Repurposing Ziyuglycoside II against Colorectal Cancer via Orchestrating Apoptosis and Autophagy

Can Bai[1], Zhe Zhang[2], Li Zhou[2], Huan-Yu Zhang[3], Yan Chen[2], and Yong Tang[1]

[1]Chengdu University of TCM
[2]Sichuan University
[3]Hainan Medical College

May 5, 2020

Abstract

Background and Purpose: Colorectal cancer (CRC) is one of the most diagnosed cancer worldwide, and effective chemotherapeutic drugs for CRC still remain a challenge. Ziyuglycoside II (Ziyu II) is one of the major active compounds of Sanguisorba officinalis L. Previous study has identified Ziyu II as a potent anti-tumor agent, but limited data on the efficacy and potential mechanism of Ziyu II in the treatment of CRC. Experimental Approach: CRC cells were used to examine the tumor suppression effect of Ziyu II alone or in combination with autophagy inhibitors in vitro and in vivo. A variety of biochemical assays were conducted to elucidate the underlying mechanisms of Ziyu II in CRC cells. Key Results: Ziyu II exhibits antitumor activity against CRC cells both in vitro and in vivo. It demonstrated that treatment with Ziyu II induced apoptosis via accumulation of reactive oxygen species (ROS). Intriguingly, Ziyu II treatment triggered complete autophagic flux in CRC cells. Inhibition of autophagy partially reversed Ziyu II-induced growth inhibition in CRC cells, suggesting a cytotoxic role of Ziyu II-induced autophagy. Mechanistic studies showed that Ziyu II induced autophagy by inhibiting Akt/mTOR pathway, and Akt reactivation partially reduced Ziyu II-induced autophagy. Notably, Ziyu II improves the sensitivity of CRC cells to the first-line chemotherapeutic drugs 5-fluorouracil. Conclusion and Implications:This study provides new insights into the molecular mechanisms of Ziyu II-mediated CRC suppression involving induction of both apoptosis and autophagy, and establishes potential applications of Ziyu II for clinical CRC treatment.

Abbreviations:

CRC: Colorectal cancer; Ziyu II: 3β-3-α-L-arabinopyranosyloxy-19-hydroxyurs-12-en-28-oic acid; ROS: reactive oxygen species; CMA: chaperone-mediated autophagy; TCM: traditional Chinese medicine; β-actin: actin, beta; ATG5: autophagy related 5; ATG7:autophagy related 7; MAP1LC3B/LC3B: microtubule associated protein 1 light chain 3 beta;3-MA:3-methyladenine;5-FU:5-fluorouracil; CQ:chloroquine;

DMSO: dimethyl sulfoxside; EdU: 5-ethynyl-20-deoxyuridine; LDH: lactate dehydrogenase; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Bullet point summary

What is already known

- Ziyu II is a potent anti-tumor agent
- Ziyu II exhibits antitumor activity in breast cancer cells *in intro*

What this study adds

- Ziyu II exhibits antitumor activity both in human colorectal cancer cells and cell line-derived xenograft
- Ziyu II-mediated colorectal cancer suppression involving induction of both apoptosis and autophagy

Clinical significance

Ziyu II would be a promising candidate for human colorectal cancer

Introduction

Colorectal cancer (CRC) has been implicated as one of the most commonly diagnosed cancers worldwide, with an estimated 1.85 million new cases in 2018, accounting for 9.2% of all cancer-related deaths [1,2]. Meanwhile, the rate of CRC is growing fast with insufficient therapeutic options. The screening of CRC remains the most important and cost-effective strategy in reducing the incidence and mortality of the disease, but it still lacks the screening programs in the developing countries [3-4]. In addition, the primary strategies for CRC treatment such as surgery, radiotherapy and adjuvant chemotherapy, have greatly improved the survival rate, but there are some obstacles in the application of chemotherapy, such as lack of selectivity, insufficient drug concentrations in tumor tissues, emergence of drug-resistant cancer cells, and inevitable systemic toxicity [5]. Therefore, novel agents with fewer or no side effects are urgent need to improve the outcome of CRC patients.

Autophagy has recently received considerable attention due to its important roles in human diseases and earned a Nobel Prize for physiology or medicine in 2016 [6]. Autophagy commonly includes three types that is macro-autophagy, micro-autophagy and chaperone-mediated autophagy (CMA) [7-9]. Macro-autophagy (hereafter referred to as autophagy) is an evolutionarily conserved biological process by which damaged organelles and macromolecules are degraded and recycled for cell survival and proliferation under physiologic conditions, such as accumulation of reactive oxygen species (ROS) and energy limiting [6,10]. Meanwhile, it also occurs frequently during tumorigenesis and cancer chemotherapy [11-12], even related to cancer drug resistance [13-14]. Due to its "self-digest" function, the role of autophagy in cancer is complex and context-dependent [15]. A growing number of reports indicate that targeting the autophagy process has been regarded as a novel therapeutic approach [16-17]. Therefore, development of novel autophagy regulators has rewired a way of cancer treatment in recent years.

Drug repurposing has recently emerged as an alternative approach to accelerate drug development for cancer treatment. Repurposing the large arsenal of non-anticancer drugs holds promise to achieve a rapid clinical practice at a lower cost than *de novo* drug development [18]. In the past decades, traditional Chinese medicine (TCM) has drawn growing attention as special drug pool for drug repurposing in the cancer management. Ziyuglycoside II (3β-3-α-L-arabinopyranosyloxy-19-hydroxyurs-12-en-28-oic acid) (Fig. 1A) is one of the major active compounds of *Sanguisorba officinalis L* , which is widely distributed in the north temperate zone of Asia and Europe, especially in China. And it has a wide range of clinical applications including antibiosis, anti-inflammation, anti-oxidation and anti-cancer. Previous study has identified Ziyu II as a potent anti-tumor agent that inhibits cancer cell proliferation by triggering apoptosis and inducing cell cycle arrest in various cancers, including breast cancer and gastric cancer [19-21]. However, the detailed mechanism underlying the anticancer effect of Ziyu II remains to be further defined.

In this study, we demonstrated that Ziyu II induces growth inhibition and obvious cell death of CRC cells by triggering autophagy and apoptosis both *in vitro* and *in vivo* . Notably, Ziyu II promotes complete autophagic flux which results from the inhibition of the Akt/mTOR signaling pathway, leading to growth inhibition of CRC cells. Moreover, Ziyu II synergistically suppresses CRC cell growth with the first-line chemotherapeutic drugs 5-fluorouracil. Together, our findings elucidated the mechanisms of Ziyu II-induced growth inhibition of CRC cell by orchestrating both apoptosis and autophagy, which may pave the way for the use of Ziyu II in clinic treatment of CRC.

Methods

Cell proliferation assay

The MTT assay was used to assess the cell growth rate. Briefly, cells were plated in 96-well plates ($4x10^3$ per well) and subjected to different treatments. The detailed procedures have been previously described by Dou et al [45]. For colony formation assay, cells were cultured in 24-well plates (800 cells/ well) and treated

with different treatments. After 2 weeks, the colonies were fixed with 4% paraformaldehyde and stained with Giemsa. The visible colonies were photographed by Molecular Imager Gel Do XR + System (Bio-Rad, Hercules, CA, USA) and counted using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The detailed procedures have been previously described by Wang et al [38]. The 5-ethynyl-20-deoxyuridine (EdU) labeling assay was performed in 24-well plates (2 x 10^4 cells) using the EdU cell Proliferation Assay Kit (Ribobio, Guangzhou, China). After different treatments, 10 μmol/L EdU was added to the cells, and the cells were incubated for 12 hours at 37^0C. Cells were fixed with 4% paraformaldehyde. DAPI was then added for nuclear staining followed by imaging with a fluorescence microscope.

Lactate dehydrogenase release assay

Lactate dehydrogenase (LDH) test kit (Beyotime Biotechnology, Nanjing, China) was used to assess the cytotoxicity of Ziyu II. Cells were cultured in 96-well plates (6x10^3 cells/well). After treatment with various concentrations of Ziyu II for 24h, cell culture supernatant was transferred to the new 96-well plate for LDH analysis.

Measurement of intracellular ROS levels

The ROS levels were quantified in accordance with the protocol of the Reactive Oxygen Species Assay Kit (Beyotime Biotechnology, China). Cells were cultured in 6-well plates (3x10^5 per well) treated with indicated concentrations of Ziyu II for 24 h and stain with Muse Oxidative Stress reagent at 37°C for 30 min. Cells were then collected for ROS analysis using FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Flow cytometry

The ratio of apoptotic cells was measured with an annexin V–FITC/propidium iodide (PI) Detection Kit (KGA108; Key Gen Biotech, Nanjing, China) according to the manufacturer's instructions. Cells were harvested and washed twice with PBS and then resuspended in 500 μl binding buffer. After adding 5 μl annexin V–FITC and 2μl PI into the cell suspension, respectively, at least 20,000 live cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed by using FlowJo software (FlowJo, Ashland, OR, USA).

Plasmids

The human AKT (NM_001014431.1) coding region with GFP-Tag was cloned using PCR and was ligated into the pEGFP-N1 vector. The origin PCR primers for AKT cds sequence were as follows: Sense primer: 5' CCG GAA TTC ATG AGC GAC GTG GCT ATT 3'; Anti-sense primer: 5'CGC GGA TCC CCG GCC GTG CCG CTG GCC GA 3'. The constitutively active form of Akt (CA-Akt, myrAkt delta4-129) was purchased from Addgene. Flag-Ubiquitin was a kind gift from Professor Changan Jiang (Sichuan University, China). The plasmids were introduced into cells using Lipofectamine 3000 according to the manufacturer's protocol.

Animal model

All animal experiments were approved by the Institutional Animal Care and Treatment Committee of Sichuan University. Female BALB/c nude mice, aged 5 weeks, were purchased from HFK Bioscience Co., Ltd (Beijing, China). The mice were housed under standard conditions. For the subcutaneous xenograft model, DLD-1 cells (1 x 10^7cells/mouse) were suspended in PBS and injected into flanks of mice subcutaneously. When the tumor volume reached ~50mm^3, mice were randomly divided into two groups. The mice were administered with 0.1 ml vehicle (10% ricinus oil, 5% DMSO, 10% ethanol, 75% physiologic saline) or Ziyuglycoside II (50 mg/kg/d) by gavage. The tumor volumes were measured every other day and evaluated according to the following formula: tumor volume(mm^3) = (length x $width^2$)/2. 4 weeks post treatment, the mice were euthanized and tumors were harvested.

Immunohistochemical analysis

Immunohistochemical analysis was performed as described previously [44]. All samples were observed under a Leica DM 2000 microscope. The percentage of positive cell area (a) was multiplied by the intensity of immunostaining (B: 0, negative; 1, weak positive; 2, positive; 3, strong positive). The final score of each slide is calculated as a × B. The final score for each slide was calculated as A×B.

Immunoblot

Cells were lysed with RIPA buffer (150 mmol/L Tris-HCl pH 7.0, 150 mmol/L NaCl, 1% NP-40, 1% Sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (Sigma, p8340) for 30 min at 4°C. Lysates were quantified by Bio-Rad protein assay. The protein samples were then subjected to SDS-PAGE and probed with the indicated antibodies to visualize the protein levels using a chemiScope 6000 Touch (Clinx, Shanghai, China).

Statistical analysis

All statistical analysis was performed using GraphPad Prism Software version 6.0. All experiments were performed three or more times independently. For two-group comparisons, the student's two-tailed t-test was used. For multiple group comparisons, one-way ANOVA analysis was used. $P < 0.05$ was defined as statistically significant.

Materials

Cell culture

Human colorectal cancer cell lines (HCT116, HT29, DLD-1, SW620 and SW480) and the human normal colonic epithelial cell line NCM460 were obtained from the ATCC (Manassas, VA, USA) and cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), 100 U/ml penicillin (Millipore Sigma, Burlington, MA, USA), and 100 U/ml streptomycin (Millipore Sigma) at 37°C in 5% CO_2.

Antibodies and reagents

Antibodies were purchased from Cell Signaling Technology (ATG5 12994S, ATG7 8558S, Akt 4685, p-Akt 4060, mTOR 2972, p-mTOR 2971, p70S6K 9202, p-p70S6K 9208, 4E-BP1 9452, p-4E-BP1 9451), Abcam (PARP ab74290, cleaved-PARP ab32064, Ki67 ab66155, Santa Cruz Biotechnology (β-actin sc-1616, horseradish peroxidase-conjugated anti-rabbit secondary antibody sc-2004, horseradish peroxidase-conjugated anti-mouse secondary antibody sc-2005), Thermo Fisher Scientific (goat anti-mouse Alexa Fluor 488, goat anti-rabbit Alexa Fluor 488, goat anti-mouse Alexa Fluor 594, goat anti-rabbit Alexa Fluor 594) and Novus (LC3 NB100-2220). DAPI (62248) and Lipofectamine 3000 (L3000015) were purchased from Thermo Fisher Scientific. Unless otherwise indicated, all commercial chemicals were purchased from MedChem Express (Monmouth Junction, NJ, USA).

Results

Ziyu II inhibits CRC cells growth both *in vitro* and*in vivo*

To examine whether Ziyu II exhibits an antitumor effect against CRC, CRC cell lines (HCT116, DLD-1, HT29, SW48, SW620, RKO) and noncancerous colorectal cell line (NCM460) were treated with different dose of Ziyu II. MTT assay showed that Ziyu II markedly inhibited the growth of CRC cells in a dose-dependent manner, whereas the IC50 value in NCM460 cells were much higher than those in CRC cells (Fig.1B). Consistently, the proliferation of CRC cells was significantly decreased upon Ziyu II treatment, as evidenced by reduced colony formation and EdU incorporation (Fig.1C-E). Then we performed LDH release assay and found that Ziyu II damaged the integrity of plasma membrane (Fig.1F). In summary, these results demonstrated that Ziyu II inhibits the growth of CRC cells *in vitro* .

To further ascertain the antitumor effect of Ziyu II on CRC growth*in vivo* , we generated a CRC xenograft model by subcutaneously inoculating DLD-1 cells into nude mice. As shown in Fig. 1G and H, Ziyu II treatment markedly decreased the size and the weight of xenografts when compared with the control group.

In addition, xenografts treated with Ziyu II grew at a slower rate than those treated with placebo (Fig. 1I). Moreover, most of Ziyu II-treated tumors displayed reduced Ki67 staining (Fig.1J and K). Moreover, we found that Ziyu II treatment had no significant effect on the pathological features of major organs, suggesting that Ziyu II has no obvious toxic or adverse effect on mice (Fig. S1). Taken together, these data suggest that Ziyu II inhibits the growth of CRC cells both *in vitro* and*in vivo* .

Ziyu II induces apoptosis in CRC cells both in *vitro* and in *vivo*

Apoptosis is a major form of cell death induced by chemotherapy drugs and has been widely documented. Previous studies suggested that Ziyu II exerted antitumor activity by inducing apoptosis in human breast cancer and gastric cancer cells [20,22]. To get more insights into the mechanism of Ziyu II-induced cell death, we detected whether Ziyu II induced apoptosis in CRC cells. Firstly, Ziyu II treatment resulted in increased levels of cleaved-caspase3, a prototypical caspase that becomes activated during apoptosis [23].and cleaved-PARP and decreased expression of anti-apoptotic protein Bcl2 (Fig. S2A). Consistently, the flow cytometry analysis exerted remarkable apoptotic effect in Ziyu II treated CRC cells (Fig. 2A). To further validate Ziyu II induced apoptosis, CRC cells were treated with Ziyu II combination with the apoptosis inhibitor-zVAD. As shown in Fig. S2B, combinational treatment resulted in reduced levels of cleaved-PARP. Consistently, the pro-apoptotic effect of Ziyu II was partially rescued under the combinational treatment, as evidenced by MTT assay and colony formation (Fig. 2B-D). As the accumulation of reactive oxygen species (ROS) plays a key role in apoptosis induction, we detected whether Ziyu II treatment promoted ROS accumulation. The flow cytometry analysis showed that Ziyu II markedly increased the level of ROS in a dose-dependent manner in CRC cells (Fig. 2E). What's more, MTT assay showed that N-acetylcysteine (NAC, a ROS scavenger) significantly reversed Ziyu II-induced growth inhibition (Fig. 2F). In brief, these results demonstrate the pro-apoptotic effect of Ziyu II in CRC cells *in vitro* .

To evaluate the pro-apoptotic effect of Ziyu II on CRC cells *in vivo* , xenografts were stained for cleaved-caspase3. As shown in Fig. S2C and D, Ziyu II-treated xenografts displayed stronger cleaved-caspase3 staining than that of placebo-treated xenografts. In summary, these data indicate that apoptosis is involved in Ziyu II against CRC cells both in *vitro* and in *vivo* .

Ziyu II induces autophagosome formation in CRC cells

As increasing evidence has highlighted the important roles of drug-induced autophagy in anticancer therapies [24-27], we thus investigated whether autophagy is involved in Ziyu II induced CRC cell death. We first examined the protein levels of autophagy related proteins in Ziyu II-treated CRC cells, and found that Ziyu II treatment promoted the turnover of LC3-I to lipidated LC3-II in a dose-dependent manner in CRC cells (Fig.3A). Also, we evaluated the expression levels of ATG5 and ATG7, two autophagy related proteins, which are notably increased in a dose dependent manner after Ziyu II treatment (Fig.3A). When combined with 3-MA, an inhibitor of class III PI3K, the elevated LC3-II levels were prominently inhibited in Ziyu II-treated CRC cells, suggesting that Ziyu II induces autophagy initiation (Fig. 3B). This was further confirmed by siRNA-mediated *ATG5* silencing, as evidenced by decreased LC3 puncta in combinatorial treatment group (Fig. S3A-E). Furthermore, both the endogenous LC3 and exogenous GFP-LC3 puncta, representing the number of autophagic vacuoles [28], were remarkably increased in Ziyu II-treated CRC cells (Fig. 3C-F). In addition, Ziyu II-treated xenografts displayed stronger LC3 staining than that of the control group (Fig. 3G and H). Taken together, these results show that Ziyu II promotes the initiation process of autophagy in CRC cells.

Ziyu II promotes autophagic flux in CRC cells

The elevated levels of LC3-II may be attributed to either autophagy initiation or blockage of autophagic flux. Thus, we determined whether Ziyu II induced complete autophagic flux. First, we examined the protein levels of LC3-II under the combinatorial treatment of Ziyu II with chloroquine (CQ), an autolysosome inhibitor. As expected, combinatorial treatment resulted in enhanced accumulation of LC3-II (Fig.4A). Moreover, we also detected the colocalization of LC3 (autophagosome marker) with LAMP1 (lysosome marker) in Ziyu II-treated CRC cells. As shown in Fig. 4B and C, CRC cells treated with Ziyu II showed obvious colocalization

of LC3 and LAMP1, suggesting that Ziyu II promotes the fusion of the autophagosome with lysosome. To further confirm the fusion of autophagosome with lysosome in Ziyu II-treated CRC cells, we used a tandem monomeric mRFP-GFP tagged LC3 construct and observed increased formation of red fluorescent autolysosomes (GFP-RFP+ signal) (Fig. 4D-F). Together, these findings suggest that Ziyu II treatment induces complete autophagic flux in CRC cells.

Ziyu II induces autophagy via inhibiting Akt/mTOR pathway

Akt/mTOR is an important signaling pathway for cell survival, which is closely associated with cancer progression and development [29]. It has been previously reported that Ziyu II treatment decreased the phosphorylation level of Akt in human umbilical vein endothelial cells [30]. Therefore, we aimed to test whether Akt/mTOR signaling was involved in Ziyu II-induced autophagy in CRC cells. As shown in Fig. 5A, Ziyu II treatment significantly inhibited Akt/mTOR signaling pathway, as evidenced by decreased phosphorylation levels of Akt, mTOR, p70S6K, and 4E-BP1. To further confirm these results, we transfected a constitutively active form of Akt to rescue Ziyu II-induced Akt/mTOR inhibition. As expected, Akt reactivation markedly reduced Ziyu II-induced LC3-II turnover and LC3 puncta accumulation (Fig. 5B-G), suggesting an important role of the Akt/mTOR signaling pathway in Ziyu II-induced autophagy. Moreover, most of Ziyu II-treated xenografts displayed reduced p-Akt staining (Fig. 5H and I). Taken together, these findings suggest that Ziyu II induces autophagy by inhibiting Akt/mTOR signaling pathway in CRC cells.

Autophagy contributes to the antitumor activity of Ziyu II in CRC cells

We next intend to evaluate whether autophagy was involved in the anti-CRC effect of Ziyu II. First, CRC cells were treated with Ziyu II combined with CQ or 3-MA. As shown in Fig. 6A and Fig. S4A, combinational use of CQ or 3-MA with Ziyu II partially restored Ziyu II-induced growth inhibition in CRC cells. In addition, LDH release assay also revealed that CQ or 3-MA counteracted Ziyu II-induced cytotoxicity (Fig. 6B and Fig. S4B). A similar increase in cell proliferation was also observed in Ziyu II-treated CRC cells in combination with CQ or 3-MA, as evidenced by colony formation analysis (Fig. 6C, D and Fig. S4C, D). Consistently, similar results were obtained by inhibition of autophagy by transfecting a constitutively active form of Akt (Fig. 6E-H and Fig. S4E, F). Collectively, these results demonstrate that autophagy is involved in Ziyu II-induced CRC growth suppression.

We further investigated whether Ziyu II enhanced the chemosensitivity of CRC cells to 5-fluorouracil (5-FU), the first-line chemotherapeutic drug for CRC treatment. As indicated, combinational treatment of Ziyu II with 5-FU markedly decreased the cell growth (Fig.S5A) and proliferation rate (Fig.S5B and C) compared with monotherapy in CRC cells, suggesting that Ziyu II effectively sensitizes CRC cells to the treatment of 5-FU.

Discussion and Conclusions

Ziyu II is one of the major active components of *S. officinalis L* . Previous studies have demonstrated the anticancer effect of Ziyu II in a variety of cancer types. In the present study, we demonstrated its anticancer effect against CRC cells both *in vitro* and *in vivo* . We showed that ROS-induced apoptosis and the autophagy induced by the inhibition of Akt/mTOR signaling both contribute to the growth inhibition of Ziyu II against CRC cells. In addition, Ziyu II acts synergistically with the first-line chemotherapeutic drugs 5-fluorouracil in the suppression of CRC cell growth, suggesting Ziyu II as a potential anticancer drug or chemosensitizer for CRC treatment.

One of the key points in the development of anti-cancer drugs is to find active ingredients from natural substances. At present, a large number of studies have shown that TCM is a kind of multi-target anti-cancer drug by inhibiting cell proliferation, causing cell cycle arrest, promoting cell apoptosis, inhibiting neovascularization, and blocking invasion and metastasis of tumors [31-33]. *S. officinalis* L. is a popular traditional Chinese medicinal plant used in the treatment of inflammation, metabolic diseases and various cancers. Ziyu II is one of the major active components of *S. officinalis L* . Until now, anticancer effects of Ziyu II have been reported in human breast and gastric cancer and the detailed underlying mechanisms

are different. Ziyu II can effectively induce G2/M phase arrest and apoptosis in MCF-7 and MDA-MB-231 human breast cells [20,21]. In addition to several breast cancer cells, Ziyu II exerts anticancer activity by inducing apoptosis or antiangiogenic, but not cell cycle arrest, in BGC-823 human gastric carcinoma cells [22]. Thus, the action of Ziyu II on cancer cells might appear differently dependent on cell types. To provide clarity, we investigated the anticancer effects of Ziyu II on colorectal cancer cells. In this study, the molecular mechanism of anti-cancer effect of Ziyu II in CRC cells is inducing both apoptosis and autophagy.

Drug repurposing has been recognized as an attractive strategy to offer more-effective options to patients with cancer, and has the substantial advantages of cheaper, faster and safer than *de novo* drug development [34]. Previous studies have demonstrated that many types of TCM have been identified as potential anticancer drugs via drug repurposing screen. For example, Shikonin is a medicinal compound extracted from *Lithospermum erythrorhizon* , an herb which has been used for centuries in Chinese medicine for the treatment of burns, cuts, and lesions caused by disease [35]. Growing evidence has demonstrated Shikonin as potential anticancer drug or chemosensitizer via inducing apoptosis and necroptosis [36, 37]. Our previous works have also identified several natural compounds which exhibit potential anticancer activities [38 -40]. Among these candidates, Ziyu II, a major active component of *S. officinalis L* used for anti-inflammation and anti-oxidation, has been found to suppress CRC growth by apoptosis and autophagy in the present study, further highlighting the important role of drug repurposing in cancer drug development.

Apoptosis and autophagy are involved in the physiological process of cells such as growth, differentiation, death and also closely related to the development of tumor [41,42]. The machinery of apoptosis and autophagy are quite complex, with many signaling pathways involved. Apoptosis can be triggered by multiple factors including ROS [43]. Meanwhile, CRC is characterized by partially inhibited apoptosis, which in turn provides a selective advantage for the survival of tumors and becomes a major obstacle to treatment [44]. In the same way, numerous evidences have suggested that many signaling pathways are involved in the regulation of autophagy, and Akt-mTOR is one of the most classical signaling pathways that negatively regulate autophagy. In the present study, we investigated the anti-cancer effect of Ziyu II in CRC cells both *in vitro* and *in vivo* . Interestingly, we found that Ziyu II induced apoptosis and autophagy through the different pathway in CRC cells. Ziyu II-induced apoptosis is caused by excessive accumulation of ROS. While Ziyu II induced autophagy through inhibition of Akt/mTOR signaling pathway as evidenced by decreased phosphorylation of Akt, mTOR and downstream substrates. Overall, our findings suggest that targeting the Akt/mTOR signaling pathway may deserve exploration as a potential therapeutic strategy for CRC treatment.

In summary, our current findings demonstrate that the Chinese herbal medicine extract-Ziyu II inhibits colorectal cancer growth by orchestrating Akt/mTOR-mediated autophagic cell death and ROS-induced apoptosis. These findings provide new insights into the mechanisms of Ziyu II induced colorectal cancer suppression and support the rational utility of Ziyu II for therapeutic treatment of colorectal cancer.

ACKNOWLEDGEMENTS

We sincerely thank L Zhang and Z Huang for useful discussions. We are grateful to the Sino-German Center (GZ919),The Project First-Class Disciplines Development (CZYHW1901) of Chengdu University of TCM, and Sichuan Science and Technology Program (2019YFH0108, 2018HH0123, 2018SZ0257) for financial support.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: CB and YT. Performed the experiments: CB, ZZ and YC. Analyzed the data: CB, HYZ and YT. Wrote the manuscript: CB, LZ and YT.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A. (2018), A Jemal. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians, 68(6): 394-424.
2. Siegel, R. L., Miller, K. D., Fedewa, S. A., Ahene, D.J., Meester, R.G.S., Barzi, A., DVW,A.J. (2017). Colorectal cancer statistics 2017. CA: a cancer journal for clinicians, 67(3): 177-193.
3. Onyoh, E. F., Hsu, W. F., Chang, L.C., Lee, Y.C., Wu, M. S., Chiu, H. M. (2019). The Rise of Colorectal Cancer in Asia: Epidemiology, Screening, and Management. Current gastroenterology reports, 21(8): 1-10.
4. Kuipers, E. J., Grady, W. M., Lieberman, D. (2015). Colorectal cancer. Nature reviews disease primers.1:15065.
5. Geng, F., Wang, Z., Yin, H., Yu, J., Cao, B. (2017). Molecular targeted drugs and treatment of colorectal cancer: recent progress and future perspectives. Cancer biotherapy and radiopharmaceuticals. 32(5): 149-160.
6. Mizushima, N. (2018). A brief history of autophagy from cell biology to physiology and disease. Nature cell biology, 20(5): 521–527.
7. Lin, L., Baehrecke E. H. (2015). Autophagy, cell death, and cancer[J]. Molecular and cellular oncology, 2(3): e985913.
8. Ruocco , N., Costantini, S., Costantini, M. (2016). Blue-print autophagy: potential for cancer treatment. Marine drugs,14(7): 138.
9. Glick, D., Barth, S., Macleod, K.F., (2010). Autophagy: cellular and molecular mechanisms. The Journal of pathology, 221(1): 3-12.
10. Mejlvang, J., Olsvik, H., Svenning, S., Bruun, J. A., Abudu, Y. P., Larsen, K. B., ... T Johansen (2018). Starvation induces rapid degradation of selective autophagy receptors by endosomal microautophagy. Journal of cell biology.217(10): 3640-3655.
11. Chen, Y., Chen, H.N., Wang, K., Zhang, L., Huang, Z., Liu, J. Y.,... Huang, C. H. (2019). Ketoconazole exacerbates mitophagy to induce apoptosis by downregulating cyclooxygenase-2 in hepatocellular carcinoma. Journal of hepatology, 70(1): 66-77.
12. Poillet-Perez, L., Xie, X., Zhan, L., Yang, Y., Sharp, D.W., Hu, S., ... White, E. (2018). Autophagy maintains tumour growth through circulating arginine. Nature, 563(7732): 569-573.
13. Jagannathan, S., Abdel-Malek, M. A. Y., Malek, E., Vad, N., Anderson, T. K. C., Driscoll J.J.(2015). Pharmacologic screens reveal metformin that suppresses GRP78-dependent autophagy to enhance the anti-myeloma effect of bortezomib. Leukemia, 29(11): 2184–2191.
14. Sun, T., Liu, H., Ming L. (2017). Multiple roles of autophagy in the sorafenib resistance of hepatocellular carcinoma. Cellular physiology and biochemistry, 44(2): 716-727.
15. Kimmelman, A. C., White, E. (2017). Autophagy and tumor metabolism. Cell metabolism, 25(5): 1037-1043.
16. Sun, X., Yan, P., Zou, C., Wong, Y. K., Shu, Y. H., Lee, Y. M., ... Zhang, J. B. (2019). Targeting autophagy enhances the anticancer effect of artemisinin and its derivatives. Medicinal research reviews, 39(6): 2172-2193.
17. Lei, Y., Zhang, D., Yu, J., Dong, H., Zhang, J., Yang S. (2017). Targeting autophagy in cancer stem cells as an anticancer therapy. Cancer letters, 393: 33-39.
18. Corsello, S. M., Bittker, J. A., Liu, Z., Gould J., McCarren, P., Hirschman, J. E., ... Golub, R. (2017). The Drug Repurposing Hub: a next-generation drug library and information resource. Nature medicine,23(4): 405-408.
19. Lkhagvasuren, K., Kim, J. K., (2019). Ziyuglycoside II induces caspases-dependent and caspases-independent apoptosis in human colon cancer cells. Toxicology in Vitro, 59: 255-262.
20. Zhu, X., Wang, K., Zhang, K., Huang, B., Zhang, J., Zhang, Y., ... Zhou, F. F. (2013). Ziyuglycoside II inhibits the growth of human breast carcinoma MDA-MB-435 cells via cell cycle arrest and induction of apoptosis through the mitochondria dependent pathway. International journal of molecular sciences, 14(9): 18041-18055.
21. Zhu, X., Wang, K., Zhang, K., Zhu, L., Zhou, F. (2014). Ziyuglycoside II induces cell cycle arrest and

apoptosis through activation of ROS/JNK pathway in human breast cancer cells. Toxicology letters, 227(1): 65-73.
22. Zhu, A. K., Zhou, H., Zhou, Xia, J. Z., Jin, H.C., Wang, K. Yan, J., ... Shan, T. (2013). Ziyuglycoside II-induced apoptosis in human gastric carcinoma BGC-823 cells by regulating Bax/Bcl-2 expression and activating caspase-3 pathway. Brazilian journal of medical and biological research, 46(8):670-675.
23. Letai, A. (2017). Apoptosis and cancer. Annual Review of Cancer Biology, 1: 275-294.
24. Poillet-Perez, L., Xie, X., Zhan, L., Yang, Y., Sharp, D.W., Hu, S., ... White, E. (2018). Autophagy maintains tumour growth through circulating arginine. Nature, 563(7732): 569-573.
25. Deng, S., Shanmugam, M. K., Kumar, A. P., Yap, C.T., Sethi G., Bishayee, A.(2019). Targeting autophagy using natural compounds for cancer prevention and therapy. Cancer, 125(8): 1228-1246.
26. Luo, P., Xu, Z., Li, G., Yan, H., Zhu, Y., Zhu, H., ... He, Q. J., (2018). HMGB1 represses the anti-cancer activity of sunitinib by governing TP53 autophagic degradation via its nucleus-to-cytoplasm transport. Autophagy, 14(12): 2155-2170.
27. Cui, Z., Zhang, Y., Xia, K., Yan, Q., Kong, H., Zhang, J., ...Fan, C.H. (2018). Nanodiamond autophagy inhibitor allosterically improves the arsenical-based therapy of solid tumors. Nature communications, 9(1): 4347.
28. Lee, Y. K., Jun, Y. W., Choi, H. E., Huh, Y.H., Kaang, B.K., Deok, J. J., Jin, A. L., (2017). Development of LC3/GABARAP sensors containing a LIR and a hydrophobic domain to monitor autophagy[J]. The EMBO journal, 36(8): 1100-1116.
29. Soleimani, A., Rahmani, F., Ferns, G. A., Ryzhikov, M., Avan, A., Hassanian, S.M. (2018). Role of Regulatory Oncogenic or Tumor Suppressor miRNAs of PI3K/AKT Signaling Axis in the Pathogenesis of Colorectal Cancer. Current pharmaceutical design, 24(39): 4605-4610.
30. Nam, S. H., Lkhagvasuren, K., Seo, H. W., Kim, J. K. (2017). Antiangiogenic Effects of Ziyuglycoside II, a Major Active Compound of Sanguisorba officinalis L. Phytotherapy Research, 31(Suppl 1).
31. Liu, C., Yang, S., Wang, K., Bao, X., Liu, Y., Zhou, S.Y., ... Yu, H. Y. (2019). Alkaloids from Traditional Chinese Medicine against hepatocellular carcinoma. Biomedicine and Pharmacotherapy, 120: 109543.
32. Nie, J., Zhao, C., Deng, L. I., Chen, J., Yu, B., Wu, X.L., Peng, P., Chen, X. Y. (2016). Efficacy of traditional Chinese medicine in treating cancer. Biomedical reports, 4(1): 3-14.
33. Xiang, Y., Guo, Z., Zhu, P., Chen, J., Huang, Y., (2019). Traditional Chinese medicine as a cancer treatment: Modern perspectives of ancient but advanced science. Cancer medicine, 8(5): 1958-1975.
34. Pushpakom, S., Iorio, F., Eyers, P. A., Escott, K. J., Hopper, S., Wells, A., ... Pirmohamed, M.,(2019). Drug repurposing: progress, challenges and recommendations. Nature reviews Drug discovery, 18(1): 41-58
35. Zhang, X., Cui, J. H., Meng, Q. Q., Li, S. S., Zhou, W., Xiao, S.(2018). Advance in anti-tumor mechanisms of shikonin, alkannin and their derivatives. Mini reviews in medicinal chemistry, 18(2): 164-172.
36. Hwang, K. E., Jeong, E. T., Park, D. S., Oh, S. H., Jun, H. Y., Yoon, K. H., Kim, H. R., Kim, Y.S. (2017). Shikonin-induced necroptosis is enhanced by the inhibition of autophagy in non-small cell lung cancer cells. Journal of translational medicine, 15(1): 123.
37. Jeung, Y. J., Kim, H. G., Ahn, J., Lee, H. J., Lee, S. B., Won, M., ... Chung, K. S. (2016). Shikonin induces apoptosis of lung cancer cells via activation of FOXO3a/EGR1/SIRT1 signaling antagonized by p300. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1863(11): 2584-2593.
38. Wang, K., Liu, R., Li, J., Mao, J., Lei, Y., Wu, J., ... Wei,Y.Q. (2011). Quercetin induces protective autophagy in gastric cancer cells: involvement of Akt-mTOR-and hypoxia-induced factor 1α-mediated signaling. Autophagy, 7(9): 966-978.
39. Huang, Y. H., Sun, Y., Huang, F. Y., Li, Y. N., Wang, C. C., Wen, L. M., ... Huang, C. H. (2017). Toxicarioside O induces protective autophagy in a sirtuin-1-dependent manner in colorectal cancer cells. Oncotarget, 8(32): 52783.
40. Sun, Y., Huang, Y. H., Huang, F.Y., Mei, W. L., Liu, Q., Wang, C. C., ... Tan, G. H. (2018). 3'-epi-12β-hydroxyfroside, a new cardenolide, induces cytoprotective autophagy via blocking the

Hsp90/Akt/mTOR axis in lung cancer cells. Theranostics, 8: 2044.
41. Fiandalo, M. V., Kyprianou, N. (2012). Caspase control: protagonists of cancer cell apoptosis. Experimental oncology, 34: 165.
42. Hussey, S., Terebiznik, M. R., Jones, N. L. (2008). Autophagy: healthy eating and self-digestion for gastroenterologists. Journal of pediatric gastroenterology and nutrition, 46: 496-506.
43. Diwanji, Neha, Bergmann, Andreas (2018). An unexpected friend- ROS in apoptosis-induced compensatory proliferation: implications for regeneration and cancer[C]//Seminars in Cell & Developmental Biology. Academic Press, 80: 74-82.
44. Yang, S. Y., Kevin, M. S., Barry, F., Alexander, M. S., Marc, Winslet, C. (2009). Apoptosis and colorectal cancer: implications for therapy. Trends in Molecular Medicine, 15:225-233.
45. Dou, Q. H., Chen, H. N., Wang, K., Yuan, K. F., Lei, Y. L., Li, K., ... Huang C. H. (2016). Ivermectin induces cytostatic autophagy by blocking PAK1/Akt axis in breast cancer. Cancer Research,76: 4457-4469.

Figures

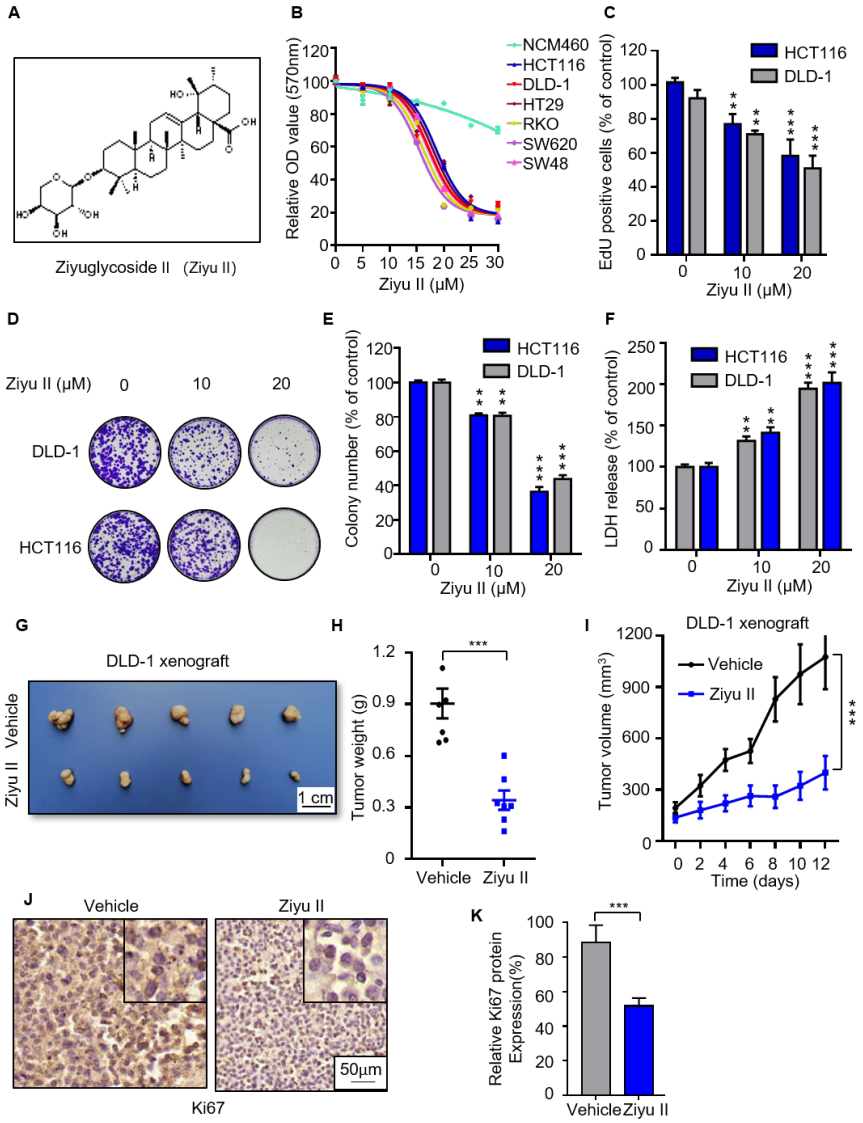


Figure. 1. Ziyuglycoside II inhibits colorectal cancer cells proliferation both *in vitro* and *in vivo*

(**A**). Chemical structure of Ziyu II. MTT assay of colorectal cancer lines treated with the indicated concentrations of Ziyu II for 24h (**B**). EdU incorporation (**C**), LDH release assay in cells treated as in (**D**). Colony formation assay of DLD-1 and HCT116 cells treated with the indicated concentrations of Ziyu II for 2wks. Representative images (**E**) and quantification of colonies (**F**) were shown. G–J, NOD-SCID mice were inoculated with DLD-1 cells and treated with Ziyu II or vehicle. Tumor volumes were measured at indicated time points (**I**). Photograph of isolated tumors derived from control or Ziyu II-treated mice (**G**). Tumor weights at time of sacrifice (**H**). Ki67 expression in tumor xenografts was examined by IHC(**J**), and relative immunohistochemical scores were shown (**K**) Scale bar, 50 μm. All data are means±SD. **,$p < 0.01$; ***, $p < 0.001$.

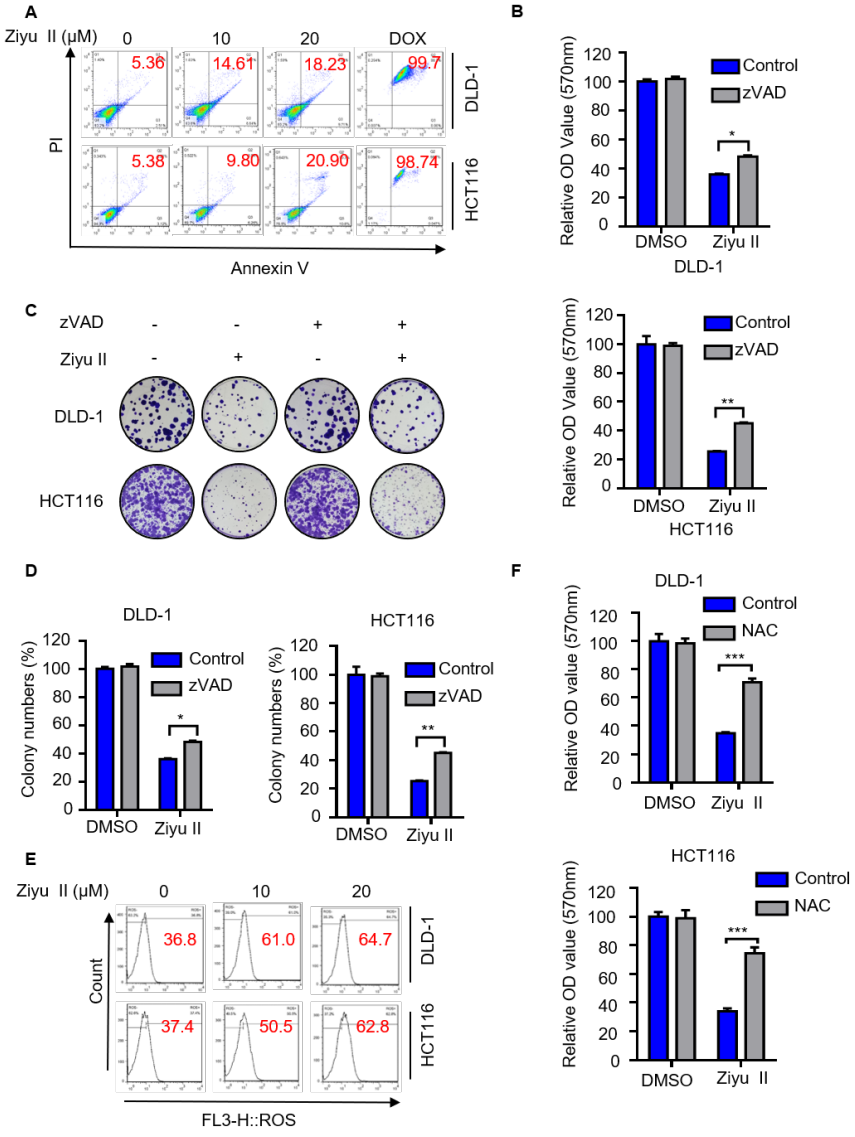


Figure 2. Ziyuglycoside II induces apoptosis in colorectal cancer cells *in vitro*

DLD-1 and HCT116 cells were subjected to Ziyu II for 24 h, and flow cytometric analysis of apoptosis (**A**). (**B**) MTT assay of DLD-1 and HCT116 cells treated with the presence or absence of Ziyu II, and in combination with or without ZVAD for 24h. Colony formation assay of DLD-1 and HCT116 cells treated with the presence or absence of Ziyu II, and in combination with or without ZVAD for 2wks. Representative images (**C**) and quantification of colonies (**D**) were shown. (**E**) Flow cytometric analysis of ROS in DLD-1 and HCT116 cells treated with the indicated concentration of Ziyu II for 24h. (**F**). MTT assay of DLD-1 and HCT116 cells treated with NAC for 24h. All data are means±SD. *, $p < 0.05$; **,$p < 0.01$; ***, $p < 0.001$.

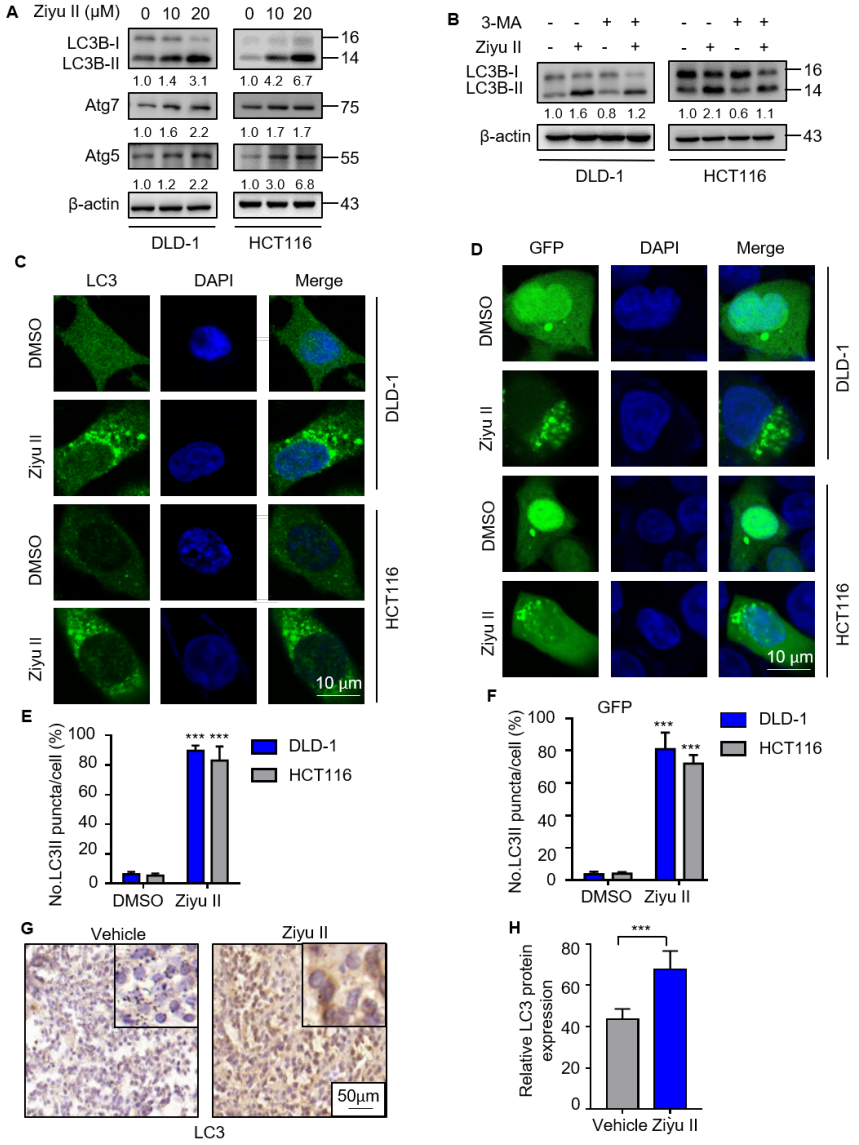


Figure 3 . **Ziyu II induces autophagy in colorectal cancer cells both *in vitro* and *in vivo***

(**A**)Immunoblotting of LC3, Atg7, Atg5 in DLD-1 and HCT116 cells treated with the indicated concentrations of Ziyu II for 24 hours. (**B**). Immunoblotting of LC3 in DLD-1 and HCT116 cells treated the presence or absence of Ziyu II, and in combination with or without 3MA for 24h. (**C**). the formation of endogenous

LC3 puncta in cells treated with DMSO or Ziyu II for 24 hours and (**E**) total number of endogenous LC3 puncta per cell. (**D**), the formation of exogenous GFP-LC3 puncta in cells treated with DMSO or Ziyu II for 24 hours. and (**F**) total number of exogenous LC3 puncta per cell. Scale bars: 10 μm. LC3 expression in orthotopic xenografts was examined by IHC. Representative images were provided as indicated (**H**), and relative immunohistochemical scores were shown (**I**). Scale bar, 50 μm. All data are means±SD. ***, $p <$ 0.001.

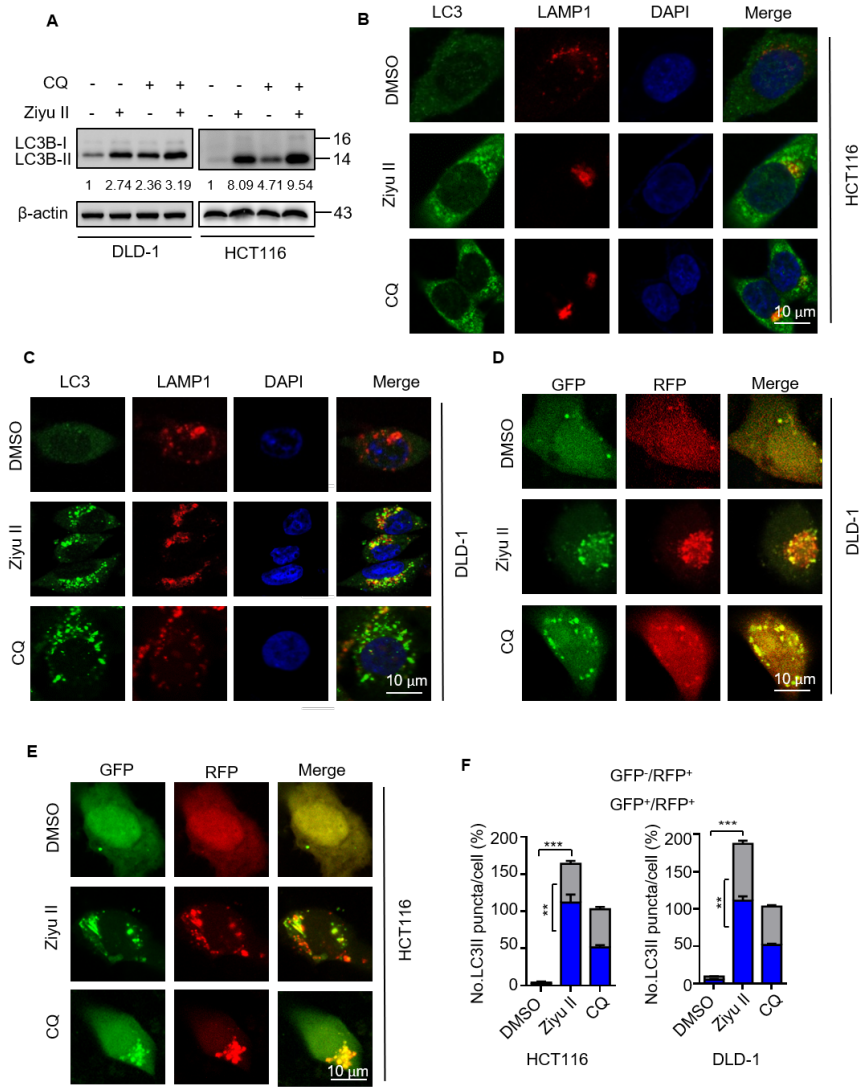


Figure 4 . Ziyu II promotes autophagy flux in colorectal cancer cells.

(**A**). Immunobloting of LC3 in cells treated in the presence or absence of Ziyu II for 24 hours. (**B** , **C**). Immunofluorescent analysis of the colocalization of endogenous LC3 with LAMP1 after treated of Ziyu II and CQ (10 mM) for 24 h, respectively. (**D** , **E** , **F**). DLD-1 and HCT116 cells were transiently transfected with an RFP-GFP tandem fluorescent-tagged LC3 (RFP-GFP-LC3) and treated with Ziyu II and 10 mmol/L chloroquine (CQ) for 24h, respectively. The formation of autophagosome (RFP-positive; GFP-positive) and autophagolysosome (RFP-positive; GFP-negative) was examined and quantified by ImageJ. Scale bars: 10 μm. All data are means±SD. $^{**}p < 0.01$, $^{***}p < 0.001$.

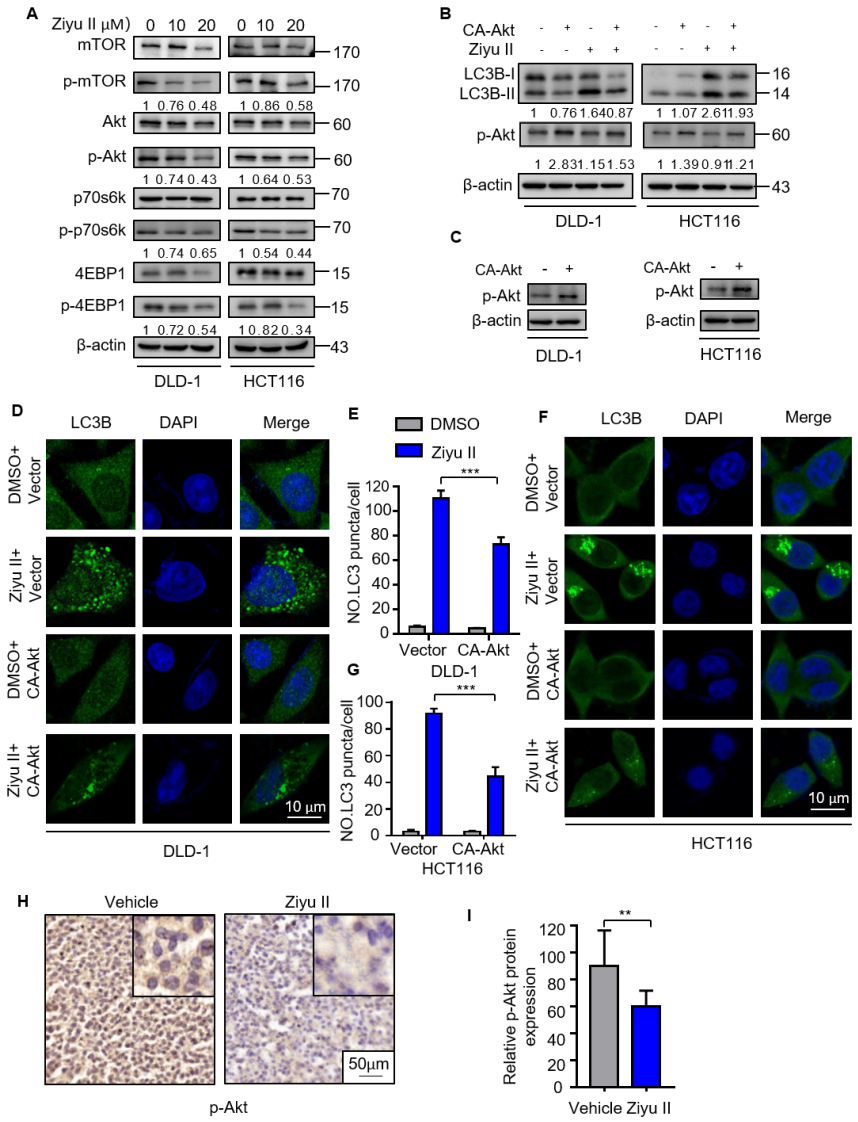


Figure 5 . Ziyu II induces autophagy by repressing the Akt/mTOR pathway in colorectal cancer cells both *in vitro*and *in vivo*

(**A**). Immunobloting of phosphorylation of Akt(S473), mTOR(S2448), p70S6K (S424/T421), and 4EBP1 (S65/T70) in cells treated with the indicated concentrations of Ziyu II for 24h. (**B**). DLD-1 and HCT116 cells were transfected with an empty vector (pECE) or with a constitutively active CA-Akt for 48h, and then cells were treated with Ziyu II for another 24h. Akt phosphorylation, and LC3 lipidation were determined by immunoblotting. (**C** and **D**) left, the formation of endogenous LC3 puncta was assessed in cells treated as in**B** . (**E** , **F**), total number of endogenous LC3 puncta per cell. Scale bars, 10 μm. All data are means±SD. ***, $p< 0.001$. Akt expression in orthotopic xenografts was examined by IHC. Representative images were provided as indicated (G), and relative immunohistochemical scores were shown (H). Scale bar, 50 μm. ***, $p < 0.001$.

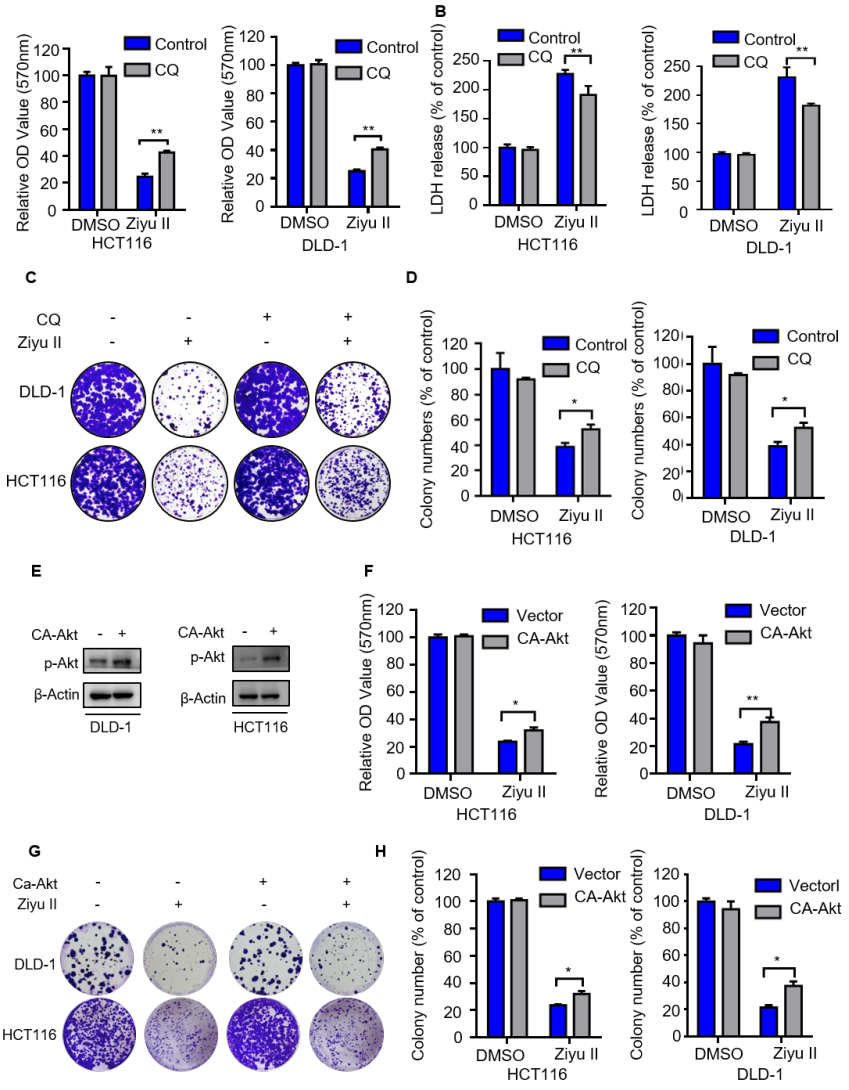
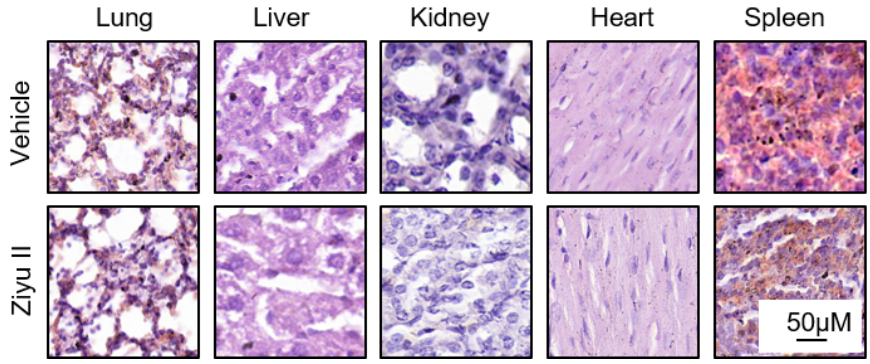


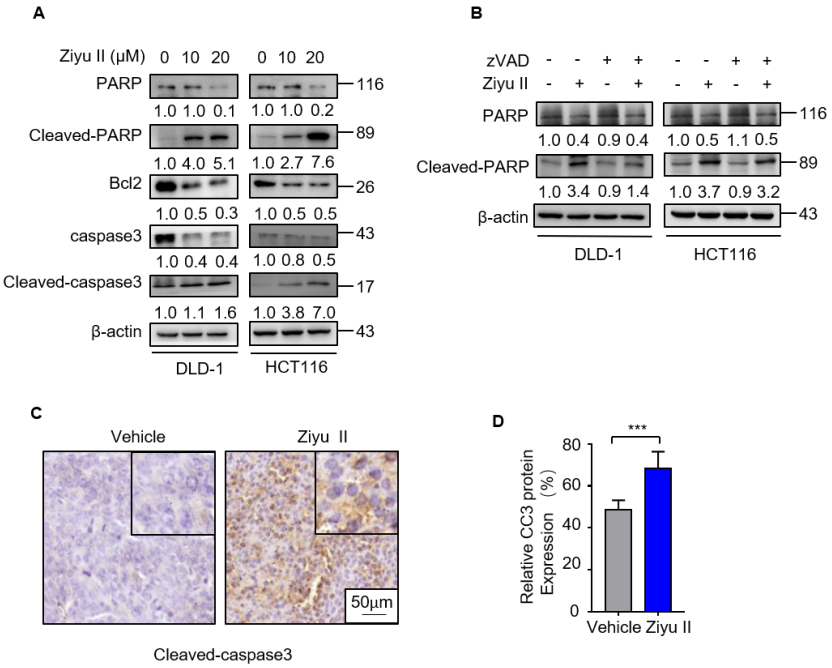
Figure 6 . Inhibition of autophagy represses the antiproliferative effect of Ziyu II in colorectal cancer cells.

(**A**). MTT assay of DLD-1 and HCT116 cells were treated with or without CQ in the presence or absence of Ziyu II. Representative images (**B**) and quantification of colonies (**C**) were shown. (**D**) LDH release assay in cells treated as in (**A**). (F) MTT assay of DLD-1 and HCT116 cells were transfected with an empty vector (pECE) or with a constitutively active CA-Akt for 48h, and then treated with Ziyu II. Colony formation assay of DLD-1 and HCT116 cells were transfected with an empty vector (pECE) or with a constitutively active CA-Akt for 48h, and then treated with Ziyu II. Representative images (**G**) and quantification of colonies (**H**) were shown. All data are means±SD. *, $p < 0.05$; **, $p< 0.01$.

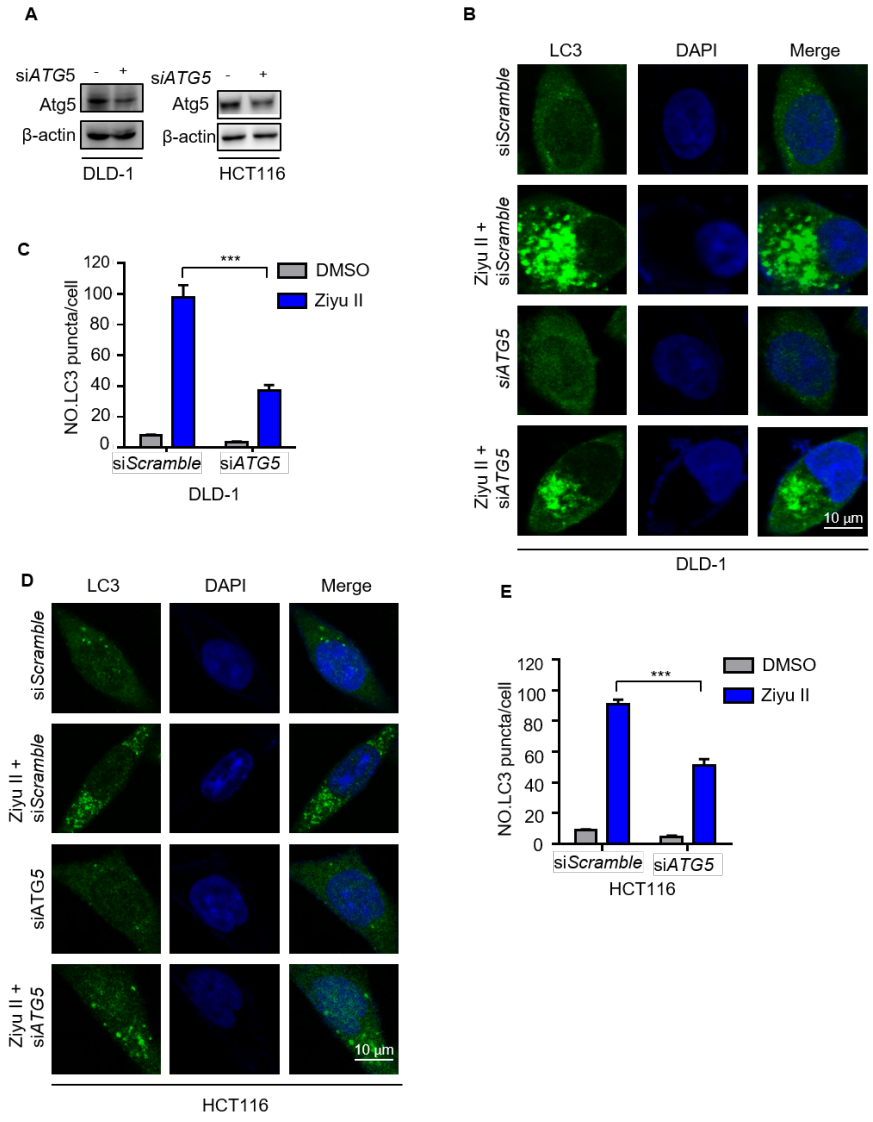
Supplementary Materials



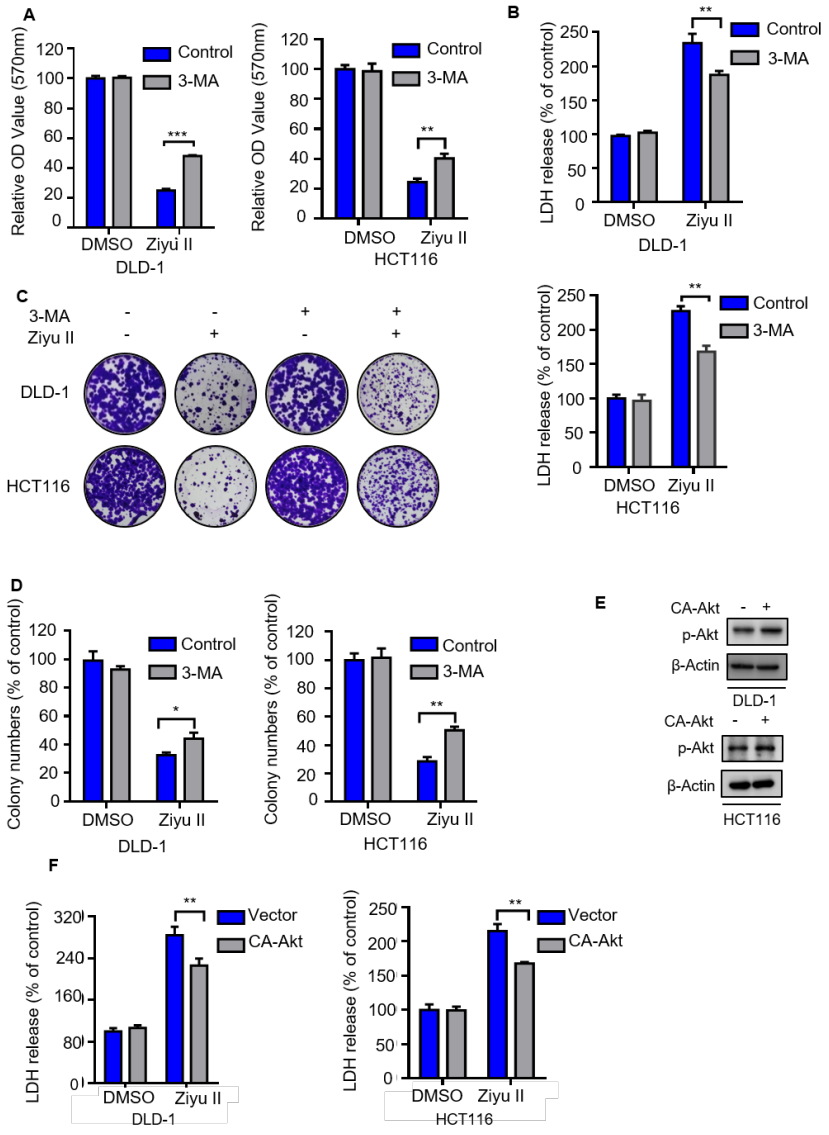
Supplementary figure 1. Ziyu II has no obvious toxicity in mice. Hematoxylin-eosin (H&E) staining of Lung, liver, kidney, heart and spleen from nude mice treated vehicle or Ziyu II. Scale bar, 50 μm.



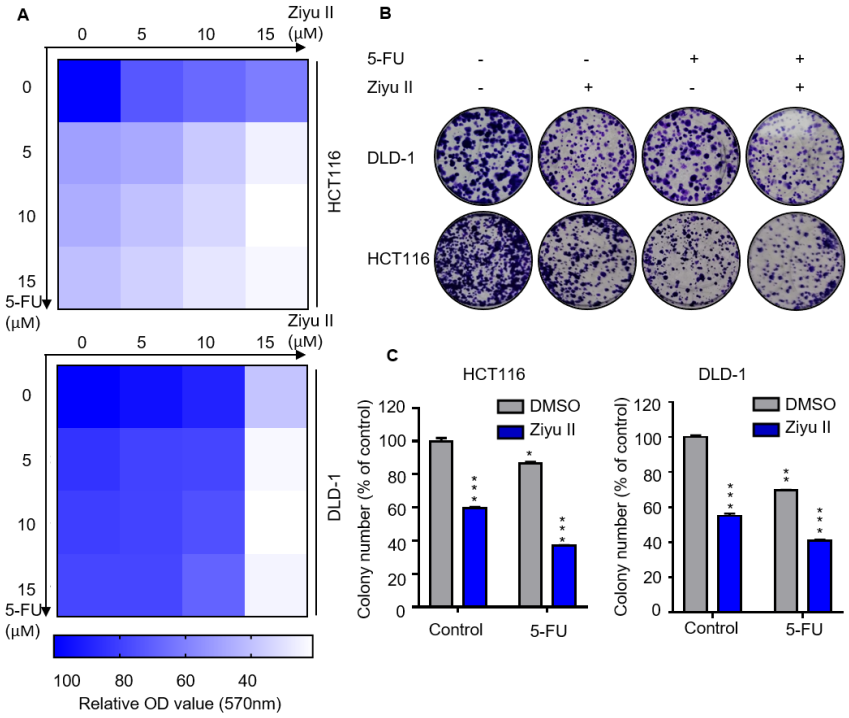
Supplementary figure 2. Ziyuglycoside II induces apoptosis in colorectal cancer cells both in *vitro* and in*vivo* Immunoblot analysis of Cleaved-PARP in cells treated with the indicated concentrations of Ziyu II for 24 hours (**A**). Immunoblot analysis of Cleaved-PARP in cells treated with ZVAD in the presence or absence of Ziyu II (20 uM) for 24h (**B**). Immunohistochemical staining of cleaved-caspase 3 (CC3) (**C**) in tumors from vehicle or Ziyu II-treated mice bearing DLD-1 subcutaneous tumor xenografts. Relative immunohistochemical scores were shown (**D**). Scale bar, 50 μm. ***, $p< 0.001$.



Supplementary figure 3. Interfering with ATG5 can inhibit the formation of LC3 puncta in CRC (**A**). Cells were transfected with an empty vector or with siAtg5 for 48 hours, and then cells were treated with Ziyu II (20 uM) for another 24 hours. Atg5 was determined by immunoblotting and (**B** , **D**) the formation of endogenous LC3 puncta was assessed in cells treated as in A and (**C** , **E**) total number of endogenous LC3 puncta per cell. Scale bars: 10 μm. All data are means±SD. ***, $p < 0.001$.



Supplementary figure4. Inhibition of autophagy represses the antiproliferative effect of Ziyu II in colorectal cancer cells. Colony formation assay of DLD-1 and HCT116 cells were treated with or without 3-MA in the presence or absence of Ziyu II. Representative images (**A**) and quantification of colonies (**C**) were shown. MTT assay (**B**) of ells were treated with or without 3-MA in the presence or absence of Ziyu II. LDH release assay in cells treated as in (**B**). (**F**). Cells were transfected with an empty vector (pECE) or with a constitutively active CA-Akt for 48 hours, and then treated with Ziyu II. the cytotoxicity was detected by the release of LDH. All data are means±SD. *, $p < 0.05$; **, $p< 0.01$.



Supplementary figure5. Ziyu II enhances the anti-cancer efficacy of 5-Fluorouracil in colorectal cancer cells (**A**). Cell growth of CRC cells treated with the indicated dose of Ziyu II in combination with 5-FU. (**B**). Colony formation assay of CRC cells treated with or without 15 μM Ziyu II in the presence or absence of 10 μM 5-FU. Representative images (**B**) and quantification of colonies **(C)** were shown. ***, $p < 0.001$.