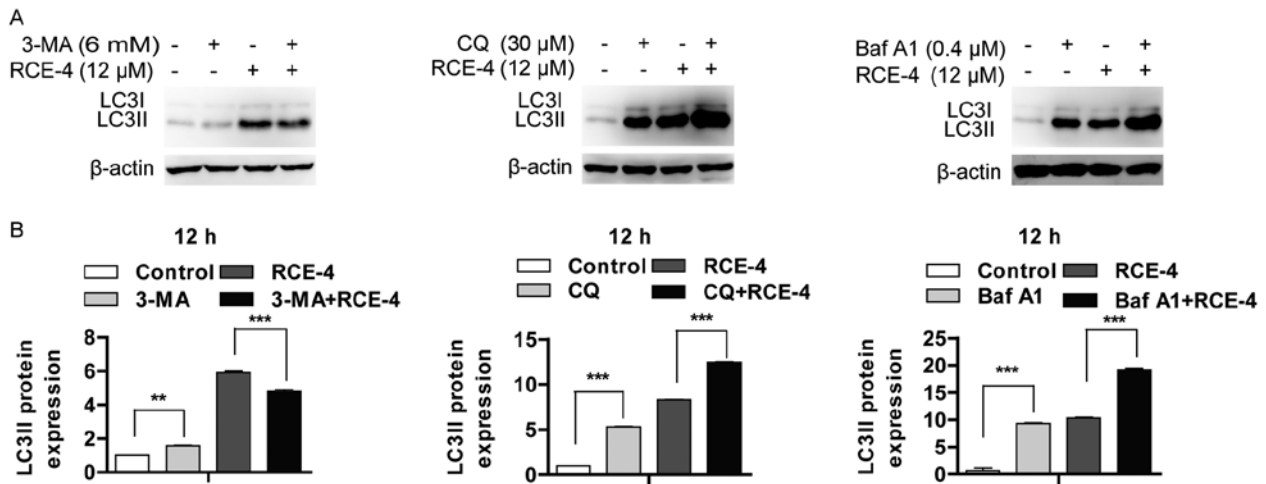


Figure 4. RCE-4 promotes the formation of autophagosomes in CaSki cells. (A) Effect of RCE-4 treatment for 12 h, with or without autophagy inhibitors, on the protein expression level of LC3II. (B) Bar graphs represent the western blot grey values, presented as the mean \pm standard deviation. ** $P < 0.01$ and *** $P < 0.001$ vs. control group (corresponding concentration of RCE-4 without autophagy inhibitor treatment). RCE-4, (1 β , 3 β , 5 β , 25S)-spirostan-1, 3-diol 1-[α -L-rhamnopyranosyl-(1 \rightarrow 2)- β -D-xylopyranoside]; LC3, microtubule-associated proteins 1A/1B light chain 3B; LC3II, lipid-modified LC3; 3-MA, 3-methyladenine; CQ, chloroquine; Baf A1, bafilomycin A1.

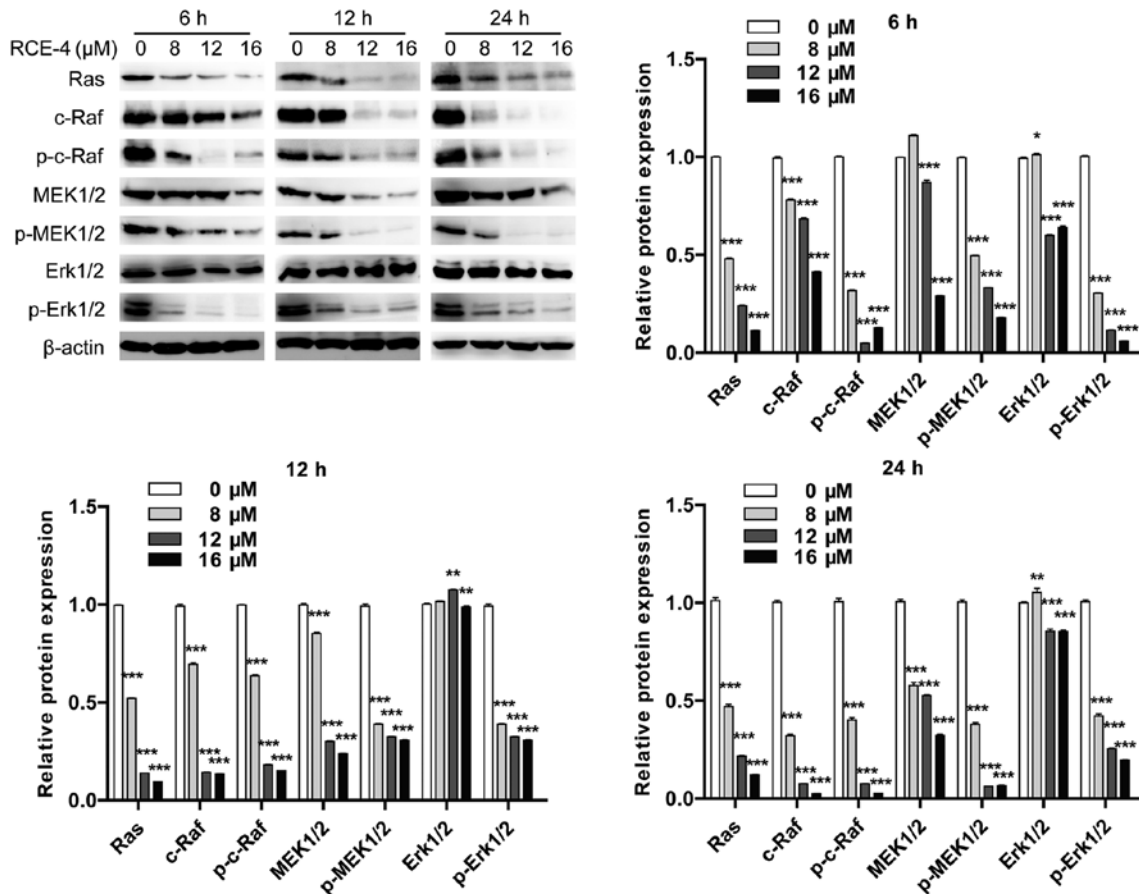


Figure 5. RCE-4 suppresses the Erk pathway signaling. Total proteins were collected from CaSki cells following treatment with various concentrations of RCE-4 (0, 8, 12 and 16 μM) for 6, 12 or 24 h. Expression levels of β-actin, Ras, total c-Raf, p-c-Raf, total MEK1/2, p-MEK1/2, total Erk1/2 and Erk1/2 were determined by western blot analysis. Bar graphs represent the western blot grey values, presented as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. 0 RCE-4 group. RCE-4, (1 β , 3 β , 5 β , 25S)-spirostan-1, 3-diol 1-[α -L-rhamnopyranosyl-(1 \rightarrow 2)- β -D-xylopyranoside]; MEK, mitogen-activated protein kinase kinase; Erk, extracellular signal-regulated kinase; p-, phosphorylated.

of autophagic components. In conclusion, RCE-4 induced autophagy after 6 and 12 h, though such autophagy may be inhibited or ineffective after 24 h of treatment.

RCE-4 regulates autophagy by altering the protein expression level of mTOR. mTOR, which forms two distinct signaling complexes [mTOR complex (C)1 and mTORC2] by binding to

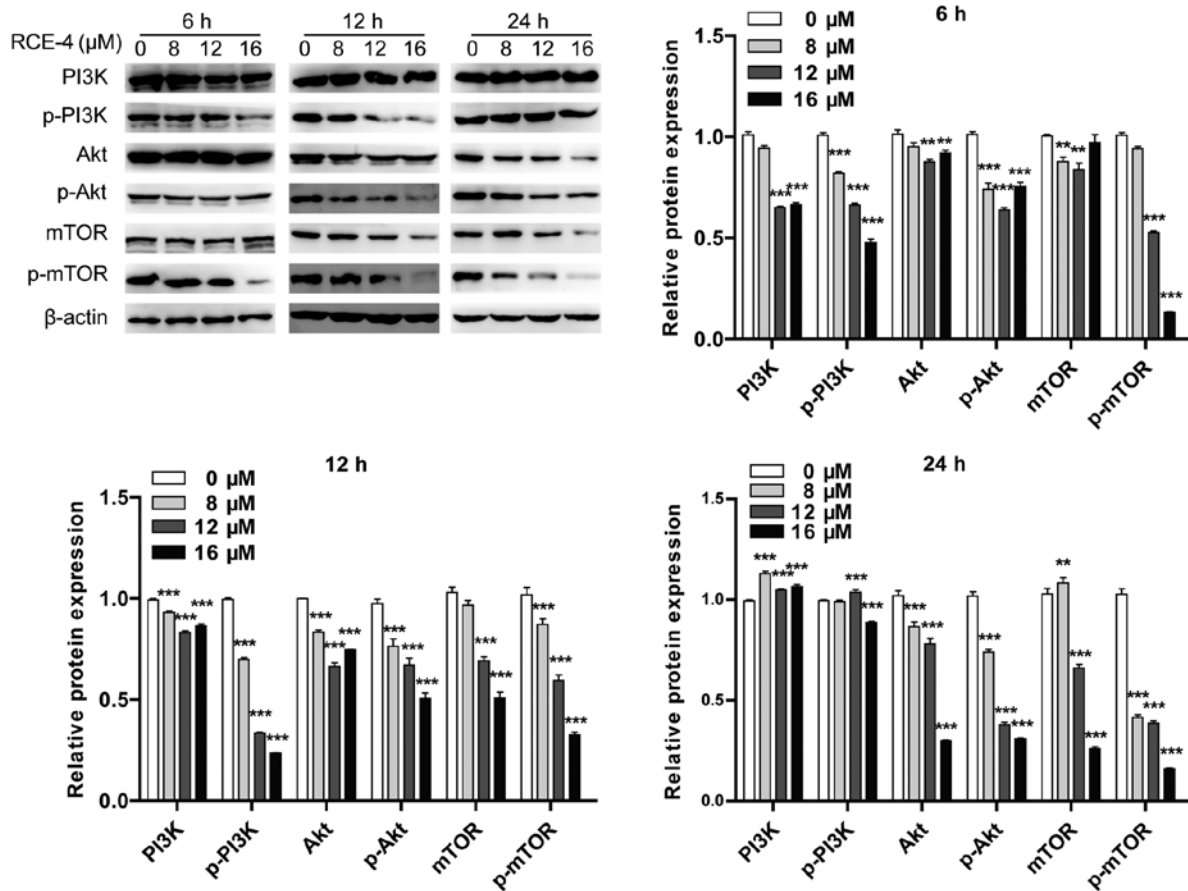


Figure 6. RCE-4 suppresses PI3K pathway signaling. Total proteins were collected from CaSki cells following treatment with various concentrations of RCE-4 (0, 8, 12 and 16 μ M) for 6, 12 or 24 h. Expression levels of β -actin, total PI3K, p-PI3K, total Akt, p-Akt, total mTOR and p-mTOR were analyzed by western blot analysis. Bar graphs represent the western blot grey values, presented as the mean \pm standard deviation. ** P <0.01 and *** P <0.001 vs. 0 RCE-4 group. RCE-4, (1 β , 3 β , 5 β , 25S)-spirostan-1, 3-diol 1-[(α -L-rhamnopyranosyl-(1 \rightarrow 2)- β -D-xylopyranoside)]; p-, phosphorylated.

multiple accompanying proteins, is a major regulator of cellular metabolism and serves a key role in autophagy regulation. The PI3K/AKT, Ras/Raf/MEK/Erk and AMPK pathways, activated by a high AMP/ATP ratio, are upstream signal cascades of mTOR (32). Western blot analysis revealed that AMPK and p-AMPK protein expression levels were upregulated at 6 and 12 h, and downregulated at 24 h post-RCE-4 treatment in CaSki cells (Fig. 3). After a 24-h period, RCE-4 had also downregulated the expression of Ras, c-Raf, p-c-Raf, MEK1/2, p-MEK1/2 and p-Erk1/2 at all of the concentrations examined (Fig. 5). In addition, the expression levels of PI3K, p-PI3K, p-Akt, and p-mTOR were inhibited at 6 h post-RCE-4 treatment at concentrations of 12 and 16 μ M; the protein expression levels of p-PI3K, p-Akt, mTOR and p-mTOR were suppressed at 12 h after post-RCE-4 treatment; the protein expression levels of Akt, p-Akt, mTOR and p-mTOR were also suppressed at 24 h after post-RCE-4 treatment (Fig. 6).

Discussion

It is well known that cervical cancer is a serious health threat to the female population worldwide. Due to the negative effects of radiotherapy on ovarian function, chemotherapy as a treatment for cervical cancer has become increasingly valued by clinicians (33). RCE-4, a potential chemotherapeutic drug for cervical cancer, was demonstrated to induce

mitochondria-mediated apoptosis (5,9). However, not all apoptosis-inducing agents are effective in complex *in vivo* systems; for example, it has been reported that have demonstrated that the tumor necrosis factor-associated apoptosis-inducing ligand was unable to initiate apoptosis in tumor cells due to compensatory inhibition by autophagy (34,35).

Autophagy and apoptosis may determine the fate of cells through cooperative or competitive pathways, depending on which process predominates (36). The aim of the present study was to assess the role of RCE-4-induced autophagy in proliferation inhibition, and the level and duration of autophagy in the CaSki cervical cancer cell line. To improve the understanding of the anti-tumor mechanisms induced by RCE-4, the molecular mechanisms of RCE-4-induced autophagy and the specific cellular targets or signaling pathways associated with its anti-proliferative activity, were additionally elucidated. It was demonstrated that RCE-4 was potently cytotoxic to CaSki cells, which was characterized by decreased levels of cell proliferation and survival. Furthermore, the use of autophagy inhibitors enhanced proliferative inhibition following RCE-4 treatment, suggesting that autophagy promoted cell survival (37). In addition, it has been reported that have also demonstrated that autophagy inhibitors restrict the pro-survival function of autophagy, restore treatment sensitivity and promote tumor cell death (38-40). This indicates that RCE-4, combined with autophagy inhibitors, may be a promising strategy for cervical

cancer treatment. Furthermore, the results of the present study support the role of RCE-4-induced autophagy and its contribution to the formation of chemoresistance.

Autophagy is a fundamental process of energy metabolism, where autophagosomes encapsulate dysfunctional cellular components, fuse with lysosomes to form autolysosomes, and facilitate degradation. Several important autophagy-associated proteins, including LC3II and p62, are involved in this process. LC3II, a classical autophagosomal marker, is located on the inner and outer membranes of autophagosomes and is often used to indicate autophagy. p62, which is inversely associated with selective autophagic degradation, serves as a bridge between LC3II and the ubiquitinated substrate to be degraded (41). Therefore, p62 and LC3II expression levels are important indicators of autophagy flux.

In the present study, it was determined that RCE-4 induced autophagy in CaSki cells. CYTO-ID staining revealed an increase in the number of autophagosomes in stained cells following an exposure period of 6, 12 or 24 h. Furthermore, using mRFP-GFP-LC3 adenoviruses and LCFM an increase in autophagosome and autolysosome formation in CaSki cells following RCE-4 treatment was revealed. These data were supported by the results from the western blot analysis, which indicated that p62 was downregulated and LC3II was upregulated after 12 h of RCE-4 treatment. Furthermore, the use of 3 autophagy inhibitors (3-MA, CQ and Baf A1) with different targeting effects altered the expression level of LC3II following 12 h of RCE-4 treatment. LC3II was downregulated in the 3-MA+RCE-4 group, but upregulated in the CQ+RCE-4 and Baf A1+RCE-4 groups, compared with the RCE-4 control group. These results indicated that the increase in LC3II protein expression level was due to increased autophagosome formation following autophagy activation, which was consistent with harmine-induced autophagy (42). To summarize, the present study demonstrated that RCE-4 induced autophagy and the formation of autolysosomes, which were subsequently degraded.

Growth factors activate downstream signaling cascades, including the PI3K and ERK pathways, and thereby suppress the tuberous sclerosis (TSC) tumor suppressor complex TSC1/TSC2. As a GTPase-activating protein, the TSC complex is the most critical upstream negative regulator of mTORC1 (43). By contrast, AMPK activates TSC complexes under stress conditions, indirectly inhibiting the activity of mTORC1. AMPK also directly suppresses mTORC1 by phosphorylating regulatory-associated protein of mTOR, which itself binds to mTOR (44). In addition, mTORC1 not only suppresses autophagy by inhibiting ULK1/2 and vacuolar protein sorting-34 complex, but also decreases the transcriptional activity of lysosomal and autophagy-associated genes by phosphorylating transcription factor EB (32). mTORC1 and AMPK are upstream signaling components of ULK1, an autophagy-initiating kinase, and autophagy is regulated by the kinase activities of AMPK, mTOR and ULK1 (45). A previous study suggested that various AMPK and mTORC1-dependent phosphorylation events overlapped at ULK1 (20), indicating that both AMPK and mTORC1 tightly controlled the function of ULK1 through protein phosphorylation. To enhance phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3) activity in complexes containing UV radiation resistance-associated gene protein (UVRAG) or

Beclin-1-associated autophagy-related key regulator (ATG14), AMPK phosphorylates Beclin-1 at Ser91 and Ser94; these ATG14- or UVRAG-containing complexes are involved in autophagy initiation. Activation of PIK3C3 complexes containing ATG14 by Beclin-1 phosphorylation was demonstrated to be necessary for autophagy induction (46). In the present study, it was demonstrated that following 12 h of RCE-4 treatment, the expression levels of AMPK, p-AMPK, ULK1, p-ULK1, p-Beclin-1 and LC3II were upregulated, and mTOR and p-mTOR expression levels were downregulated, indicating that autophagy had been initiated. It was also revealed that when treated with RCE-4, the expression levels of p-PI3K, p-Akt, mTOR, p-mTOR, Ras, c-Raf, p-c-Raf, MEK1/2, p-MEK1/2 and p-Erk1/2 were decreased. Therefore, RCE-4-induced autophagy was concluded to be closely associated with the AMPK pathway, and the dual blockade of the ERK and PI3K pathways.

Notably, in the present study, after 24 h of treatment, RCE-4-mediated autophagy was inhibited, or RCE-4 may have prevented normal lysosomal functioning, which was indicated by a percentage decrease in autolysosome staining, the downregulation of AMPK, p-AMPK, ULK1, p-ULK1 and p-Beclin-1, and the upregulation of p62 expression levels. Wang *et al* (29) and Feng *et al* (30) reported that the inhibition of autophagy was associated with a decreased percentage of autolysosomes and an upregulated expression of p62. p-AMPK, p-ULK1 and p-Beclin-1 exhibited similar time- and concentration-dependent changes. This is supported by the previous observation of direct phosphorylation and activation of ULK1 and Beclin-1 kinases by AMPK (47). Although the results from the present study demonstrated that the activation of autophagy was regulated by the AMPK pathway, the RCE-4-induced protective autophagy in CaSki cells was not conducive to the anti-cervical cancer effect of RCE-4; however, autophagy may have been inhibited by prolonged treatment times. Furthermore, the downregulation of Beclin-1 levels indicated that crosstalk and feedback mechanisms between RCE-4-induced apoptosis and autophagy may be involved (48).

Activation of the ERK and PI3K pathways promotes tumor cell proliferation and inhibits apoptosis (3,18,25). RCE-4 has a stronger inhibitory effect on CaSki cells by inducing the dual blockade of the Ras/Raf/MEK/Erk and PI3K/Akt/mTOR pathways, compared with a single pathway inhibitor. These pathways are the most frequently dysregulated kinase cascades in human cancer (49), and both PI3K/Akt/mTOR and Ras/Raf/MEK/Erk cascade inhibitors have been studied for their possible chemotherapeutic use (50). The present study suggested that the cytotoxicity of RCE-4 was closely associated with the suppression of PI3K/AKT and Ras/Raf/MEK/Erk signaling. However, it is unclear how RCE-4 mediates both pathways, and this requires further investigation.

To conclude, the present study revealed that RCE-4 inhibited the proliferation of cervical cancer cells, and activated protective autophagy. In addition, RCE-4 activated the AMPK pathway, and inhibited PI3K and ERK signaling pathways by regulating the associated proteins involved in autophagy. These data may provide novel insights into the molecular mechanisms of the potential anti-cervical cancer effects of RCE-4, and assist in developing this potential candidate for cervical cancer treatment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon request.

Authors' contributions

JFC conceived and designed the research, supplied reagents, materials, analysis tools and designed certain experimental methods. WXiang performed the experiments and wrote the manuscript. GLJ, XFX and WXi collected *Reineckia carnea* herbs in the field and completed their extraction and separation. FC and JZW purified and identified RCE-4 compounds. RJZ, LT and SJT assisted in the analysis of data and provided key commentary. All authors read the results and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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