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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