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Dramatic suppression of colorectal cancer cell growth by the dual mTORC1 and mTORC2 inhibitor AZD-2014

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ABSTRACT

Colorectal cancer is a major contributor of cancer-related mortality. The mammalian target or rapamycin (mTOR) signaling is frequently hyper-activated in colorectal cancers, promoting cancer progression and chemo-resistance. In the current study, we investigated the anti-colorectal cancer effect of a novel mTOR complex 1 (mTORC1) and mTORC2 dual inhibitor: AZD-2014. In cultured colorectal cancer cell lines, AZD-2014 significantly inhibited cancer cell growth without inducing significant cell apoptosis. AZD-2014 blocked activation of both mTORC1 (S6K and S6 phosphorylation) and mTORC2 (Akt Ser 473 phosphorylation), and activated autophagy in colorectal cancer cells. Meanwhile, autophagy inhibition by 3-methyladenine (3-MA) and hydroxychloroquine, as well as by siRNA knocking down of Beclin-1 or ATG-7, inhibited AZD-2014-induced cytotoxicity, while the apoptosis inhibitor had no rescue effect. *In vivo*, AZD-2014 oral administration significantly inhibited the growth of HT-29 cell xenograft in SCID mice, and the mice survival was dramatically improved. At the same time, in xenografted tumors administrated with AZD-2014, the activation of mTORC1 and mTORC2 were largely inhibited, and autophagic markers were significantly increased. Thus, AZD-2014 inhibits colorectal cancer cell growth both *in vivo* and *in vitro*. Our results suggest that AZD-2014 may be further investigated for colorectal cancer therapy in clinical trials.

1. Introduction

The colorectal cancer is one of the main contributors of cancer-related death in China and around the world [1,2]. Around 103,170 new cases of colorectal cancer are diagnosed annually, causing 51,690 deaths [3]. Chemotherapy has been widely used for colorectal cancer, however drug resistance and/or off-target toxicity limit the efficiency of current chemo-drugs [4–6]. Meanwhile, five-year survival of advanced or metastatic colorectal cancer has not been significantly improved using traditional therapies [4–6]. Thus, the development of novel anti-colorectal cancer agent is urgent and extremely important.

In colorectal cancer, the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target or rapamycin (mTOR) pathway is frequently dysregulated as a result of several gene mutations [7]. Several mTOR signaling components including mTOR, p70-S6 Kinase 1 (S6K), and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) were highly expressed and activated in glandular elements of colorectal cancers [8]. Significantly, mTOR inhibition using a specific mTOR siRNA resulted in considerably decreased cancer cell growth both *in vitro* and *in vivo* [8]. Thus, mTOR signaling pathway is a target for the colorectal cancer treatment [8]. Din et al. found that aspirin inhibits mTOR signaling, and induces autophagic death in colorectal cancer cells [9]. Adiponectin was shown to inhibit colorectal cancer cell growth through inhibiting mTOR activation [10].

Significant achievements have been made in understanding the role of mTOR in cancer development and progression. Activation of mTOR signaling is vital for regulation of cellular survival, metabolism, growth, and proliferation [11–13]. mTOR exists as two distinct functional complexes known as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [11–13]. The early mTOR inhibitors including rapamycin and its analogues (RAD001, CCI-779, AP-23573) suppress the signaling to the downstream targets S6K and 4E-BP1 [13]. However, studies have shown that rapamycin and its analogues activate Akt and mitogenic Erk/mitogen-activated protein kinase (MAPK) [14] signalings, due to a feedback response of inhibition of mTORC1 [11]. Thus, dual mTORC1 and 2 inhibitors that inhibit Akt signaling could offer greater clinical benefits [13]. As a matter of fact, a number of these dual mTOR inhibitors have been developed [13,15]. Of these inhibitors, AZD-

Abbreviations: 3-MA, 3-methyladenine; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; mTOR, mammalian target or rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; S6K, p70-S6 Kinase 1; LC3B, light chain 3B.

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2014 showed potent and selective efficiency to inhibit both mTORC1 and mTORC2 [15,16].

In the current study, we examined the potential effects of AZD-2014 against colorectal cancer cells, and studied the underlying mechanisms. We found that AZD-2014 dramatically inhibits colorectal cancer cell growth through autophagy both *in vivo* and *in vitro*.

2. Materials and methods

2.1. Cell culture

As reported [14], colorectal cancer cell lines DLD-1 and Caco-2, purchased from CAS Shanghai Biological Institute, were maintained in RPMI (Gibco, Shanghai, China) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Shanghai, China), penicillin/streptomycin (1:100, Sigma) in a humidified incubator at 37 °C and 5% CO_2.

2.2. Reagents and chemicals

AZD-2014 was obtained from Selleck China (Shanghai, China); 3-methyaldenine (3-MA), hydroxychloroquine and mouse monoclonal antibody against tubulin were purchased from Sigma (Louis, MO). The general caspase inhibitor Z-VAD-fmk was purchased from Calbiochem (Darmstadt, Germany). Anti-rabbit and mouse IgG-horseradish peroxidase (HRP) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other antibodies used in this study were obtained from Cell Signaling Tech (Shanghai, China).

2.3. Cell death detecting by trypan blue staining

As reported [14], after treatment, the number of dead (trypan blue positive) cells was counted. The death rate (%) was calculated by the number of the trypan blue positive cells divided by the total number of the cells.

2.4. Clonogenicity assay

As described previously [14], HT-29 cells (2×10^3) were suspended in 1 ml of DMEM containing 0.25% agar (Sigma, St. Louis, MO), 10% FBS and with indicated treatments or vehicle controls. The cell suspension was then added on top of a pre-solidified 0.25% agar in a 100 mm culture dish. The medium was replaced every two days. After 6 days of incubation, colonies were photographed at 4×. The number of large colonies (larger than 40 μm in diameter) was manually counted.

2.5. MTT cell viability assay

As described previously [14], cell viability after indicated treatment/s was measured by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay. Different seeding densities were optimized at the beginning of the experiments.

2.6. Flow cytometric analyses of cell cycle distribution

After treatment, both detached and adherent HT-29 cells were collected and centrifuged at 1000 g for 5 min at 4 °C. Pellets were rinsed with ice-cold PBS and fixed with 70% ethanol for 2 h. Cells were then stained with staining buffer (PBS containing 20 μg/ml of propidium iodide (PI), 100 μg/ml RNase A, and 0.1% Triton X-100) for 15 min at 37 °C in the dark. Cell cycle distribution in these cells were then analyzed by a flow cytometer (BD Bioscience).

2.7. Cell apoptosis assay through Annexin V staining

After treatment, cell apoptosis was detected by the Annexin V staining (Roche Molecular Biochemicals, Indianapolis, IN, USA) with FACS sorting as reported [14].

2.8. Quantification of apoptosis by enzyme-linked immunosorbent assay (ELISA)

As described previously [14], the Cell Apoptosis ELISA Detection Kit Plus (Roche, Palo Alto, CA) was used to quantify HT-29 cell apoptosis according to the manufacturer's protocol. Briefly, the cytoplasmic histone/DNA fragments from cells with treatments were extracted and bound to immobilized anti-histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was then added for the detection of immobilized histone/DNA fragments. After addition of substrate for peroxidase, the spectrophotometric absorbance of the samples was determined sing a plate reader at a test wavelength of 405 nm with a reference wavelength of 590 nm.

2.9. Caspase-3 activity assay

After treatment, cytosolic proteins from approximately 3×10^6 HT-29 cells were extracted in hypotonic cell lysis buffer (25 mm HEPES, pH 7.5, 5 mm $MgCl_2$, 5 mm EDTA, 5 mm dithiothreitol, 0.05% phenylmethylsulfonyl fluoride). The protein concentration of samples was determined by using a Bio-Rad Bradford protein assay kit (Bio-Rad, Shanghai, China). Twenty micrograms of cytosolic extracts were added to caspase assay buffer (312.5 mm HEPES, pH 7.5, 31.25% sucrose, 0.3125% CHAPS) with benzyloxycarbonyl-DEVD-7-amido-4-(trifluoromethyl) coumarin as the substrate (Calbiochem, Darmstadt, Germany). The release of 7-amido-4-(trifluoromethyl)coumarin (AFC) was quantified, after 2 h of incubation at 37 °C, using a Fluoroskan system (Thermo-Labsystems, Helsinki, Finland) set to an excitation value of 355 nm and emission value of 525 nm. The results were expressed as relative fluorescence units/μg of protein.

2.10. Western blots

After treatment, aliquots of 30 μg of lysed protein (lysed by 40 mM HEPES [pH 7.5], 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.5 mM orthovanadate, EDTA-free protease inhibitors [Roche] and 1% Triton) from each sample was separated by 10% SDS polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). After blocking with 10% instant non-fat dry milk for 1 h, the membrane was incubated with the specific antibody overnight at 4 °C followed by incubation with secondary antibody for 1 h. The bolt was visualized by ECL (enhanced chemiluminescence) machine.

2.11. Transfection and RNA interference

Beclin 1 siRNA-1 (CUCAGGAGAGGAGCCAUUU) [17] and Beclin 1 siRNA-2 (GAUUGAAGACACAGGAGGC) [17] were synthesized by Kaiji BioTech (Nanjing, China). ATG-7 siRNA-1 (sequence: GGAGUCACAGCUCUUCCUU [18,19]) and ATG-7 siRNA-2 (sequence: CAGAAGGAGUCACAGCUCUUCCUUA) [20] were also synthesized. HT-29 cells were cultured in six-well plates and transfected at 60% confluence with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. One hundred nanoMolar of indicated siRNA was transfected. After 3 h transfection, 2% FBS was added, and cells were cultured for another 48 h before they were trypsinized and

used for experiments. After treatment, Western blot was utilized to test the target protein to insure the transfection efficiency.

2.12. In vivo antitumor efficacy evaluation

Tumor growth inhibition studies were performed in SCID mice. Briefly, 2×10^6 viable HT-29 cells in 100 μL of growth medium were subcutaneously inoculated, and mice bearing ~300 mm^3 tumors were randomly divided into two groups and were treated with 5 mg/kg AZD-2014 (PO, QD × 14; $n = 10$, the dose was based on results from pre-experiments) or vehicle (saline, PO, QD × 14; $n = 10$). Tumor volume was shown, which was calculated by the modified ellipsoid formula: $(\pi/6) \times AB^2$, where A is the longest and B is the shortest perpendicular axis of an assumed ellipsoid corresponding to tumor mass [21,22]. Mice survival and body weight were also recorded every week.

2.13. Statistical analysis

All data were normalized to control values of each assay and were presented as mean ± standard deviation (SD). Data were analyzed by one-way ANOVA followed by a Scheffe's f-test by using SPSS software (SPSS Inc., Chicago, IL, USA). Significance was chosen as $p < 0.05$ or $p < 0.01$.

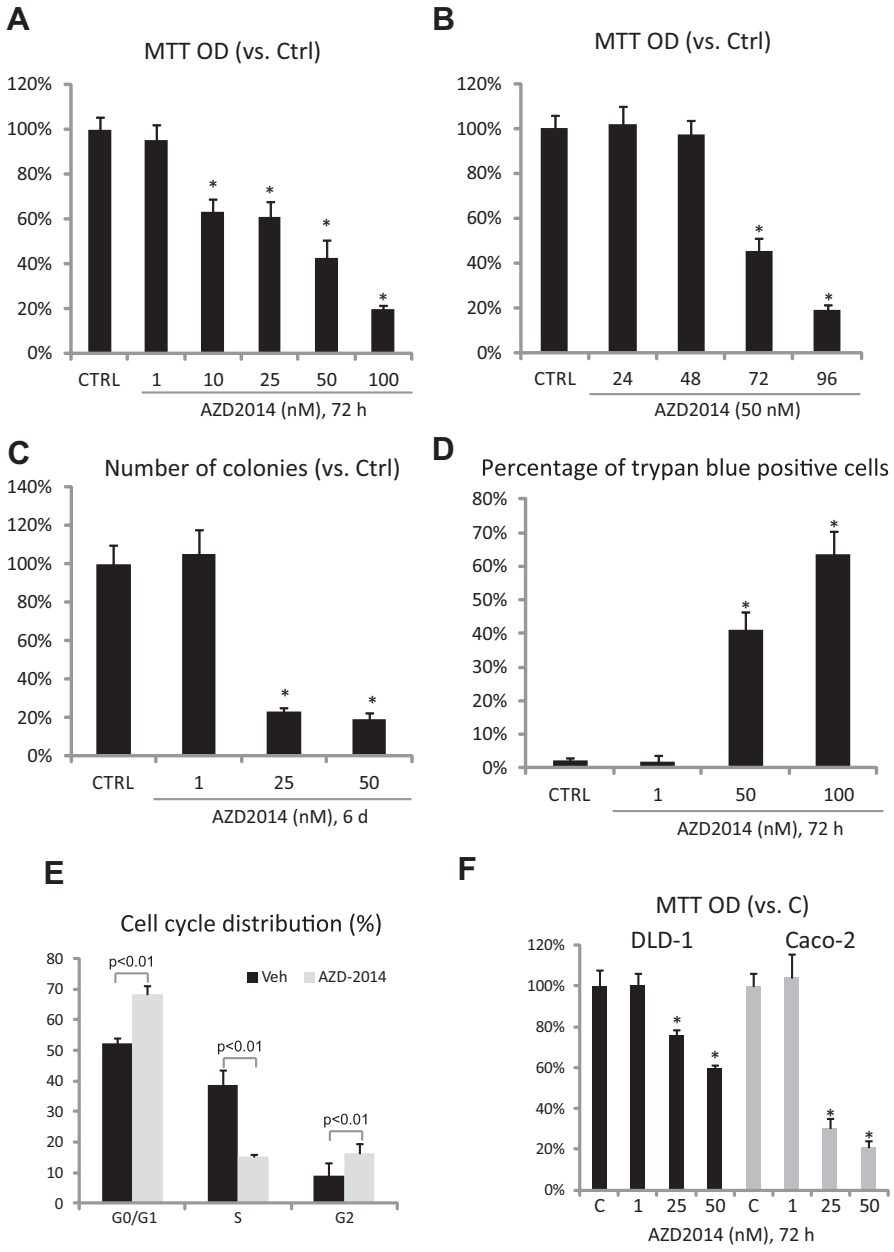

Fig. 1. AZD-2014 exerts significant cytotoxic effects in colorectal cancer cells. HT-29 cells were treated with vehicle ("Ctrl") or different concentrations of AZD-2014 for 72 h (A and D), or treated with 50 nM of AZD-2014 for indicated time (B), cell viability was analyzed by MTT assay, and the percentage of trypan blue positive ("dead" cells) was also recorded (D). HT-29 cells were treated with different concentration of AZD-2014 for 6 days, the number of the viable colonies (diameter >40 μm) was counted and normalized to "Ctrl" group (C). HT-29 cells were treated with vehicle or AZD-2014 (50 nM) for 72 h, cell cycle distribution was analyzed by FACS (E). MTT results of DLD-1 and Caco-2 cells treated with vehicle or indicated concentration of AZD-2014 for 72 h (F). The data shown were mean ± SD. Experiments in this figure were repeated three times. *$p < 0.05$ vs. group "Ctrl".

3. Results

3.1. AZD-2014 exerts significant cytotoxic effects in colorectal cancer cells

To test AZD-2014s effect on colorectal cancer cell growth, HT-29 cells were exposed to different concentration of AZD-2014 (1–100 nM) and cultured for indicated time. The MTT cell viability assay and clonogenicity assay were utilized to tested cell growth after treatment [14]. Results in Fig. 1A and C demonstrated that AZD-2014 dose-dependently inhibited HT-29 cell growth, and the MTT OD (Fig. 1A) and the number of viable colonies (Fig. 1C) decreased significantly with AZD-2014 (>10 nM) treatment. Note that the low concentration of AZD-2014 (1 nM) did not induce significant growth inhibition (Fig. 1A and C). 50 nM of AZD-2014 induced around 50% of cell viability loss (Fig. 1A). Thus, this concentration was used for further mechanistic study. AZD-2014 (50 nM) took at least 72 h to cause significant cell viability loss, indicating a time-dependent effect (Fig. 1B). Results in Fig. 1D demonstrated that AZD-2014 (50 and 100 nM) promoted HT-29 cell death, reflected by increased trypan blue positive cells. MTT cell viability assay results in Fig. 1F showed AZD-2014 inhibited cell growth of two other colorectal cancer cell lines (DLD-1 and Caco-2). These results together demonstrated the cytotoxic effects of AZD-2014 in cultured colorectal cancer cells.

3.2. AZD-2014-induced cytotoxicity is unlikely through apoptosis in colorectal cancer cells

Results in Fig. 1 confirmed the cytotoxic effect of AZD-2014 in colorectal cancer cells. We then examined whether cell apoptosis was involved in this process. As described [14], three independent methods including Histone-DNA apoptosis ELISA assay, Annexin V FACS assay and caspase-3 activity assay were applied to test HT-29 cell apoptosis after AZD-2014 treatment. Results from all these assays (Fig. 2A–C) showed that AZD-2014 failed to induce significant cell apoptosis in HT-29 cells. And the apoptosis ELISA OD, number of Annexin V positive cells and caspase-3 activity were almost unchanged (or slightly changed) after cytotoxic AZD-2014 treatment (Fig. 2A–C). The time response of AZD-2014 (0, 12, 24, 48 and 72 h) on cell apoptosis was also tested, and again no significant apoptosis was observed (data not shown). On the other hand, camptothecin induced a dramatic HT-29 cell apoptosis (Fig. 2A–C). To further role out the requirement of apoptosis in AZD-2014-induced cytotoxic effects, the apoptosis inhibitor was applied. Our results demonstrated that the general caspase inhibitor Z-VAD-fmk [23] had almost no effects on AZD-2014-induced cell viability loss (Fig. 2D) and colony number decrease (Fig. 2E) in HT-29 cells. Z-VAD-fmk also failed to rescue DLD-1 and Caco-2 cells from AZD-2014. Thus, AZD-2014-induced cytotoxicity is unlikely through apoptosis in colorectal cancer cells.

3.3. AZD-2014 blocks mTORC1 and mTORC2 activation and induces autophagic death in colorectal cancer cells

Our lab has been focusing on autophagic cell death. In a recent study, we found that ceramide (C6)-induced death of HT-29 cells requires cell autophagy, but not apoptosis [14]. Here we observed that AZD-2014-induced cytotoxicity in cultured colorectal cancer cell was unlikely due to cell apoptosis. Thus, we tested the possible involvement of autophagy. We first examined autophagy activation in AZD-2014-treated HT-29 and DLD-1 cells. Results in

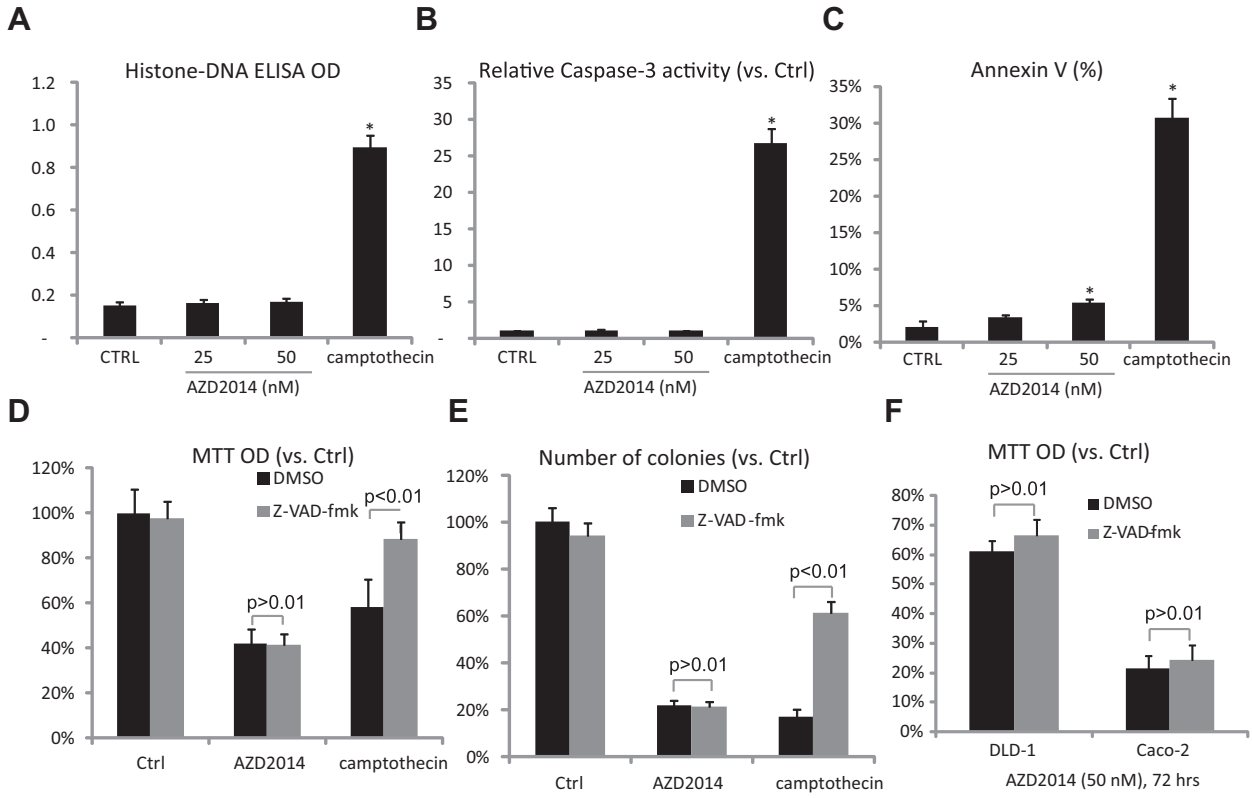

Fig. 2. AZD-2014-induced cytotoxicity is unlikely through apoptosis in colorectal cancer cells. Cultured HT-29 cells were treated with vehicle ("Ctrl"), AZD-2014 (25/50 nM) or camptothecin (250 nM) and cultured for 60 h, cell apoptosis was examined by Histone-DNA Apoptosis ELISA assay (A), caspase-3 activity assay (B) and FACS Annexin V assay (C). Cultured HT-29 cells, DLD-1 cells or Caco-2 cells were pre-treated with z-VAD-fmk (50 μM) for 2 h, followed by AZD-2014 (50 nM) or camptothecin (250 nM) stimulation, cells were further cultured for 72 h, cell viability was then analyzed by MTT assay (D and F), cell survival was examined by the clonogenicity assay (E). The data shown were mean ± SD. Experiments in this figure were repeated three times. $^{*}p < 0.05$ vs. "Ctrl" group (A–C).

Fig. 3A and B showed that AZD-2014 induced significant Beclin-1 and light chain 3B (LC3B)-II expressions in above cell lines, indicating autophagy activation. Importantly, autophagy inhibitors 3-methyaldenine (3-MA) [24] and hydrogenchloroquine (Cq) [25] significantly inhibited AZD-2014-induced HT-29 cell viability loss (Fig. 3C) and colonies decrease (Fig. 3D). To further confirm that autophagy contributes to cell death induced by AZD-2014, we utilized siRNAs to selectively knockdown Beclin-1 or ATG-7, two autophagy regulators [26,27]. Western blot results showed that Beclin-1 or ATG-7 was significantly downregulated by the targeted siRNAs (two non-overlapping siRNAs), as compared with that in control and scramble siRNA transfected HT-29 cells (Fig. 3G). Importantly, AZD-2014-induced loss of cell viability (Fig. 3E) and reduction of survival colonies (Fig. 3F) were dramatically inhibited by Beclin-1 or ATG-7 siRNA knockdown. Note that the siRNAs alone had no effect on HT-29 cell survival or colony formation (data not shown). These results confirmed that autophagy is required for AZD-2014-mediated cytotoxicity. For the mechanism study, we tested activation mTORC1 and mTORC2 in colorectal cancer cells before and after AZD-2014 treatment, and results showed that AZD-2014 blocked both mTORC1 and mTORC2 activation in HT-29 and DLD-1 cells (Fig. 3H), mTORC1 activation was

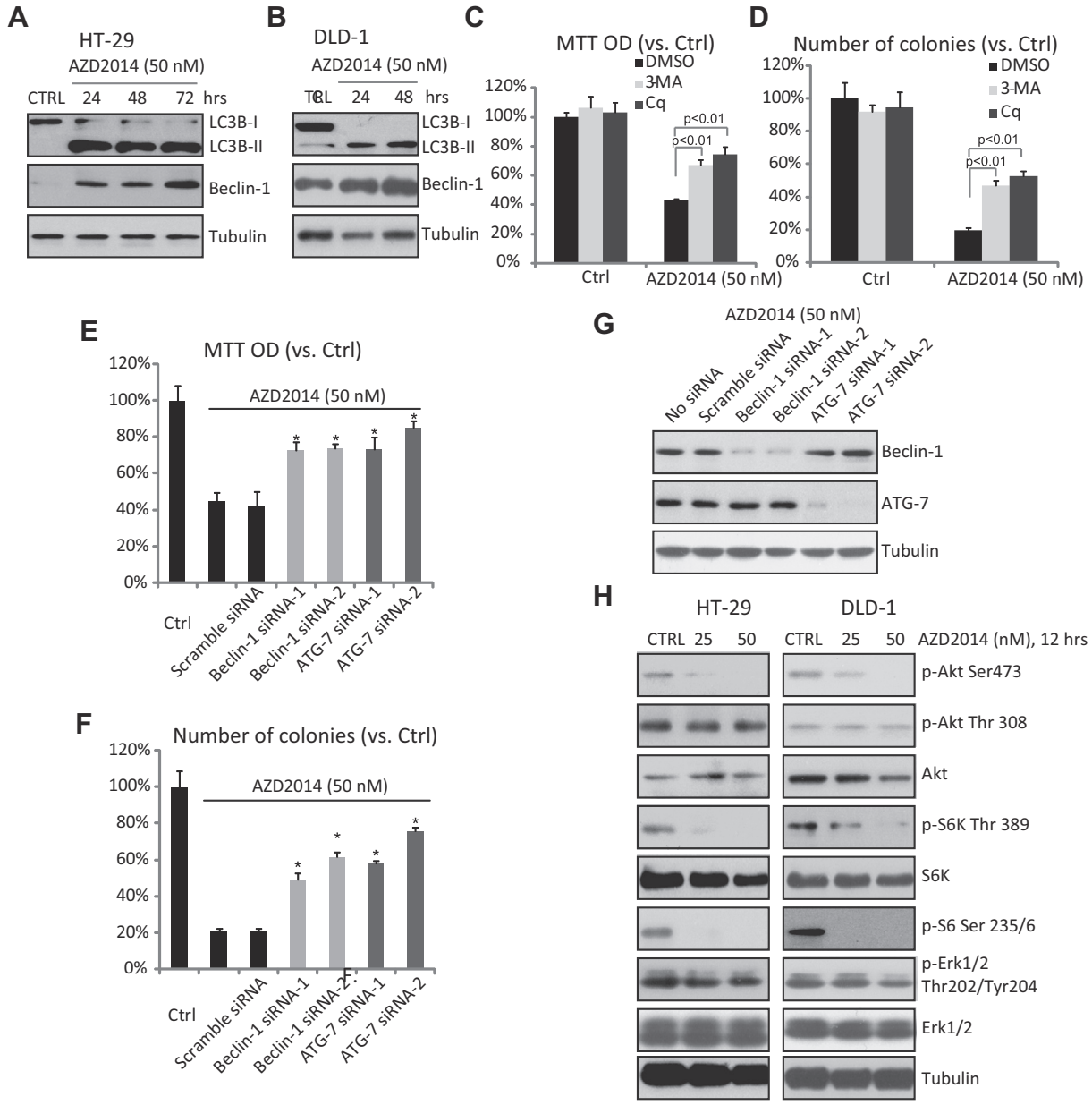

Fig. 3. AZD-2014 blocks mTORC1 and mTORC2 activation and induces autophagic death in colorectal cancer cells. HT-29 and DLD-1 cells were treated with vehicle or AZD-2014 (50 nM) for indicated time, expressions of Beclin-1, LC3-B and tubulin (loading control) were examined by Western blots (A and B). Cultured HT-29 cells were pretreated with autophagy inhibitors 3-MA (10 mM) or hydroxychloroquine (Cq, 10 mM) for 1 h, followed by AZD-2014 (50 nM) treatment, MTT assay was performed to test cell viability after 72 h (C), cell death was tested by the clonogenicity assay after 6 days (D). Control HT-29 cells, or HT-29 cells transfected with scramble siRNA, Beclin-1 siRNA (−1 or −2), or ATG-7 siRNA (−1 or −2) (100 nM each) were left untreated, or treated with AZD-2014 (50 nM). Cell viability was analyzed by MTT assay 72 h after AZD-2014 stimulation (E), and clonogenicity survival assay was also performed 6 days after AZD-2014 treatment (F). The expression of Beclin-1, ATG-7 and tubulin in AZD-2014-treated cells (6 days) were tested by Western blots (G). HT-29 and DLD-1 cells were treated with vehicle or AZD-2014 (25/50 nM) for 12 h, activation of Akt, mTORC1/2 and Erk/MAPK was tested by Western blots using antibodies listed (H). The data shown were mean ± SD. Experiments in this figure were repeated three times with similar results. $^{*}p < 0.05$ vs. group of control HT-29 cells with AZD-2014 stimulation (E and F).

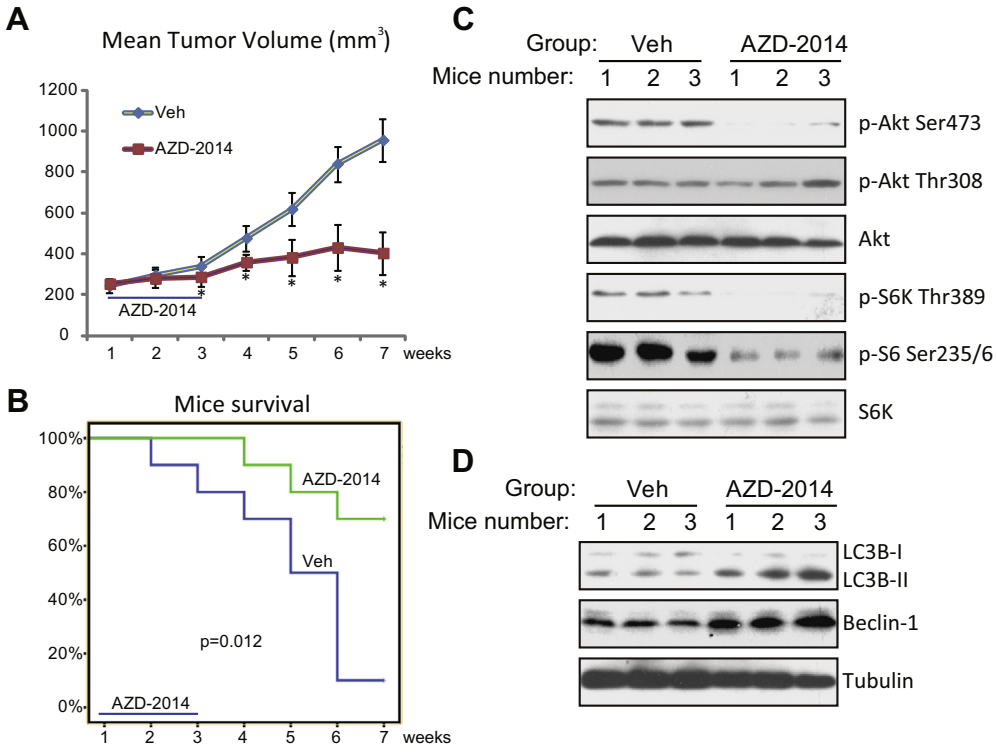

Fig. 4. Oral administration of AZD-2014 suppresses HT-29 cell growth *in vivo*. (A) Mean tumor growth curves for mice (n = 10 mice per group) bearing HT-29 human colon cancer xenografts following administration of AZD-2014 (5 mg/kg, PO, QD × 14) or vehicle (saline). (B) The survival curve of mice (n = 10 mice per group) bearing HT-29 human colon carcinoma xenografts following administration of AZD-2014 (5 mg/kg, PO, QD × 14) or vehicle. After AZD-2014 administration, tumor tissues were isolated and lysed, activation of Akt and mTORC1/2 was tested by Western blots (C), autophagy markers (LC3B-II and Beclin-1) in isolated xenograft tissues were also tested (D).The data shown was mean ± SD. Experiments in this figure were repeated three times with similar results.

reflected by phosphorylation of S6K and S6, while mTORC2 activation was indicated by Akt Ser 473 phosphorylation (Fig. 3H). Note that Akt Thr 308 phosphorylation and Erk/MAPK activation were almost unaffected by AZD-2014 in both cell lines (Fig. 3H).

3.4. Oral administration of AZD-2014 suppresses HT-29 cell growth in vivo

To determine whether AZD-2014 could serve as a therapeutic agent against colorectal cancer formation, we established a tumor model in SCID mice bearing HT-29 xenografts. As compared to vehicle group, oral administration of AZD-2014 (5 mg/kg, P.O. 14 days) dramatically inhibited the growth of HT-29 xenografts (Fig. 4A). Meanwhile, the mice survival was dramatically improved with AZD-2014 administration (Fig. 4B), while mice body weight was not significantly affected (data not shown). In tumors administrated with AZD-2014, the activation of mTORC1 (p-S6K and p-S6) and mTORC2 (p-Akt Ser 473) was both inhibited, which was showed by Western blot (Fig. 4C), while p-Akt Thr 308 was not affected (Fig. 4C). Autophagy was also induced in AZD-2014-treated tumors, as the expressions of LC3B-II and Beclin-1 were significantly upregulated (Fig. 4D). Thus, oral AZD-2014 administration inhibits mTORC1/2 activation and HT-29 cell growth, whiling inducing autophagy *in vivo*.

4. Discussion

MTOR is composed of at least two distinct multi-protein complexes: mTORC1 and mTORC2 [12,13]. mTORC1, a complex of mTOR, regulatory-associated protein of mTOR (Raptor), mLST8, Deptor, and proline-rich Akt substrate 40 (PRAS40), is regulated by diverse environmental signals. The PI3K/Akt pathway is an important upstream activator of mTORC1. Activation of mTORC1 promotes cell growth and proliferation by phosphorylation of ribosomal protein S6K1 and 4E-BP1. mTORC1 can be inhibited by rapamycin allosterically [28]. On the other hand, mTORC2 is a complex consisting of mTOR, rapamycin-insensitive companion of mTOR (Rictor), mLST8, Protor, Deptor, and mammalian stress-activated protein kinase interacting protein (mSIN1) [13]. This complex also plays an important role in regulation of proliferation, survival, and nutrient uptake in cancer cells, mainly due to the fact that mTORC2 is a major hydrophobic kinase that phosphorylates Akt on the Ser 473 residue [29], which is critical for its fully activation.

The limitations of rapamycin-based therapies in the clinical settings have led to development of the so-called "second generation" of mTOR inhibitors, or the ATP-competitive mTOR kinase inhibitors. Unlike rapamycin drugs, these inhibitors target the mTOR kinase domain, and inhibit its catalytic activity [13,15]. Therefore, the key advantage of these drugs is to inhibit the kinase activity of both the mTORC1 and mTORC2, thus also blocking the feedback activation of Akt or Erk signalings [13]. In the current study, we observed that AZD-2014 blocked both mTORC1 and mTORC2 activation, while leaving Erk unaffected in colorectal cancer cells. Thus, AZD-2014 is an ideal drug to target these pathways.

Autophagy, a catabolic process where cell degradates its own components through the lysosomal machinery, is important for regulation of cell survival, growth, proliferation, and death [30]. In the apoptosis resistant cancer cells (i.e. colorectal cancer cells), autophagic cell death is known as one major contributor of growth inhibition activated a number of chemo-drugs. For example, aspirin induces autophagic cell death in colorectal cancer cells through inhibiting mTOR signaling [9,31]. In the current study, we found that autophagic cell death, but not apoptotic cell death,

contributed to AZD-2014-mediated growth inhibition and cytotoxicity in colorectal cancer cells. In summary, AZD-2014 inhibits colorectal cancer cell growth both *in vivo* and *in vitro*. Our results suggest that AZD-2014 should be further investigated for colorectal cancer therapy in clinical trials.

Conflict of Interest

The authors have no conflict of interests.

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