Tumor Biol.
DOI 10.1007/s13277-016-5018-x

ORIGINAL ARTICLE

Autophagy inhibition sensitizes WYE-354-induced anti-colon cancer activity in vitro and in vivo

Lijun Wang[1] · **Yun-Rong Zhu**[2] · **Shaowei Wang**[1] · **Song Zhao**[3]

Received: 13 January 2016 / Accepted: 18 March 2016

Abstract Mammalian target of rapamycin (mTOR) complex 1 (mTORC1) and mTORC2 are frequently dysregulated in human colon cancers. In the present study, we evaluated the potential anti-colon cancer cell activity by a novel mTORC1/2 dual inhibitor WYE-354. We showed that WYE-354 was anti-survival and anti-proliferative when adding to primary (patient-derived) and established (HCT-116, HT-29, Caco-2, LoVo, and DLD-1 lines) colon cancer cells. In addition, WYE-354 treatment activated caspase-dependent apoptosis in the colon cancer cells. Mechanistically, WYE-354 blocked mTORC1 and mTORC2 activation. Meanwhile, it also induced autophagy activation in the colon cancer cells. Autophagy inhibitors (bafilomycin A1 and 3-methyladenine), or shRNA-mediated knockdown of autophagy elements (Beclin-1 and ATG-5), remarkably sensitized WYE-354-mediated anti-colon cancer cell activity in vitro. Further studies showed that WYE-354 administration inhibited HT-29 xenograft growth in severe combined immunodeficient (SCID) mice. Importantly, its activity in vivo was further potentiated with co-administration of the autophagy inhibitor 3-MA. Phosphorylations of Akt (Ser-473) and S6 were also decreased in WYE-354-treated HT-29 xenografts. Together, these pre-clinical results demonstrate the potent anti-colon cancer cell activity by WYE-354, and its activity may be further augmented with autophagy inhibition.

Keywords Colon cancer · mTORC1/2 · WYE-354 · Autophagy · Chemo-sensitization

✉ Shaowei Wang
wangshaoweidr@sohu.com

[1] Department of General Surgery, Tianjin Hospital, No 406 Jiefang South Road, Tianjin 300211, China

[2] Department of Orthopedics, The Affiliated Jiangyin Hospital of Medical College of Southeast University, Jiangyin City 214400, China

[3] Department of Pathophysiology, Hebei Medical University, Shijiazhuang, China

Introduction

The colon cancer is one leading cause of cancer-related mortalities [1–3]. The incidence of this devastating disease is still increasing [1, 2]. Over the past decades, significant improvement has been achieved in treatment for the colon cancer [2, 4]. Yet, for the patients with advanced, metastatic or recurrent colon cancers, the overall survival is still far from satisfactory [1–3]. Therefore, groups all over the world are developing novel and more efficient anti-colon cancer agents [2, 4–6].

The molecular heterogeneity encumbers the uniform application of specific molecularly targeted agents for the treatment of the colon cancer [2, 5–7]. Studies have shown that mammalian target of rapamycin (mTOR) signaling is often dysregulated in colon cancers, which is associated with cancer progression, chemo-resistance, and recurrence [8–12]. On the other hand, mTOR inhibitors have been developed [10–12]. These inhibitors have displayed promising anti-cancer results in pre-clinical and clinical cancer studies [10–12].

mTOR is in two distinct multi-protein complexes, including mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [13, 14]. The traditional mTORC1 phosphorylates ribosomal protein S6 kinase 1 (S6K1) and the eukaryotic translation initiation factor eIF4E-binding protein 1 (4E-BP1) [13, 14]. Both of them are vital for protein translation [13, 14]. On the other hand, the recently-discovered mTORC2 directly phosphorylates Akt at Ser 473, which is required for Akt activation [13, 14]. In addition, existing evidences have shown

Published online: 28 March 2016

that mTORC2 phosphorylates PKCα to regulate actin cytoskeleton [15]. Activation of both mTORC1 and mTORC2 is important for several cancerous behaviors, including cancer cell growth, proliferation, and survival, as well as cell migration, and apoptosis resistance [13, 16]. In the current study, we investigated the potential anti-colon cancer cell activity by a novel, specific, and highly-potent mTORC1/2 dual inhibitor WYE-354 [17].

Materials and methods

Chemicals, reagents, and antibodies

WYE-354 (catalog number: S1266) was provided by Selleck China (Shanghai, China). The pan caspase inhibitor z-VAD-fmk (catalog number: 627610) and the caspase-3 inhibitor z-DEVD-cho (Catalog number: 235420) were purchased from Calbiochem (Darmstadt, Germany). The autophagy inhibitors bafilomycin A1 (Baf A1, catalog number: B1793) and 3-methyladenine (3-MA, catalog number: M9281) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Anti-light chain 3B-II (LC3B-II, sc-28266), Beclin-1 (sc-48341), p62 (sc-28359), and (β-) tubulin (sc-55529) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Autophagy-related protein (ATG)-5 (2630) antibody, p-Akt (Ser 473) antibody (9271), p-Akt (Thr 308) antibody (4056), Akt1 antibody (2938), p-S6 ribosomal protein (S6, Ser 235/236) antibody (2211), S6 antibody (2317), p-p44/42 MAPK (Erk1/2) (4695), and Erk1/2 antibody (9102) were purchased from Cell Signaling Technologies (Beverly, MA).

Culture of established cell lines

Human colon cancer cell lines, including HCT-116, HT-29, Caco-2, LoVo and DLD-1, and the non-cancerous colon epithelial NCM460 line [18], were all purchased from Shanghai Biological Institute (Shanghai, China). The cells were placed into tissue culture flasks and grown in RPMI-1640/DMEM medium plus 10 % heat-inactivated FBS. DNA fingerprinting and profiling were performed every 6 months to confirm the origin of the cell line and to distinguish the cell line from cross-contamination. All cell lines were subjected to mycoplasma and microbial contamination examination every month. Population doubling time, colony forming efficiency, and morphology under phase contrast were measured to confirm the phonotype of cell lines. All culture reagents were obtained from Gibco (Beijing, China).

Primary culture of patient-derived colon cancer cells

Surgery-separated human colon cancer tissues were washed and digested for 45 min in collagenase A (300 units/mL; Sigma, C0130), Dnase I (Sigma, D5025) and hyaluronidase (100 units/mL; Sigma, H1115000). Samples were then mechanically dissociated and filtered through a 70-μm strainer, washed twice in 1× PBS, and used for culture in complete medium: DMEM/F12 supplemented with 10 % FCS, plus 10 ng/mL EGF. The protocols were approved by the Internal Review Board (IRB) of all authors' institutions and were conducted according to the principles expressed in the Declaration of Helsinki. A total of three colon cancer patients (named patient 1 or "P1," "P2," and "P3," all male, 45, 61, and 55 years old) were enrolled. The written-informed consent was obtained from each patient.

MTT assay of cell survival

Colon cancer cells were seeded onto 96-well plates (4000 cells per well). After treatment, cell survival was evaluated via MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Sigma) with manufacturer's instructions. Absorbance was measured at 550 nm through a Microplate Reader. The OD value of treatment group was normalized to that of the untreated control group.

Clonogenicity assay of cell proliferation

Colon cancer cells were counted and seeded in triplicate at 1000 cells/well in six-well plates pre-coated with 1 % agarose (Sigma). After 12 days of incubation in complete media with or without WYE-354, we assessed the anchorage-dependent growth, by staining with 0.005 % crystal violet (Sigma), observing and manually counting the colonies formed under a microscope. The number of viable colonies in WYE-354-treated group was normalized to that of untreated control group.

Assay of caspase-3 activity

As previously described [19], following the indicated WYE-354 treatment, 20 μg of cytosolic protein extracts of colon cancer cells was added to caspase assay buffer [19] together with the caspase-3 substrate Ac-DEVD-AFC (15 μg/mL) (Calbiochem). After 1 h incubation at 37 °C, the released AFC was measured using a spectrofluorometer (Thermo-Labsystems, Helsinki, Finland) with excitation of 380 nm and emission wavelength of 460 nm.

TUNEL staining assay of apoptosis

TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) In Situ Cell Death Detection Kit (Roche, Shanghai, China) was applied to quantify colon cancer cell apoptosis. Apoptosis percentage was reflected by the TUNEL ratio (TUNEL/DAPI × 100 %) detected under a

fluorescence microscope (Zeiss). A total of ten random views (under 1:100 magnification) of each condition were included to count TUNEL percentage.

Histone-DNA enzyme-linked immunosorbent assay (ELISA) assay

Cell apoptosis was also quantified by Histone-DNA ELISA PLUS kit (Roche) according to the manufacturer's protocol [20–22]. ELISA OD at 405 nm was recorded as a quantitative indicator of cell apoptosis [23].

Western blot analysis

Protein expression was determined using the SDS-PAGE and immunoblotting as previously described [23]. Briefly, quantified protein samples (30 μg per sample) were separated by 8–15 % SDS-PAGE gel and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Beijing, China). The blots were then incubated with designated primary antibodies. Secondary antibodies were added afterwards. Detection was accomplished by chemiluminescence with ECL (GE Healthcare). Quantification of bands was performed via ImageJ software [24].

Stable shRNA knockdown of Beclin-1 or ATG-5

The lentiviral particles packaged with Beclin-1 shRNA (catalog number: sc-29797-V) or scramble control shRNA ("sc shRNA", catalog number: sc-108065) were obtained from Santa Cruz Biotech (Santa Cruz, CA). The human ATG-5 shRNA lentivirus were designed by Genechem (Shanghai, China) based on previously described sequence [25]. The lentivirus (10 μL/well) were added to cultured colon cancer cells for 12 h. Afterwards, the culture medium was replaced with puromycin (2.0 μg/mL, Sigma)-containing fresh medium, conditional medium was renewed every 2–3 days until resistant stable colonies were formed (2–3 weeks). Expression of target proteins (ATG-5 and Beclin-1) was always determined by Western blots in stable cells.

In vivo tumor xenograft study

In the present study, 5–6-week-old female severe combined immunodeficient (SCID) nude mice were inoculated with HT-29 cells (3×10^6/0.2 mL/mice) in the left flanks and maintained in a pathogen-free environment. After 20 days, SCID mice bearing HT-29 tumors (around 200–300 mm^3 in total volume) were randomly divided into four groups: WYE-354 (10 mg/kg, formulated in 5 % ethanol, 5 % polysorbate 80, 5 % polyethylene glycol-400, i.p. injection, once daily, for 15 days) [17], 3-MA (20 mg/kg, i.p. injection, every 4 days, for 20 days) [26], WYE-354 plus 3-MA co-administration, or the vehicle alone [17]. Tumor volumes were measured once every 10 days according to the formula: tumor volume (mm^3) = $L \times W^2 / 2$ (where L is the length and W is the width). Three days after initial drug administration, HT-29 xenograft tumors ($n = 2$ per group) were isolated via surgery. Half of the xenograft samples were homogenized and were subjected to Western blot assay to test signaling markers. Meanwhile, the cryostat sections (3 μm) of another half of HT-29 xenografts were also prepared for the immunohistochemistry (IHC) assay. The slides were incubated with anti-p-Akt-Ser473 (1:50, Cellular Signaling Tech)/anti-p-S6(1:200, Cellular Signaling Tech) and were then stained with HRP-coupled secondary antibody (Santa Cruz). The peroxidase activity was then visualized through 3-amino-9-ethyl-carbazol (AEC) and counterstained tissues with MAYER'S hemalaun solution (Merck). The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). When analyzing mice survival, humane endpoints were always applied to minimize mice suffering. All injections were performed under the 2,2,2-tribromoethanol anesthesia method.

Statistical analysis

Data were expressed as mean ± SD. All statistical analyses were performed using GraphPad software. Analysis of variance (ANOVA) was utilized to assess the statistical significance of the differences. Differences were considered statistically significant at $p < 0.05$.

Results

WYE-354 is cytotoxic and anti-proliferative when adding to cultured human colon cancer cells

First, we studied the potential effect of WYE-354 on colon cancer cell survival. MTT assay was performed. As shown in Fig. 1a, WYE-354 decreased MTT OD of two well-established human colon cancer cell lines: HCT-116 and HT-29. WYE-354 demonstrated a dose-dependent response in inhibiting the cancer cells (Fig. 1a). In addition, the anti-survival activity by WYE-354 was time-dependent (Fig. 1b). It took 48–72 h for WYE-354 (100 nM) to exert a significant anti-survival effect (Fig. 1b). Interestingly, same WYE-354 treatment was non-cytotoxic to cultured NCM460 cells, the latter is a non-cancerous human colon mucosal epithelial cell line [18, 27] (Fig. 1a). Clonogenicity assay results in Fig. 1c showed that WYE-354 (50/100 nM) treatment significantly decreased the number of HCT-116/HT-29 colonies. Thus, the mTOR inhibitor is anti-proliferative to these colon cancer cells.

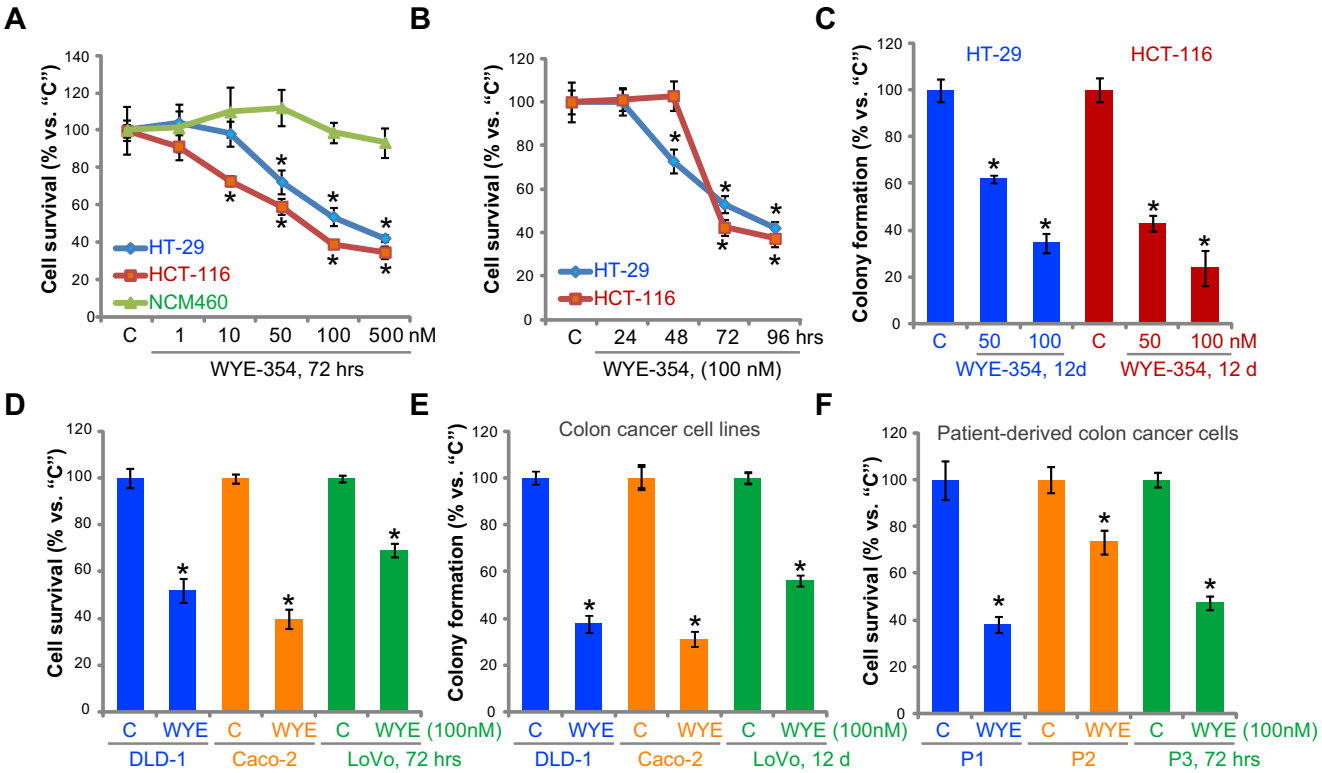


Fig. 1 WYE-354 is cytotoxic and anti-proliferative when adding to cultured human colon cancer cells. Established human colon cancer cell lines (HCT-116, HT-29, Caco-2, LoVo, and DLD-1), three lines of primary human colon cancer cells (P1, P2, and P3), or non-cancerous NCM460 colon epithelial cells were treated with applied concentration of WYE-354 ("WYE") for indicated time period, cell survival was tested by MTT assay (**a**, **b**, **d**, **f**), and cell proliferation was evaluated by clonogenicity assay (**c**, **e**). The data in this and all following figures were representatives of three different experiments. $n = 5$ for each assay. The values were expressed as the means ± SD (same for all figures). "C" stands for untreated control group (same for all figures). *$p < 0.05$ vs. "C" group

The potential effect of WYE-354 to other established colon cancer cells was also tested. MTT results in Fig. 1d showed that WYE-354 ("WYE", 100 nM) was anti-survival/cytotoxic to three other human colon cancer cell lines: DLD-1, Caco-2, and LoVo. Through the colony formation assay, we showed that the proliferation of above cells was also inhibited by WYE-354 ("WYE", 100 nM) (Fig. 1e). To study the role of WYE-354 on primary cells, three primary colon cancer cell lines (derived from affected patients) were established. These cells were also treated with WYE-354. Results showed that WYE-354 ("WYE", 100 nM) inhibited survival of all three lines of primary cancer cells (Fig. 1f). Together, these results demonstrate that WYE-354 inhibits survival and proliferation of human colon cancer cells.

WYE-354 induces apoptosis activation in human colon cancer cells

Above results demonstrated that WYE-354 inhibited survival and proliferation of human colon cancer cells. Next, we studied the possible involvement of apoptosis activation in the process. Various apoptosis assays were performed, including Histone-DNA ELISA assay, TUNEL staining assay, and caspase-3 activity assay. Results of these assays demonstrated that WYE-354 induced apoptosis activation in HCT-116 cells and HT-29 cells (Fig. 2a–c). The effect of WYE-354 on cancer cell apoptosis was also concentration-dependent (Fig. 2a–c). The apoptosis ELISA OD (Fig. 2a), the TUNEL percentage (Fig. 2b), and the caspase-3 activity (Fig. 2c) were all increased following 50–500 nM of WYE-354 treatment. Significantly, same WYE-354 treatment failed to induce significant apoptosis activation in the non-cancerous NCM460 epithelial cells (Fig. 2a–c). The results again pointed out a selective activity of WYE-354 only to cancerous cells.

To study the role of apoptosis in WYE-354-mediated anti-colon cancer activity in vitro, the caspase inhibitors were applied. As demonstrated, co-treatment with the caspase-3-specific inhibitor z-DEVD-cho ("DEVD") or the pan caspase inhibitor z-VAD-fmk ("VAD") attenuated WYE-354-induced cytotoxic and anti-proliferative activities against HT-29 cells (Fig. 2d, e). Similar results were also achieved in HCT-116 cells (data not shown). The results of the Histone-DNA ELISA assay showed that WYE-354 ("WYE", 100 nM) was also pro-apoptotic in the three lines of primary (patient-

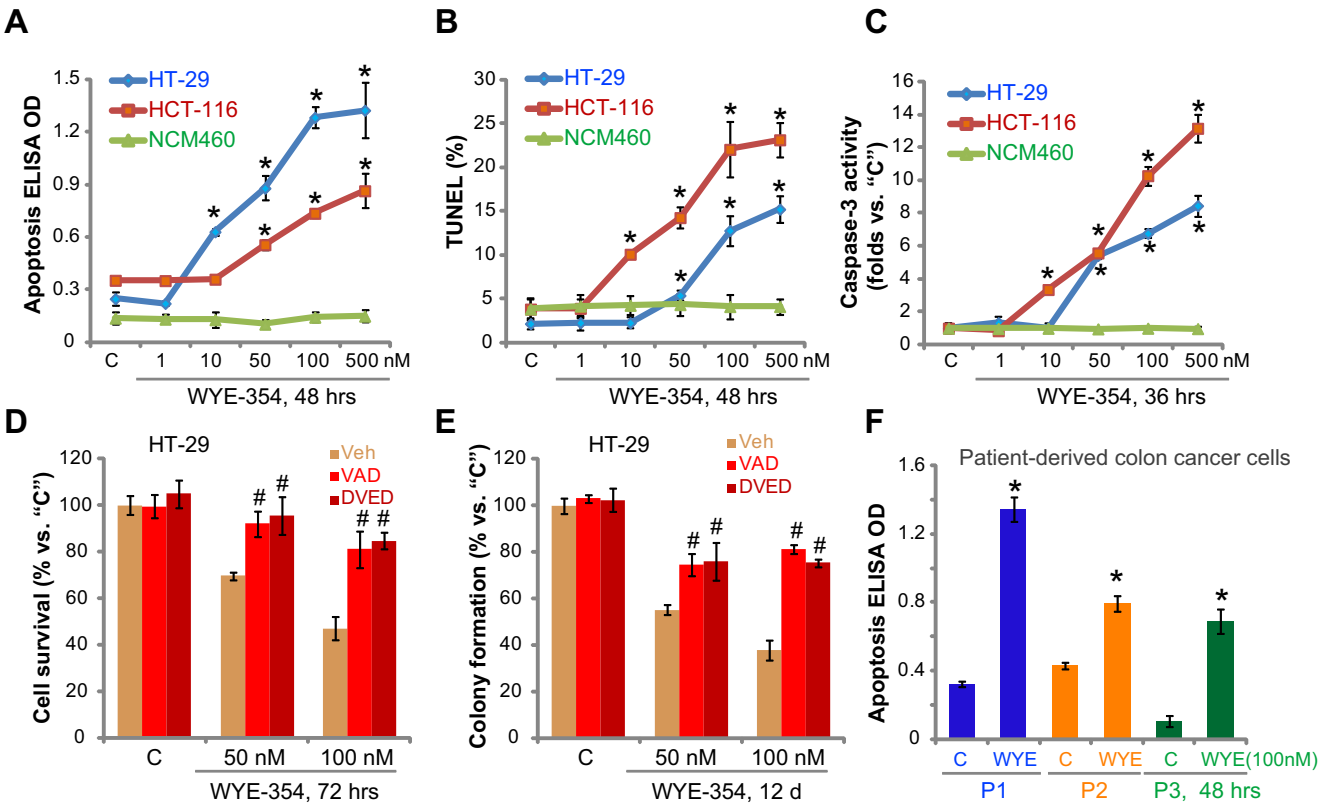


Fig. 2 WYE-354 induces apoptosis activation in human colon cancer cells. Colon cancer cell lines (HCT-116 and HT-29), three lines of primary human colon cancer cells (P1, P2, and P3), or non-cancerous NCM460 cells were treated with applied concentrations of WYE-354 for indicated time, cell apoptosis was tested by listed assays (**a**–**c**, **f**). HT-29 cells were pre-incubated with the caspase-3-specific inhibitor z-DEVD-cho ("DEVD", 50 μM) or the pan caspase inhibitor z-VAD-fmk ("VAD", 50 μM) for 1 h, followed by WYE-354 (50 and 100 nM) treatment for applied time, cell survival was tested by MTT assay (**d**), and cell proliferation was evaluated by clonogenicity assay (**e**). "Veh" stands for 0.1 % of DMSO (**d**, **e**). $^{*}p < 0.05$ vs. "C" group (**a**–**c**, **f**). $^{\#}p < 0.05$ vs. "Veh" group (**d**, **e**)

derived) colon cancer cells (Fig. 2f). Together, we indicate that WYE-354 induces caspase-dependent apoptotic death in colon cancer cells.

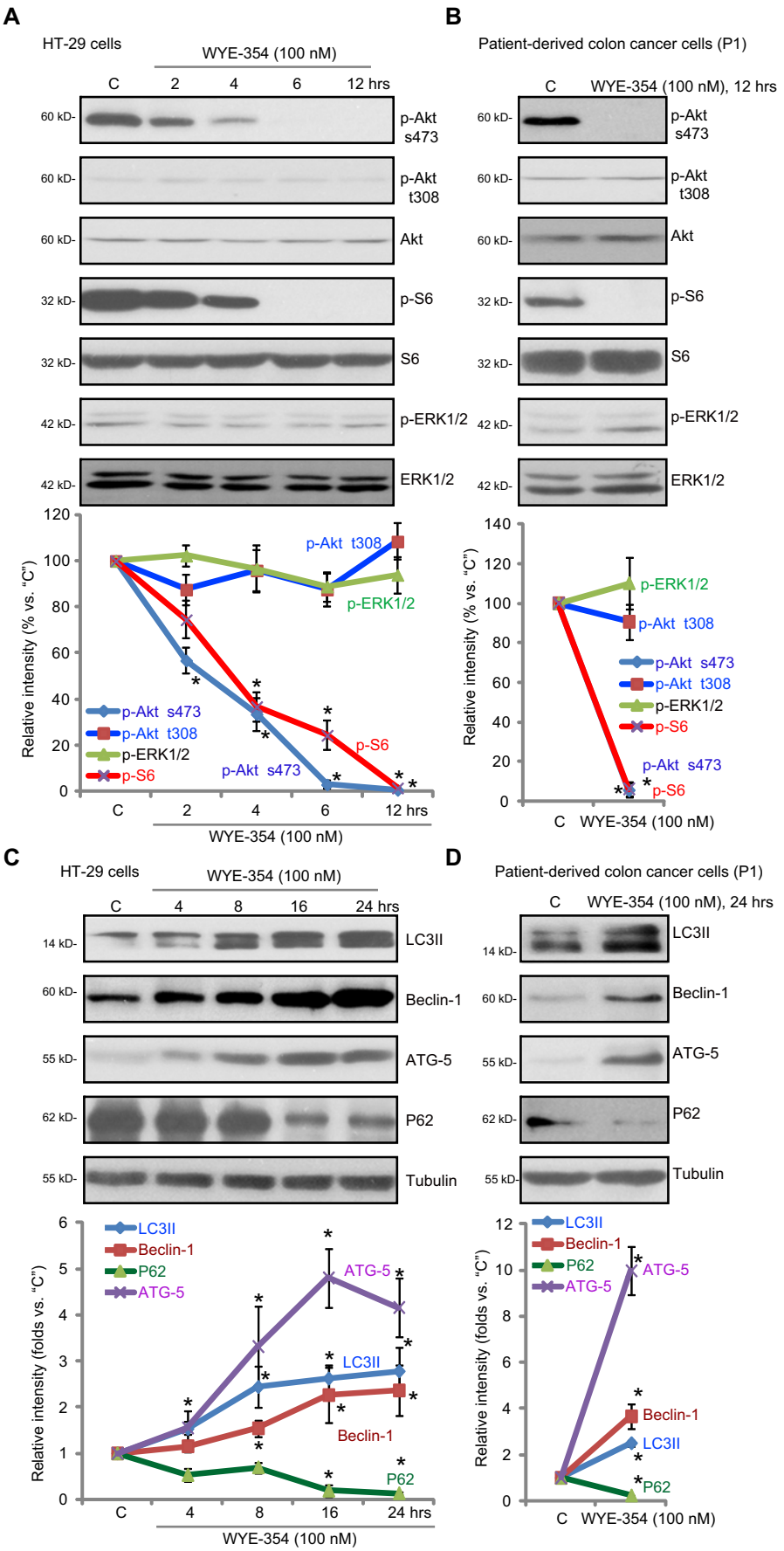
mTORC1 and mTORC2 inhibition and autophagy activation in WYE-354-treated colon cancer cells

WYE-354 is a newly developed mTOR kinase inhibitor [17]. We thus tested mTOR signaling in colon cancer cells with WYE-354 treatment. In HT-29 cells, WYE-354 (100 nM) time-dependently inhibited phosphorylations of Akt (Ser-473) and S6 (Ser-235/236), indicating that both mTORC1 and mTORC2 were inactivated [28]. On the other hand, p-Akt at Thr-308 and p-Erk1/2 were not affected by same WYE-354 treatment (Fig. 3a, lower panel, $n = 5$). Similar results were also observed in HCT-116 cells (data not shown). In primary colon cancer cells ("Patient 1" or "P1"), WYE-354 similarly blocked mTORC1 (p-S6) and mTORC2 (p-Akt Ser-473), yet leaving p-Akt Thr-308 and p-Erk1/2 unaffected (Fig. 3b). Same results were obtained in two other primary lines ("P2/P3", data not shown). These results demonstrate that WYE-354 simultaneously blocks mTORC1 and mTORC2 activation in colon cancer cells.

One important consequence of mTOR inhibition is autophagy activation, which often exerts cytoprotective and anti-apoptotic roles [29–32]. As shown in Fig. 3c, WYE-354 (100 nM) induced LC3B-II, Beclin-1, and ATG-5 expression, yet downregulated p62 in HT-29 cells. These results indicated autophagy activation [32]. Autophagy activation by WYE-354 was also time-dependent, and it was slightly after mTORC1/2 blockage (Fig. 3a, c, lower panels). We observed similar results in HCT-116 cells (data not shown) and in primary colon cancer cells (Fig. 3d, "P1"). These results confirmed autophagy activation in WYE-354-treated colon cancer cells.

Inhibition of autophagy could sensitize WYE-354-induced anti-colon cancer cell activity in vitro

Next, we studied the potential role of autophagy in WYE-354-mediated anti-colon cancer cell activity. Two well-established autophagy inhibitors, bafilomycin A1 (Baf A1) and 3-methyladenine (3-MA), were applied in the study. MTT assay


A
HT-29 cells
WYE-354 (100 nM)
C 2 4 6 12 hrs
60 kD- p-Akt s473
60 kD- p-Akt t308
60 kD- Akt
32 kD- p-S6
32 kD- S6
42 kD- p-ERK1/2
42 kD- ERK1/2
Relative intensity (% vs. "C")
p-Akt t308
p-ERK1/2
p-Akt s473
p-Akt t308
p-ERK1/2
p-S6
p-S6
p-Akt s473
C 2 4 6 12 hrs
WYE-354 (100 nM)
B
Patient-derived colon cancer cells (P1)
C WYE-354 (100 nM), 12 hrs
60 kD- p-Akt s473
60 kD- p-Akt t308
60 kD- Akt
32 kD- p-S6
32 kD- S6
42 kD- p-ERK1/2
42 kD- ERK1/2
Relative intensity (% vs. "C")
p-ERK1/2
p-Akt t308
p-Akt s473
p-Akt t308
p-ERK1/2
p-S6
p-Akt s473
p-S6
C WYE-354 (100 nM)
C
HT-29 cells
WYE-354 (100 nM)
C 4 8 16 24 hrs
14 kD- LC3II
60 kD- Beclin-1
55 kD- ATG-5
62 kD- P62
55 kD- Tubulin
Relative intensity (folds vs. "C")
LC3II
Beclin-1
P62
ATG-5
ATG-5
LC3II
Beclin-1
P62
C 4 8 16 24 hrs
WYE-354 (100 nM)
D
Patient-derived colon cancer cells (P1)
C WYE-354 (100 nM), 24 hrs
14 kD- LC3II
60 kD- Beclin-1
55 kD- ATG-5
62 kD- P62
55 kD- Tubulin
Relative intensity (folds vs. "C")
LC3II
Beclin-1
P62
ATG-5
ATG-5
Beclin-1
LC3II
P62
C WYE-354 (100 nM)

◀ **Fig. 3** mTORC1 and mTORC2 inhibition and autophagy activation in WYE-354-treated colon cancer cells. HT-29 cells and primary human colon cancer cells (patient 1, P1) were treated with WYE-354 (100 nM) for indicated time period, expressions of listed proteins were tested Western blots (**a**–**d**, *upper panels*); indicated proteins were quantified, and their relative intensity (vs. "C") was shown (**a**–**d**, *lower panels*, $n = 5$). *$p < 0.05$ vs. "C" group

results showed that co-treatment with 3-MA or Baf-A1 significantly potentiated WYE-354-induced anti-survival activity against HT-29 cells (Fig. 4a). In addition, the two inhibitors enhanced WYE-354-induced HT-29 cell apoptosis. The latter was tested by Histone-DNA apoptosis ELISA assay (Fig. 4b). Notably, treatment of HT-29 cells with 3-MA or Baf-A1 alone also induced minor but significant anti-survival and pro-apoptotic effect (Fig. 4a, b). The results indicated that basal autophagy activation is also important for HT-29 cell survival. Similar WYE-354-sensitization activity by 3-MA/Baf-A1 was also observed in HCT-116 cells (data not shown).

Both 3-MA and Baf-A1 are well-known autophagy inhibitors, the two may also exert off-targeted effects [30, 33, 34]. To exclude these possibilities, shRNA method was applied. Stable HT-29 cells expressing Beclin-1-shRNA or ATG-5-shRNA were established (see "Materials and methods"). Western blot results in Fig. 4c confirmed efficient Beclin-1/ATG-5 knockdown by the targeted-shRNA. Significantly, as shown in Fig. 4d, e, WYE-354-induced viability reduction and apoptosis were both dramatically augmented in Beclin-1/ATG-5-shRNA expressing cells. As compared to cells expressing scramble control shRNA (Src shRNA), HT-29 cells with Beclin-1/ATG-5-shRNA showed decreased survival and increased apoptosis (Fig. 4d, e). Above experiments were also repeated in HCT-116 cells, and similar results were obtained (data not shown). In primary human colon cancer cells, 3-MA and Baf-A1 similarly enhanced WYE-354-induced anti-survival activity (Fig. 4f). These results suggest that mTORC1/2 blockage by WYE-354 may induce feedback activation of autophagy, which exerts a cytoprotective role to inhibit WYE-354's cytotoxicity. Pharmacological or genetic inhibition of autophagy therefore sensitizes WYE-354's activity against colon cancer cells.

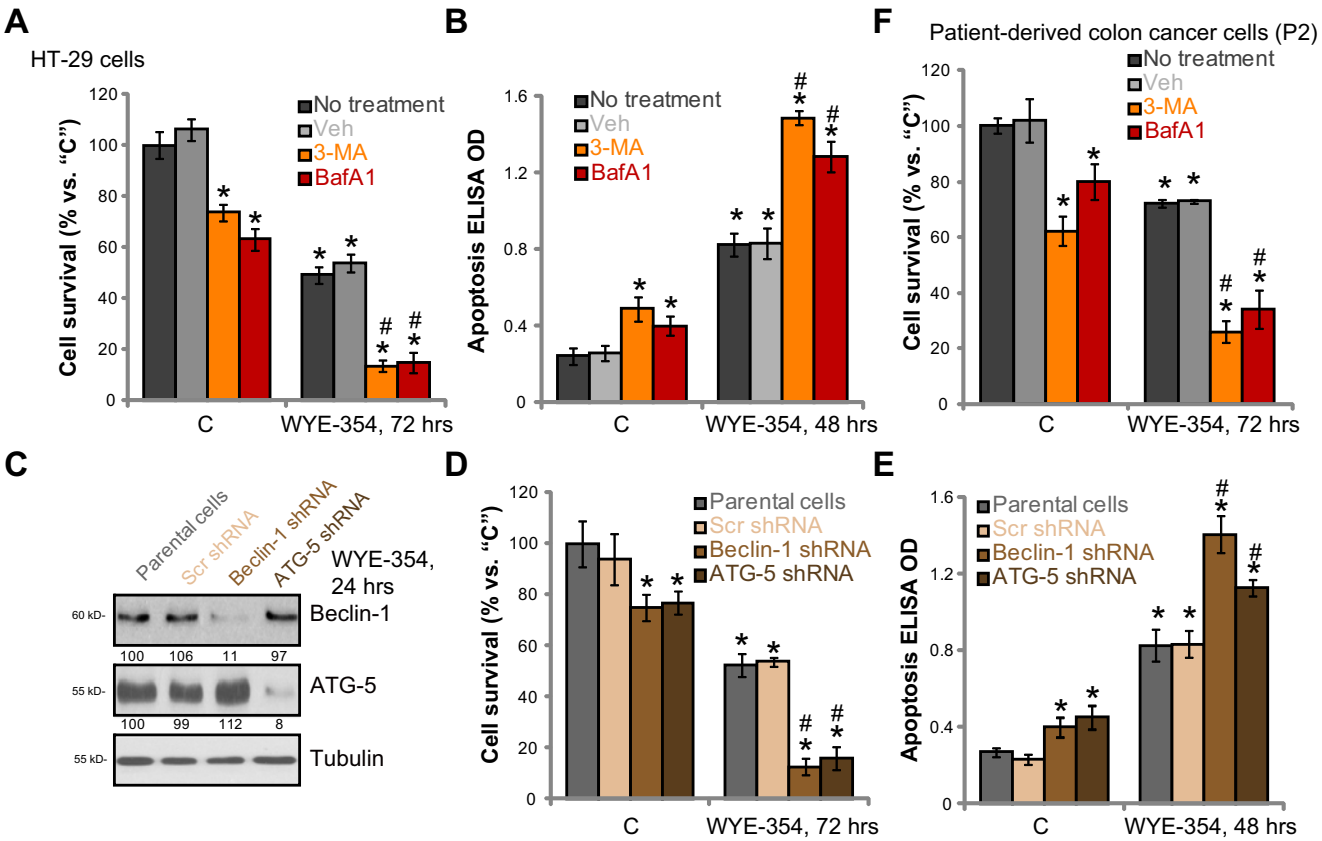


Fig. 4 Inhibition of autophagy could sensitize WYE-354-induced anti-colon cancer cell activity in vitro. HT-29 cells or and primary human colon cancer cells (patient 2, P2) were treated with WYE-354 (100 nM), in the presence or absence of 3-methyladenine (3-MA, 10 mM) or bafilomycin A1 (Baf A1, 1 μM) for applied time period; cell survival and apoptosis were tested by MTT assay (**a** and **f**) and Histone-DNA ELISA assay (**b**, for HT-29 cells), respectively. Stable HT-29 cells expressing scramble control shRNA (Src shRNA), Beclin-1 shRNA, or ATG-5 shRNA, as well as their parental cells were treated with WYE-354 (100 nM) for indicated time period, expression of targeted proteins was tested by Western blots (**c**, relative Beclin-1 and ATG-5 expression was quantified), cell survival (**d**) and apoptosis (**e**) were also tested. *$p < 0.05$ vs. "C" group. #$p < 0.05$ vs. "WYE-354" only group

The in vivo anti-colon cancer cell activity by WYE-354 alone or in combination with 3-MA

The in vivo anti-cancer cell activity by WYE-354 was also examined. As described, HT-29 cells were inoculated into the left flanks of the SCID mice. After 20 days of growth, the xenografted tumors were established. Results in Fig. 5a demonstrated that WYE-354 administration (10 mg/kg, i.p. injection) inhibited HT-29 tumor growth in SCID mice. However, the in vivo anti-cancer activity by WYE-354 as a single agent was moderate (Fig. 5a). In line with the in vitro findings, co-administration with the autophagy inhibitor 3-MA (20 mg/kg) significantly potentiated WYE-354-induced anti-HT29 xenograft activity in vivo. 3-MA alone also showed minor but significant effect in inhibiting HT-29 tumors (Fig. 5a). When analyzing mice survival, we showed that mice administrated with WYE-354 and 3-MA combo were all alive (10 out of 10, 50 days after initial drug administration). On the other hand, the majority of mice (7 out of 10) were dead in vehicle control group (Fig. 5b). Mice-treated with WYE-354 or 3-MA alone survived longer (Fig. 5b). The results in Fig. 5c showed that the single or combined treatment had no significant effect on mice body weights; thus, the tested regimens in the study were generally safe to the mice.

Western blot and IHC staining assays were also performed to test the potential effect of WYE-354 on the signaling changes in vivo. As demonstrated, the levels of p-Akt (Ser-473) and p-S6 were both dramatically decreased in xenografted tumors with WYE-354 administration (Fig. 5d, e). 3-MA showed no significant effect on Akt/S6 phosphorylations in the xenografts (Fig. 5d, e). Therefore, in line with the in vitro findings, these results suggested that WYE-354 suppressed mTORC1 (p-S6) and mTORC2 (p-Akt-Ser-473) activation in vivo. Together, these results show that autophagy inhibition could possibly sensitize WYE-354-induced anti-colon cancer cell activity in vivo.

Discussions and conclusions

The two mTOR complexes, mTORC1 and mTORC2, are formed and regulated by different although some overlapping

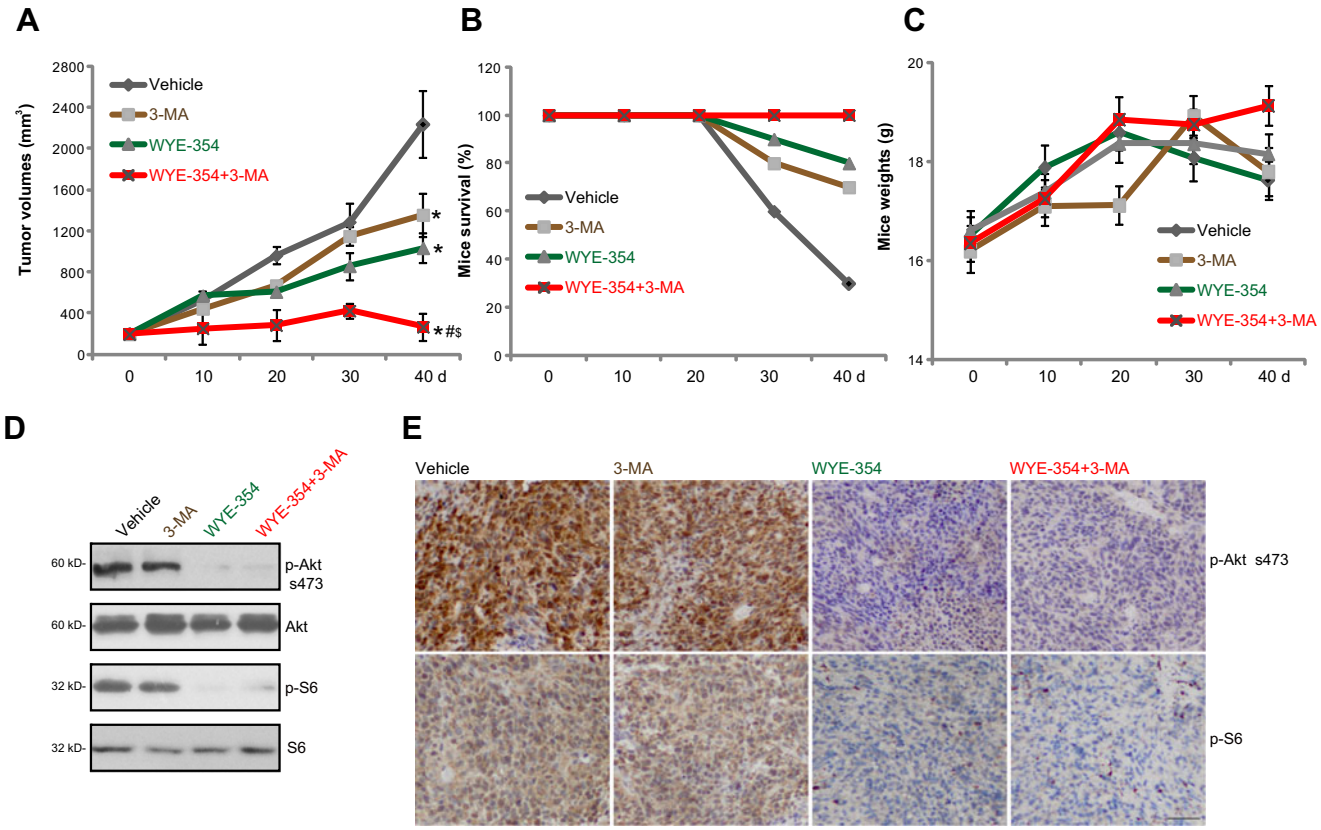


Fig. 5 The in vivo anti-colon cancer cell activity by WYE-354 alone or in combination with 3-MA. HT-29 tumor bearing SCID mice (10 mice per group) were treated vehicle control (5 % ethanol, 5 % polysorbate 80, 5 % polyethylene glycol-400, i.p. injection, once daily, for 15 days), WYE-354 (10 mg/kg, i.p. injection, once daily, for 15 days), and/or 3-MA (20 mg/kg, i.p. injection, once every 4 days, for 20 days). Tumor volumes, mice survival, and mice body weights were recorded every 10 days for a total of 40 days. Three days after initial drug administration, HT-29 xenograft tumors ($n = 2$ per group) were isolated. Expression of listed proteins was tested by Western blot assay (**d**) or IHC staining assay (**e**). $^{*}p < 0.05$ vs. vehicle group. $^{\#}p < 0.05$ vs. WYE-354 only group. $^{\$}p < 0.05$ vs. 3-MA only group. *Bar* = 100 μm (**e**)

proteins. The two are driven by multiple compensatory feedback loops [35]. Rapamycin and its analogs (i.e., RAD001, rapalogs) are the first generation of mTOR inhibitors, which only partially inhibit mTORC1 [36]. Due to the lack of activity on mTORC2, the anti-tumor activity by these rapalogs is relatively weak [35]. Meanwhile, rapalogs-induced mTORC1 inhibition could result in feedback activation of several key pro-survival/anti-apoptotic pathways, including PI3K-Akt and ERK-MAPK signalings. These drawbacks will further limit their clinical applications [35, 37].

Therefore, in recent years, groups have developed mTOR kinase inhibitors, [36]. These second generation of mTOR inhibitors target mTOR's ATP kinase domain, thus blocking mTORC1 and mTORC2 simultaneously [36]. These inhibitors have displayed promising anti-cancer activity in multiple pre-clinical caner models [36]. In the present study, we showed that WYE-354 significantly inhibited colon cancer cell growth both in vivo and in vitro. At the molecular level, WYE-354 simultaneously blocked mTORC1 and mTORC2 activation, without provoking Akt or ERK signaling in tested colon cancer cells. Phosphorylations of Akt (Ser-473) and S6 were also significant decreased in WYE-354-treated HT-29 xenografts. Thus, the concurrent blockage of mTORC2 together with mTORC1 appeared more efficient in inhibiting colon cancer cells.

Existing evidences have shown that mTOR inhibition could lead to unwanted cytoprotective autophagy activation, which attenuates the cytotoxic and pro-apoptotic activity by mTOR inhibition [38–40]. Reversely, concurrent autophagy inhibition via genetic or pharmacologic methods could significantly sensitize the anti-cancer activity by the mTOR inhibitors [38–40]. Both mTORC1 and mTORC2 are important for sequester autophagy. mTORC1 regulates autophagy through phosphorylating unc-51-like kinase 1 (ULK1) [29] and autophagy-related protein 13 (ATG-13) [41]. On the other hand, mTORC2 activation is shown to block FoxO3a nuclear translocation and autophagy induction [42]. In the current study, we showed that autophagy was activated in WYE-354-treated colon cancer cells where mTORC1 and mTORC2 were blocked. Inhibition of autophagy, through autophagy inhibitors (Baf A1 and 3-MA) or shRNA-mediated downregulation of Beclin-1/ATG-5, remarkably augmented WYE-354-induced anti-colon cancer cell activity in vitro. In vivo, WYE-354 administration inhibited HT-29 xenograft growth in SCID mice. Its activity was sensitized when combing with the autophagy inhibitor 3-MA. These results suggest that autophagy inhibition could be a valuable strategy to sensitize WYE-354-induced anti-colon cancer activity.

The colon cancer/colorectal cancer incidence remains high in China and around the world [1, 2, 5, 43]. Several antibodies and molecules have been developed for treating this disease recently, yet surgical removal remains the only curative treatment option [1, 2, 5, 43]. The results of this study indicate that WYE-354, alone or in combination of autophagy inhibitors, could be further investigated as valuable anti-colon cancer strategy.

Compliance with ethical standards

Conflicts of interest None

Author contributions Lijun Wang and Yun-Rong Zhu are equal contributors.

References

1. Hubbard JM, Grothey A. Colorectal cancer in 2014: progress in defining first-line and maintenance therapies. Nat Rev Clin Oncol. 2015;12:73–4.
2. Schmoll HJ, Stein A. Colorectal cancer in 2013: towards improved drugs, combinations and patient selection. Nat Rev Clin Oncol. 2014;11:79–80.
3. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA Cancer J Clin. 2014;64:9–29.
4. Palta M, Czito BG, Willett CG. Colorectal cancer: adjuvant chemotherapy for rectal cancer-an unresolved issue. Nat Rev Clin Oncol. 2014;11:182–4.
5. McCarthy N. Colorectal cancer: editing an invasion. Nat Rev Cancer. 2014;14:297.
6. Kerr D. Clinical development of gene therapy for colorectal cancer. Nat Rev Cancer. 2003;3:615–22.
7. Kuipers EJ, Rosch T, Bretthauer M. Colorectal cancer screening—optimizing current strategies and new directions. Nat Rev Clin Oncol. 2013;10:130–42.
8. Ekstrand AI, Jonsson M, Lindblom A, Borg A, Nilbert M. Frequent alterations of the pi3k/akt/mtor pathways in hereditary nonpolyposis colorectal cancer. Familial Cancer. 2010;9:125–9.
9. Zhang YJ, Dai Q, Sun DF, Xiong H, Tian XQ, Gao FH, et al. Mtor signaling pathway is a target for the treatment of colorectal cancer. Ann Surg Oncol. 2009;16:2617–28.
10. Francipane MG, Lagasse E. Mtor pathway in colorectal cancer: an update. Oncotarget. 2014;5:49–66.
11. Pandurangan AK. Potential targets for prevention of colorectal cancer: a focus on pi3k/akt/mtor and wnt pathways. Asian Pac J Cancer Prev. 2013;14:2201–5.
12. Metzger-Filho O, Moulin C, Awada A. Molecular targeted therapy in prevalent tumors: learning from the past and future perspectives. Curr Clin Pharmacol. 2010;5:166–77.
13. Shimobayashi M, Hall MN. Making new contacts: the mtor network in metabolism and signalling crosstalk. Nat Rev Mol Cell Biol. 2014;15:155–62.
14. Fruman DA, Rommel C. Pi3k and cancer: lessons, challenges and opportunities. Nat Rev Drug Discov. 2014;13:140–56.
15. Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, et al. Ablation in mice of the mtorc components raptor, rictor, or mlst8 reveals that mtorc2 is required for signaling to akt-foxo and pkcalpha, but not s6k1. Dev Cell. 2006;11:859–71.
16. Dancey J. Mtor signaling and drug development in cancer. Nat Rev Clin Oncol. 2010;7:209–19.
17. Yu K, Toral-Barza L, Shi C, Zhang WG, Lucas J, Shor B, et al. Biochemical, cellular, and in vivo activity of novel atp-competitive and selective inhibitors of the mammalian target of rapamycin. Cancer Res. 2009;69:6232–40.

18. Alcarraz-Vizan G, Sanchez-Tena S, Moyer MP, Cascante M. Validation of ncm460 cell model as control in antitumor strategies targeting colon adenocarcinoma metabolic reprogramming: Trichostatin a as a case study. Biochim Biophys Acta. 1840;2014: 1634–9.
19. Zhu YR, Xu Y, Fang JF, Zhou F, Deng XW, Zhang YQ. Bufotalin-induced apoptosis in osteoblastoma cells is associated with endoplasmic reticulum stress activation. Biochem Biophys Res Commun. 2014;451:112–8.
20. Chen MB, Shen WX, Yang Y, Wu XY, Gu JH, Lu PH. Activation of amp-activated protein kinase is involved in vincristine-induced cell apoptosis in b16 melanoma cell. J Cell Physiol. 2010;226:1915–25.
21. Chen MB, Wei MX, Han JY, Wu XY, Li C, Wang J, et al. Microrna-451 regulates ampk/mtorc1 signaling and fascin1 expression in ht-29 colorectal cancer. Cell Signal. 2014;26:102–9.
22. Chen MB, Zhang Y, Wei MX, Shen W, Wu XY, Yao C, et al. Activation of amp-activated protein kinase (ampk) mediates plumbagin-induced apoptosis and growth inhibition in cultured human colon cancer cells. Cell Signal. 2013;25:1993–2002.
23. Li C, Cui JF, Chen MB, Liu CY, Liu F, Zhang QD, et al. The preclinical evaluation of the dual mtorc1/2 inhibitor ink-128 as a potential anti-colorectal cancer agent. Cancer Biol Ther. 2015;16: 34–42.
24. Chen MB, Jiang Q, Liu YY, Zhang Y, He BS, Wei MX, et al. C6 ceramide dramatically increases vincristine sensitivity both in vivo and in vitro, involving amp-activated protein kinase-p53 signaling. Carcinogenesis. 2015;36:1061–70.
25. Ma J, Becker C, Lowell CA, Underhill DM. Dectin-1-triggered recruitment of light chain 3 protein to phagosomes facilitates major histocompatibility complex class ii presentation of fungal-derived antigens. J Biol Chem. 2012;287:34149–56.
26. Li J, Hou N, Faried A, Tsutsumi S, Kuwano H. Inhibition of autophagy augments 5-fluorouracil chemotherapy in human colon cancer in vitro and in vivo model. Eur J Cancer. 2010;46:1900–9.
27. Moyer MP, Manzano LA, Merriman RL, Stauffer JS, Tanzer LR. Ncm460, a normal human colon mucosal epithelial cell line. In Vitro Cell Dev Biol Anim. 1996;32:315–7.
28. Sabatini DM. Mtor and cancer: insights into a complex relationship. Nat Rev Cancer. 2006;6:729–34.
29. Kim J, Kundu M, Viollet B, Guan KL. Ampk and mtor regulate autophagy through direct phosphorylation of ulk1. Nat Cell Biol. 2011;13:132–41.
30. Marino G, Niso-Santano M, Baehrecke EH, Kroemer G. Self-consumption: the interplay of autophagy and apoptosis. Nat Rev Mol Cell Biol. 2014;15:81–94.
31. Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. Nat Rev Cancer. 2013;13:714–26.
32. Rubinsztein DC, Codogno P, Levine B. Autophagy modulation as a potential therapeutic target for diverse diseases. Nat Rev Drug Discov. 2012;11:709–30.
33. Seglen PO, Gordon PB. 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. Proc Natl Acad Sci U S A. 1982;79:1889–92.
34. Kim KH, Lee MS. Autophagy—a key player in cellular and body metabolism. Nat Rev Endocrinol. 2014.
35. Vilar E, Perez-Garcia J, Tabernero J. Pushing the envelope in the mtor pathway: the second generation of inhibitors. Mol Cancer Ther. 2011;10:395–403.
36. Zhou HY, Huang SL. Current development of the second generation of mtor inhibitors as anticancer agents. Chin J Cancer. 2012;31: 8–18.
37. Chen XG, Liu F, Song XF, Wang ZH, Dong ZQ, Hu ZQ, et al. Rapamycin regulates akt and erk phosphorylation through mtorc1 and mtorc2 signaling pathways. Mol Carcinog. 2010;49:603–10.
38. Zheng B, Mao JH, Qian L, Zhu H, Gu DH, Pan XD, et al. Preclinical evaluation of azd-2014, a novel mtorc1/2 dual inhibitor, against renal cell carcinoma. Cancer Lett. 2015;357:468–75.
39. Chen B, Xu M, Zhang H, Xu MZ, Wang XJ, Tang QH, Tang JY. The antipancreatic cancer activity of osi-027, a potent and selective inhibitor of mtorc1 and mtorc2. DNA Cell Biol. 2015.
40. Li Q, Song XM, Ji YY, Jiang H, Xu LG. The dual mtorc1 and mtorc2 inhibitor azd8055 inhibits head and neck squamous cell carcinoma cell growth in vivo and in vitro. Biochem Biophys Res Commun. 2013;440:701–6.
41. Jung CH, Jun CB, Ro SH, Kim YM, Otto NM, Cao J, et al. Ulk-atg13-fip200 complexes mediate mtor signaling to the autophagy machinery. Mol Biol Cell. 2009;20:1992–2003.
42. Mammucari C, Milan G, Romanello V, Masiero E, Rudolf R, Del Piccolo P, et al. Foxo3 controls autophagy in skeletal muscle in vivo. Cell Metab. 2007;6:458–71.
43. Leake I. Colorectal cancer: understanding the routes of metastasis in colorectal cancer. Nat Rev Gastroenterol Hepatol. 2014;11:270.