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Original Paper

A Novel Small-Molecule YLT205 Induces Apoptosis in Human Colorectal Cells *via* Mitochondrial Apoptosis Pathway *In Vitro* and Inhibits Tumor Growth *In Vivo*

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Key Words
YLT205 • Human colorectal cancer • Apoptosis • Mitochondrial pathway

Abstract
Background: Colorectal cancer continues to be one of the most common causes of cancer death, and the poor survival rates and liver metastases at the time of diagnosis urgently need more effective strategy for colorectal cancer treatment. ***Methods:*** The activities of N-(5-bromopyridin-2-yl)-2-((6-(2-chloroacetamido)benzo[d]thiazol-2-yl)thio)acetamide (YLT205), which is a novel small molecule compound synthesized by us, were investigated using MTT assay, flow cytometry, western blotting and mice tumor xenograft models. ***Results:*** YLT205 induced apoptosis of human colorectal cell lines in a dose-dependent manner. The occurrence of apoptosis was associated with activation of caspases-9 and -3, down-regulation of Bcl-2 and up-regulation of Bax in HCT116 cells. Moreover, YLT205 disrupted mitochondrial membranes and induced the release of cytochrome c into cytosol. Impaired phosphorylation of p44/42 mitogen-activated protein kinase was also observed while the expression of phosphorylated protein kinase B (Akt) was not affected. Furthermore, in HCT116 and SW620 tumor-bearing nude mice models, YLT205 dose-dependently inhibited tumor growth without obvious adverse effects. Immunohistochemistry analyses revealed YLT205 also induced apoptosis and inhibited tumor cell proliferation *in vivo*. ***Conclusion:*** These studies suggested that YLT205 might be a potential drug candidate for human colorectal cancer therapy.

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Introduction

Colorectal cancer (CRC) continues to be one of the most common causes of cancer death both in men and women, being responsible for about 10% of total cancer-related mortality [1]. According to the report, there were over 102,480 new diagnoses and 50,830 deaths only in the United States in 2013 [2]. Chemotherapy, radiotherapy and surgery are the mainly strategies for colorectal cancer treatment at present, but not yet satisfactory for poor survival rates and liver metastases at the time of diagnosis [3]. Therefore, more effective strategy for CRC treatment is still an urgent need. Recently, the treatment of CRC is mainly focused on the deregulation of cell proliferation and apoptosis [4, 5].

Apoptosis (programmed cell death) plays an important role in the balance between cell proliferation and cell death, and the deregulation of the apoptosis can lead to diseases, such as cancer [6]. Targeting apoptosis is a promising strategy in anticancer drug discovery [4, 7], for cancer cells are highly dependent on aberration of the apoptosis signaling pathways to stay alive, and cancers are often resistant to chemotherapeutic agents that primarily work by inducing apoptosis [8, 9]. Apoptosis signaling pathways can be divided into two pathways: cell death receptor-mediated extrinsic pathway and mitochondrial-mediated intrinsic pathway [10]. The intrinsic apoptosis pathway mainly act through mitochondria, and the important events include the loss of mitochondrial transmembrane potential, the release of cytochrome c, and the participation of pro- and anti-apoptotic Bcl-2 family proteins [11, 12]. Intracellular death signals, such as DNA damage, toxins or depletion of ATP, can induce the release of cytochrome c from the inner membrane of mitochondria, which can subsequently interact with the Apaf-1 and ATP, which binds to pro-caspase-9, resulting in the cleavage of pro-caspase-9 and activation the effector caspase-3 and/or caspase-7 [13]. In the extrinsic pathway, replace with the death receptors by the ligands (e.g. TNFα and TNFR, FasL and Fas binding) leads to caspase-8 activation, and then cleavage and activation of downstream effector caspases, such as caspase-3 and caspase-7, resulting in chromatin condensation, DNA laddering and formation of apoptosis [14].

Our research group found a novel benzothiazole derivative, N-(5-bromopyridin-2-yl)-2-((6-(2-chloroacetamido)benzo[d]thiazol-2-yl)thio)acetamide (YLT205), which displayed anti-proliferative activity and induced apoptosis *in vitro* [15]. In this investigation, we demonstrate that YLT205 can induce apoptosis in colon cancer cells *via* the mitochondrial apoptotic pathway. By using xenograft models in athymic mouse, we determined that YLT205 also inhibited tumor growth *in vivo* by inducing apoptosis without causing toxic effects.

Materials and Methods

Drugs and regents

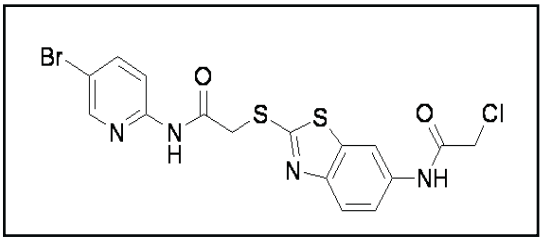
YLT205 was synthesized by our group (State Key Laboratory of Biotherapy, Sichuan University, Sichuan, China) [15], and its structural formula was shown in Fig. 1. The structure of YLT205 was determined by ^{1}H-NMR, ^{13}C-NMR and ESI-MS analysis. The DMSO stocks of YLT205 was 20 mM and stored at -20 ℃. For all *in vitro* assays, YLT205 was diluted to appropriate concentrations with the cell culture medium at final DMSO concentration of 0.1% (*V/V*). For *in vivo* experiment, YLT205 was dissolved in ultrapure water and Cremophor EL/ethanol (50:50, Sigma Cremophor EL, 100% ethyl alcohol).

3-(4,5-dimethyl-2-thiazolyl)-2,5-di-phenyl-2H-tetrazolium bromide (MTT), propidium iodide (PI), Rhodamine-123 (Rh123), Hoechst 33358, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co (St. Louis, MO). Annexin V-FITC apoptosis detection kit was obtained from BD Biosciences (San Diego, CA). The antibodies against caspase-3 (17, 19, 35 kDa), caspase-9 (35, 37, 47 kDa), cytochrome c, Bcl-2 (26 kDa), Bax (20 kDa), Akt/p-Akt (Ser473, 60 kDa) and p44/42 MAPK/p-p44/42 MAPK (Tyr202/Tyr204, 44, 42 kDa) were purchased from Cell Signaling Technology Company (Beverly, MA). Antibody against β-actin was acquired from Santa Cruz Biotechnology Company (Santa Cruz, CA). The caspase inhibitor Z-VAD-FMK and PI3K/Akt inhibitor LY294002 were purchased from Beyotime (Beijing, China).

Cell culture

Human colon cancer cell lines HCT116, SW620, SW480, DLD-1, HCT-15, LS174T and other cells were purchased from American Type Culture Collection (ATCC, Manassas, VA), H1975 and SMMC-7721 cell lines were obtained from the China Center for Type Culture Collection (CTCCC, Wuhan, China). These cells were cultured in Dulbecco's modified Eagle medium (DMEM) or RPIM 1640 supplemented with 10% fetal bovine serum (Gibco, Auckland, N.Z), 100 units/mL penicillin, and 100 μg/mL streptomycin in a humid chamber at 37 ℃ under 5% CO_2 in atmosphere.

Fig. 1. The chemical structure of YLT205.



Cell viability assay

The MTT assay was used to measure the cell viability after YLT205 treatment. Briefly, cells were plated on 96-well microplates at a density of 2-5×10^3 cells per well and cultured for 24 h. After various concentrations of YLT205 treatment for 48 h, 20 μL of MTT solution (5 mg/mL) was added to each well and incubation for 2-4 h at 37 ℃. Then the medium was discarded and 150 μL of DMSO was added to each well to dissolve the formazan crystal produced by living cells. Ten minutes later, 96-well microplates were read on Spectra MAX M5 microplate spectrophotometer (Molecular Devices) at 570 nm for O.D. values [16].

Clonogenic survival assay

For clonogenic survival assay, 400 to 1000 cells were seeded in a six-well plate, and treated with various concentrations of YLT205 (0~0.625 μM) after 24 h, then the cells were re-fed once every 3 day for another 13 days with YLT205 and then fixed and stained with crystal violet.

Morphological analysis by Hoechst staining

Hoechst 33358 staining was used to detect morphological changes associated with apoptosis. After treatment with YLT205 for 24 h, cells were washed twice with PBS (Phosphate Buffered Saline, PH=7.4) and fixed with paraformaldehyde for 15 minutes. Then the cells were stained with 5 μg/mL Hoechst 33358 solutions. Morphological changes typical of apoptosis were observed under nuclear fluorescence microscopy (Zeiss, Axiovert 200, Germany).

Analysis of apoptosis by Flow Cytometry (FCM)

To investigate the apoptosis-inducing effect of YLT205, we analyzed the percentage of apoptotic cells by FCM with propidium iodide (PI) staining or Annexin V-FITC/PI dual labeling. After treatment with YLT205 for 24 or 48 h as described above, harvested cells were washed twice with cold PBS and then stained with Annexin V-FITC and PI according to manufacturer's instructions, and then analyzed on a flow cytometer (Becton-Dickinson, USA) immediately. We also studied the apoptosis by PI single staining. Cells were harvested and fixed with 75% ice-cold ethanol, and then stained with hypotonic fluorochrome solution composed of PI at 50 μg/mL plus 0.1% Triton X-100 and then analyzed by FCM. Finally, the data was analyzed using Flow Jo software. Moreover, to investigate whether caspase and p44/42 MAPK are involved in YLT205-induced apoptosis in HCT116 cells, we treated cells with/without 2.5 μM YLT205 combined with 20 μM Z-VAD-FMK (caspase inhibitor) or 50 μM PD98059 (MAPK inhibitor), respectively. PI-stained was performed and analyzed by FCM. Finally, the data was analyzed using Flow Jo software.

Mitochondrial membrane potential (ΔΨm) assay by FCM

The changes in mitochondrial transmembrane potential (ΔΨm) were analyzed by staining with Rh123 and detected by FCM as described previously [17]. Cell culture and drug treatment were done as described above. The harvested HCT116 cells were washed with cold PBS after incubation with Rh123 (5 μg/mL) at 37 ℃ for 30 min in the dark and then measured by flow cytometry.

Immunofluorescence of cytochrome c

Cells were treated with vehicle or YLT205 for 24 h. After being fixed with 4% paraformaldehyde for 15 min, the cells were incubated with blocking buffer (PBS containing 3% bovine serum albumin, 2% goat

serum and 0.2% Triton X-100) for 1 h and incubated with antibodies against cytochrome c overnight. After washing twice with PBS, the cells were incubated with corresponding fluorescence-conjugated secondary antibodies for 1 h. Then changes in cytochrome c level were detected using nuclear fluorescence microscopy (Zeiss, Axiovert 200, Germany) [18].

Western blotting analysis

To explore the mechanism of YLT205 on cell proliferation and cell apoptosis, we detected the change of protein using Western blot assay. Briefly, after treatment with YLT205 for 24 h, cells were washed twice with PBS after harvesting and centrifuged at 13,000 rpm for 15 min, followed by lysis in RIPA buffer for 30 min. The proteins were separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were blocked for 1~2 h at room temperature in 5% nonfat dry milk and incubated overnight at 4 °C with recommended dilute solution of primary antibodies, followed by incubation with horseradish peroxidase conjugated secondary antibody. Peroxidase-labeled bands were visualized using an ECL kit (Amersham, Piscataway, NJ). A monoclonal anti-β-actin antibody was used as a control.

Xenograft nude mice model studies

All animal experiments were approved by the Institutional Animal Care and Treatment Committee of Sichuan University (Sichuan University, Chengdu, China). Female 5- to 6-week-old BALB/c athymic nude mice (Beijing HFK Bioscience Co. ltd., Beijing, China) were inoculated subcutaneously into the right flank regions with 1×10^7 HCT116 cells or 1×10^7 SW620 cells/mouse suspended in 100 μL of medium without serum and antibiotics. About a week later, mice bearing tumors around 100 mm^3 were selected and then randomly divided into groups (8 mice per group). The mice were administrated in a dose schedule of YLT205 at 37.5, 75 and 150 mg/kg, or vehicle once a day by intraperitoneal injection. The mice were observed daily, and tumor size and body weight were measured every 3 days. Tumor volumes were calculated according to the formula: Tumor Volume = 0.5 × length × $width^2$. Growth inhibition was calculated from the start of treatment by comparison of the change in tumor volume for vehicle and treated group as before [19].

Analysis of HCT116 cell apoptosis by Immunohistochemistry and TUNEL assay in vivo

After treated with YLT205 for 14 days, tumors of HCT116 xenograft nude mice models were collected, fixed, processed, and embedded in paraffin for immunohistochemical analysis and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. The tumor tissue was stained with H&E or treated with specific antibodies, like cleaved caspase-3. To explore whether YLT205 inhibited tumor growth by inducing apoptosis, apoptotic cells in tumor tissue was detected by TUNEL assay as described previously [20]. Three tumors per group were analyzed.

Toxicity evaluation

To investigate the potential side effects or toxicity of YLT205 on mice during the treatment, the etiological symptoms of the mice including mortality, body weight, diarrhea, anorexia and skin ulceration were monitored continuously [21]. Serological analysis, hematological analysis, and histological examination were carried out after dissection. Serological analysis and hematological analysis were detected by Hitachi 7200 Blood Chemistry Analyzer and a Nihon Kohden MEK-5216K Automatic Hematology Analyzer, respectively. Heart, liver, spleen, lung, and kidney of the mice were stained with hematoxylin and eosin (H&E).

Statistical analysis

Statistical analyses were carried out in Microsoft Excel or GraphPad Prism. *P* values were calculated by unpaired *t* test, and were considered significant if $P < 0.05$. Data are displayed as means±SD/SEM from three independent experiments in the figures.

Results

Effects of YLT205 on human cancer cells viability in vitro

To test the effect of YLT205 on cancer cell viability, a panel of cancer cell lines of different histological types was treated with YLT205 for 48 h, and the IC_{50} values were expressed.

Table 1. Proliferation inhibitory effect of YLT205 on human cancer cells. Each cell line was treated with various concentrations (0~20 μM) of YLT205 for 48h. Cell viability was detected by MTT assay and IC_{50} values were expressed as mean±SD from three experiments

Cell line	Cancer type	IC_{50}(μM)
HCT116	Colon	0.59±0.01
SW620	Colon	0.63 ±0.07
SW480	Colon	0.63±0.04
HCT-15	Colon	0.71±0.04
DLD-1	Colon	0.74±0.05
Ls174T	Colon	0.78±0.06
Hep G2	Liver	4.77±0.11
SMMC-7721	Liver	2.06±0.17
Hela	Cervix	0.76±0.10
MDA-MB-231	Breast	0.68±0.04
MDA-MB-468	Breast	1.83±0.01
CFPAC-1	Pancreas	6.70±0.62
PANC-1	Pancreas	4.84±0.76
A549	Lung	0.69±0.46
PC-9	Lung	4.7±0.07
NCI-H1975	Lung	0.98±0.02
SPC-A1	Lung	0.88±0.03
A2780S	Ovary	0.84±0.11
THP-1	Peripheral blood	13.18±1.95
MV4-11	Peripheral blood	1.26±0.15
U251	Glioblastoma	1.04±0.04
A431	Epidermal	1.8±0.03
A375	Melanoma	1.19±0.17

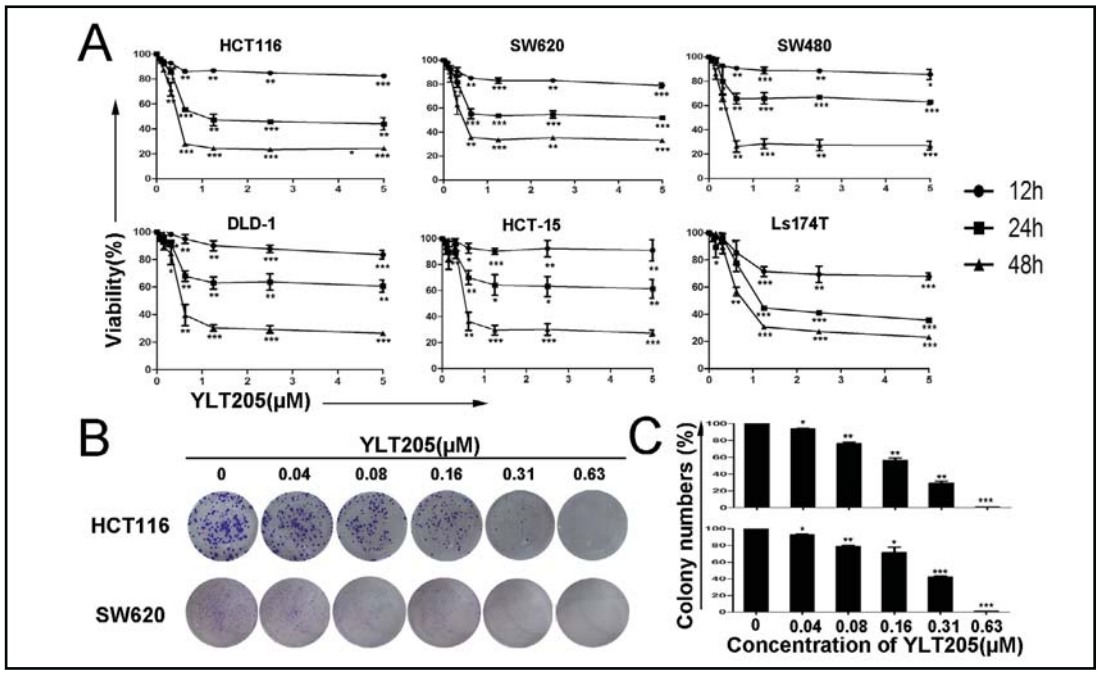


Fig. 2. Effects of YLT205 on the cell proliferation in human colon cancer cell lines. (A) Colon cancer cell lines were treated with different concentrations of YLT205 for 12, 24 or 48 h and cell viability was measured by the MTT assay. Each point represents the mean±SD for at least 3 independent experiments (*p<0.05; **p<0.01; ***p<0.001 vs vehicle control). (B) Effects of YLT205 (0~0.625 μM) on colony formation in HCT116 and SW620 cells. (C) The percentage of inhibition in colony-forming assays was expressed using vehicle treated cells at 100%. Data are expressed as mean±SD for at least 3 independent experiments (*p<0.05; **p<0.01; ***p<0.001).

As shown in Table 1, IC_{50} values ranged from 0.59 to 13.18 μM, and a comparison among the examined 23 cell lines showed that human rectal cancer cell lines were most sensitive to YLT205. Thus, we chose human colorectal cancer cells to further study for the YLT205

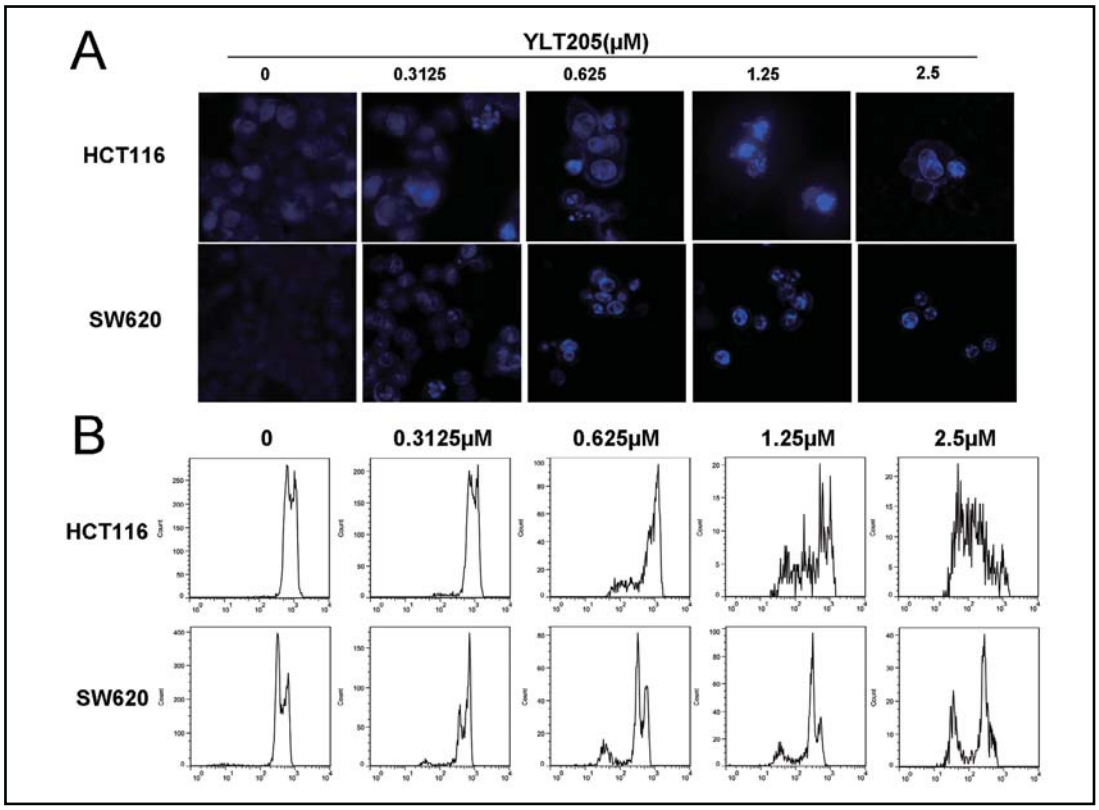


Fig. 3. Effects of YLT205 on cell morphology and viabilities of cancer cells. HCT116 cells were incubated with YLT205 for 24 h, and SW620 cells for 48 h. (A) The fluorescence microscopic appearance of Hoechst 33358-stained HCT116 and SW620 cells (×400). (B) Flow cytometric analysis of PI-stained CRC cell lines.

exerted antitumor mechanism. Furthermore, exposure of HCT116, SW620, SW480, DLD-1, Ls174T and HCT-15 cells to YLT205 for 12 h, 24 h and 48 h, respectively, resulted in decrease of the cell proliferation (Fig. 2A). These data suggested that YLT205 inhibited colorectal cancer cells proliferation in a concentration- and time-dependent manner.

YLT205 inhibited clonogenicity of human colorectal cancer cells

To further determine the effect of YLT205 on cell growth, we conducted clonogenic assay after YLT205 treatment. Fig. 2B and Fig. 2C show that colony formation of HCT116 and SW620 cell lines were inhibited in a concentration-dependent manner after exposure to YLT205. When the concentration reached to 0.625 μM, nearly no colony formation was found. These results were consistent with the MTT data and further suggested that YLT205 could inhibit cell growth of colorectal cancer cell lines.

YLT205 induced apoptosis of CRC cells

To examine the possible mechanism of YLT205 to inhibit cell viability, apoptosis was examined after YLT205 treatment of colorectal cancer cells. To such regard, we applied flow cytometric analysis and Hoechst 33358 nuclear staining. As shown in Fig. 3B, the percentage of sub-G1 cells in HCT116 cells increased from 2.13% to 4.42%, 21.30%, 49.55% and 83.70% after 0.3125 μM, 0.625 μM, 1.25 μM, 2.5 μM YLT205 treatment for 24 h, respectively, indicating that YLT205 was able to induce apoptosis in a concentration-dependent manner. Similarly, in SW620 cells, the percentage of apoptotic cells was increased from 3.60% to 5.13%, 19.03%, 25.36% and 44.88% after treatment various concentration YLT205 for 48 h (Fig. 3B), respectively. Furthermore, as shown in Fig. 3A, the features of apoptotic cells were shown after YLT205 treatment in HCT116 and SW620 cells. For instance, brilliant-blue

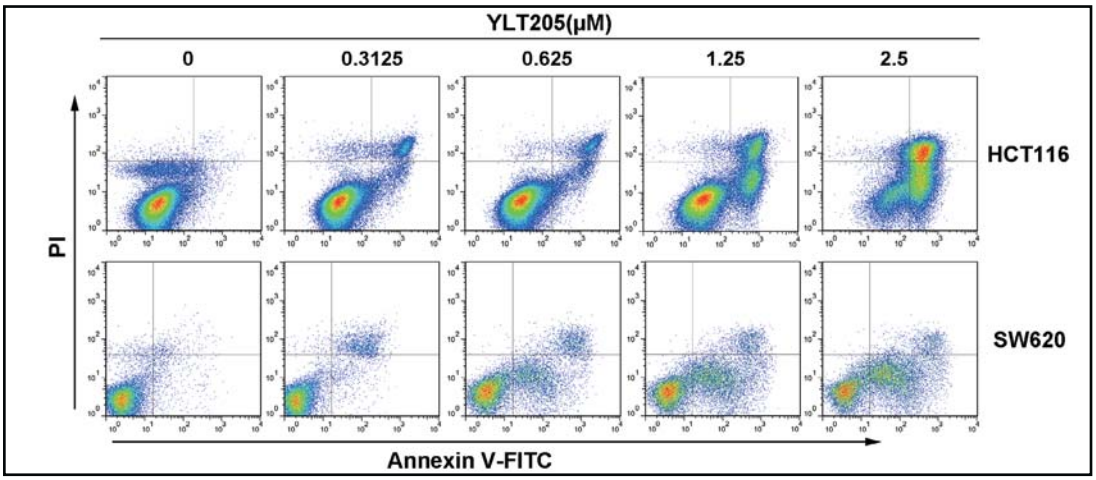


Fig. 4. HCT116 cells were incubated with YLT205 for 24 h, and SW620 for 48 h. Cells stained with Annexin V/PI after incubated with various concentrations (0~2.5 μM) of YLT205 were measured by flow cytometry.

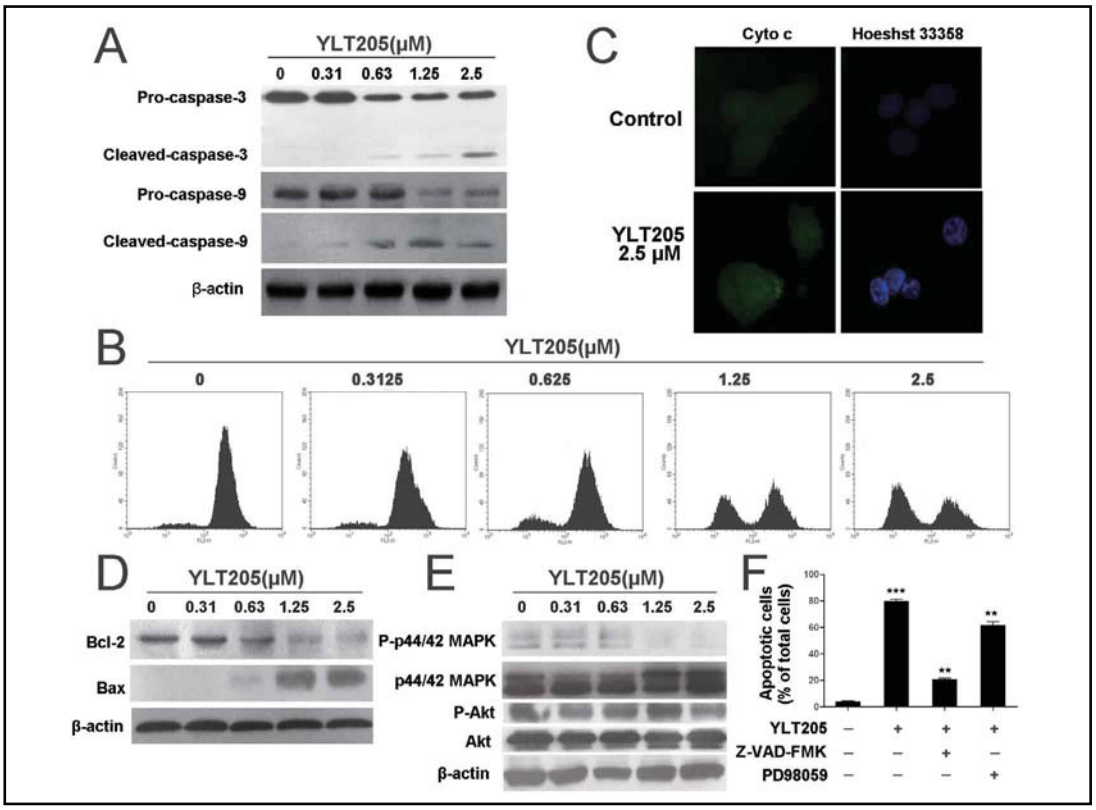


Fig. 5. Effects of YLT205 on the expression of apoptosis-related proteins and the mitochondrial membrane potential (ΔΨm) of HCT116. (A) The expression of pro-caspase-3, -9, and their activated forms was detected by western blot. (B) Changes of the mitochondrial membrane potential were determined by FCM. (C) YLT205 increased the release of cytochrome c into cytosol by immunofluorescence. The expression levels of Bcl-2, Bax (D), Akt, p44/42 MAPK and their phosphorylated forms (E) were also assayed by western blot. (F) HCT116 cells were treated with 2.5 μM YLT205 alone or in combination with Z-VAD-FMK (caspase inhibitor) or PD98059 (MEK/ERK inhibitor). Data are expressed as mean±SEM for at least 3 independent experiments (*p<0.05; **p<0.01; ***p<0.001).

fluorescence stained and condensed nuclei, cytoskeletal collapse, reduction of cell count and nuclear fragmentation were evident.

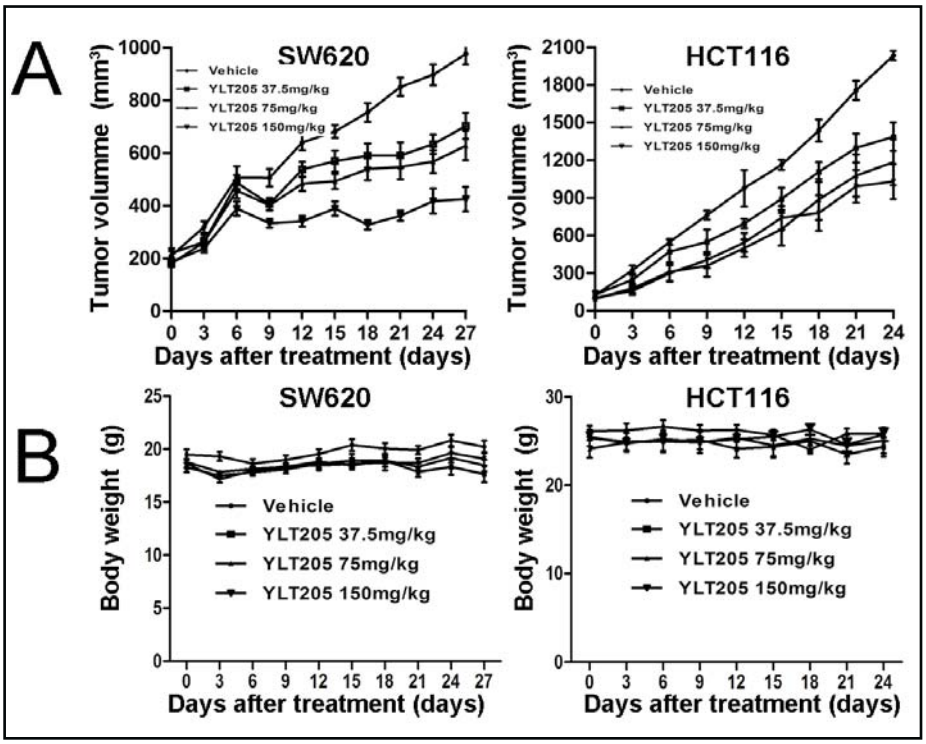


Fig. 6. YLT205 inhibits tumor xenograft model growth of human colon cancer model (SW620 and HCT116). Established tumor xenografts were treated with 37.5, 75 and 150 mg/kg/day YLT205 or vehicle. Tumor volumes (A) and body weights (B) were measured every 3 days.

To further confirm the apoptosis in the cell death result, we applied another method by Annexin V-FITC combined with PI fluorescence staining, which was determined by flow cytometry. As shown in Fig. 4, YLT205 dose-dependently increased the percentage of apoptotic cells (positive Annexin V-FITC and negative PI staining) after exposure to various concentrations of YLT205 for 24 h in HCT116 cells and 48 h in SW620 cells as well.

Effects of YLT205 on loss of ΔΨm and release of cytochrome c

Rh123, a fluorescence dye, was used to detect the ΔΨm change of cells after YLT205 treatment, and cytochrome c release was examined through immunofluorscence. As shown in Fig. 5B, YLT205 treatment for 24 h led the loss of ΔΨm potential in HCT116 cells in a dose dependent manner, showing a decreased ΔΨm from 95.29% in the vehicle to 58.64% in the 2.5 μM treatment group. The loss of mitochondrial membrane potential would be the release of cytochrome c from the mitochondria to the cytosol. The result of the immunofluorscence detection of cytochrome c showed that the level of cytochrome c significantly increased in the cytosols of HCT116 cells after treatment with 2.5 μM YLT205 (Fig. 5C), which was in line with the ΔΨm change.

Effects of YLT205 on apoptosis-related proteins

To further conform the characterization of YLT205-induced apoptosis, some apoptosis-related proteins were detected by Western blot, including caspase family proteins, Bcl-2 family proteins, Akt and MAPK. Firstly, we examined changes in the proteolytic processing of caspase-3 and caspase-9. As shown in Fig. 5A, expression of pro-caspase-3 and pro-caspase-9 in HCT116 cells was decreased significantly and the levels of their cleaved forms were increased after YLT205 exposure for 24 h. Next, we examined Bcl-2 family proteins, including Bcl-2 and Bax. Fig. 5D showed that the expression of Bcl-2 was decreased significantly while that of Bax was increased after YLT205 treatment.

In addition, the expression levels of p44/42 MAPK and Akt, which play important roles in cell proliferation and apoptosis, were examined. As shown in Fig. 5E, YLT205 treatment suppressed the expression of phosphorylated p44/42 MAPK without affecting its total expression level, but there was no clearly change in the level of phosphorylated Akt.

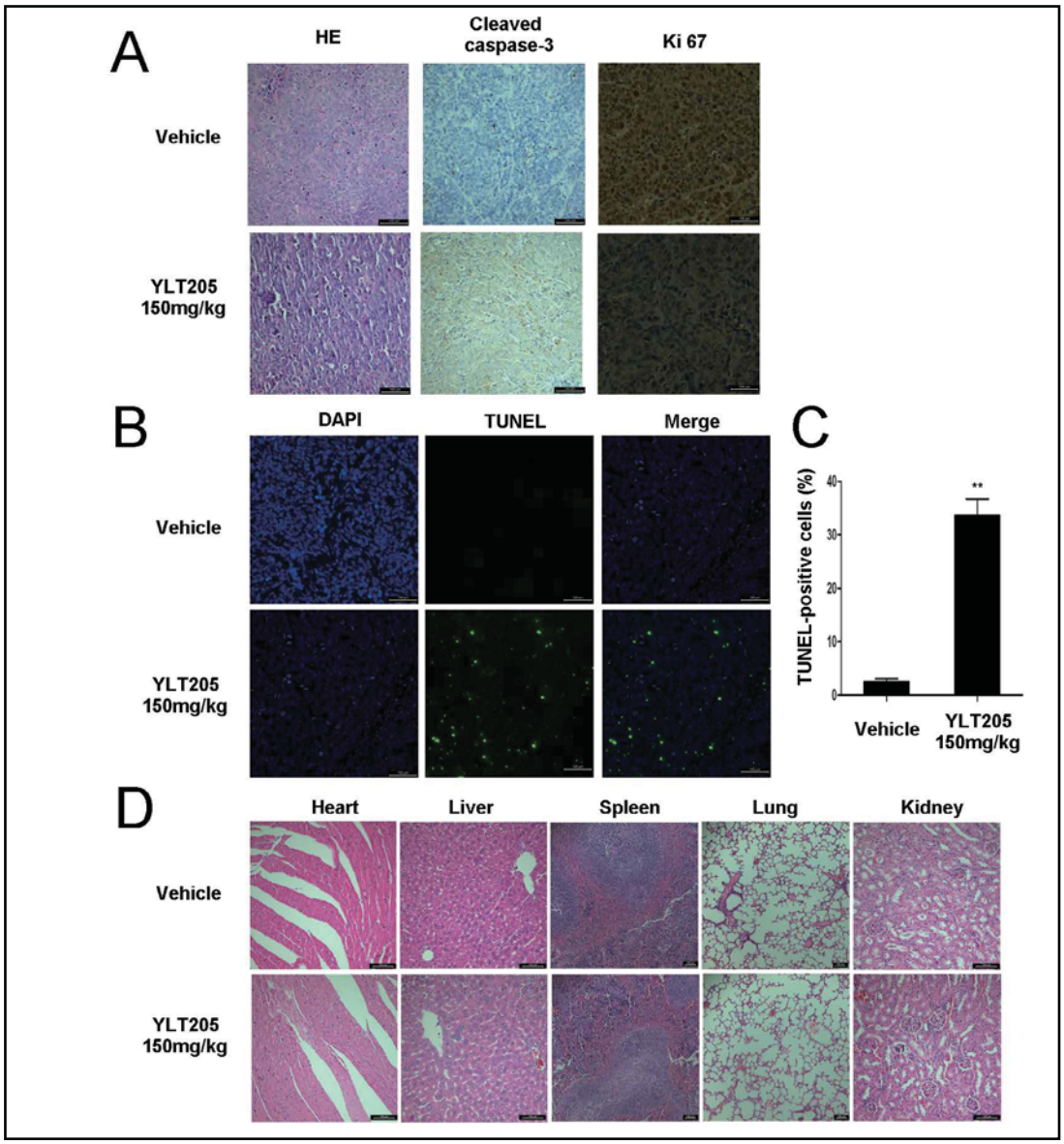


Fig. 7. Effects of YLT205 on human colon cancer xenograft model. After two weeks of YLT205 treatment, tumor sections (3 per group) from SW620 models treated with 150 mg/kg YLT205 or vehicle were collected separately. The tumor sections were examined by immunohistochemical analysis with anti-Ki67, caspase-3 antibody and H&E (A), and cell apoptosis in tumor was detected by TUNEL analysis (B). (C) The statistical data of TUNEL-positive cell number in the tumors of SW620 xenograft models. **, $P < 0.01$ vs. the control. (D) After the treatment of SW620 xenograft, heart, liver, spleen, lung and kidney of the mice were isolated and stained with H&E.

Effects of caspase and MAPK inhibitors on YLT205-induced apoptosis in vitro

To explore which type of apoptotic pathway is a predominant role in YLT205-induced apoptosis, Z-VAD-FMK (caspase inhibitor) and PD98059 (MER/ERK inhibitor) were used. As shown in Fig. 5F, compared with YLT205 treatment alone, treatment with 2.5 μM YLT205 combined with 20 μM Z-VAD-FMK decreased the percentage of apoptotic cells from 80.38% to 20.58% while combination with 50 μM PD98059 had only a slight effect on apoptosis induced by YLT205 treatment.

Table 2. Evaluation of side effects of YLT205 in mice. After the treatment of SW620 xenograft in nude mice, hematological parameters and serum biochemical values of mice were measured (n=8 for both vehicle and treated). Values are indicated in mean±SD

Hematological and Serum Biochemical Parameters	Vehicle	YLT205 150mg/kg	P value
WBC (10^9/L)	6.9±2.5	7.1±2.9	0.33
RBC (10^{12}/L)	10.3±1.2	9.6±1.6	0.06
HGB (g/L)	119.8±13.6	118.2±20.3	0.16
HCT (%)	47.5±4.7	49.8±8.5	0.37
TP (g/L)	63.7±4.8	63.6±3.6	0.48
ALB (g/L)	30.8±3.7	29.4±2.9	0.30
ALP (U/L)	55.5±4.6	52.7±7.6	0.23
CREA (μM)	2.6±1.0	2.2±0.9	0.31
TG (mM)	1.0±0.2	0.9±0.2	0.17
CHO1 (mM)	2.1±0.3	2.1±0.3	0.46
HDL (mM)	1.0±0.2	1.0±0.2	0.46

Antitumor efficacy of YLT205 in relevant human tumor xenograft models

To study the antitumor activity of YLT205 *in vivo*, HCT116 or SW620 tumor bearing BALB/c nude mice were established and treated with YLT205 at doses of 37.5, 75 and 150 mg/kg/day, respectively. As shown in Fig. 6A, compared with the vehicle group, YLT205 substantially suppressed tumor growth in a dose-dependent manner, with the inhibition rate of tumor volumes at 68.8% (SW620) and 51.3% (HCT116) at the dose of 150 mg/kg. Moreover, YLT205 treatment was well tolerated and body weight of mice was change slightly (Fig. 6B), and no other adverse effects, such as toxic death and skin ulcerations, were observed in YLT205 groups.

To elucidate the mechanism of YLT205 to inhibit tumor growth *in vivo*, immunohistochemical analysis and TUNEL staining were done. As shown in Fig. 7A, hematoxylin-eosin staining (H&E) revealed the regression of tumors in YLT205 treated animals. Besides, YLT205 remarkably decreased the number of proliferating cells in tumor tissues as indicated by cell cycle marker Ki67 and increased the number of cleaved caspase-3-positive cells in tumors compared with vehicle group. Moreover, TUNEL-positive cells in YLT205-treated group were higher than that in vehicle group with the percentage rise from 2% to 33% (Fig. 7B and Fig. 7C), Showing that YLT205 suppress tumor growth *in vivo* through inducing cell apoptosis.

Safety profile of YLT205

To further investigate the safety profile of YLT205, all the mice in SW620 models sacrificed after 28 days treatment followed by toxicity evaluations. Serum biochemistry and hematological of the mice did not show any pathological changes (Table 2). Moreover, microscopic examination of heart, liver, spleen, lung and kidney revealed that there were no toxic pathological changes after YLT205 treatment (Fig. 7D).

Discussion

YLT205 is a novel chemical structure which differs from the apoptosis inhibitors currently in clinical research [15]. This study firstly demonstrated that YLT205 can induce apoptosis in human colorectal adenocarcinoma cells *via* the mitochondrial apoptotic pathway *in vitro* and inhibited tumor growth *in vivo*.

There are two classic apoptotic pathways, including the extracellular pathway and the intracellular pathway. In both pathways, caspase-3 plays a central role [22] and can be activated by upstream effector proteins, such as caspase-9, which could arouse the activation of mitochondrial intrinsic pathway [23]. The cleavage of caspase-9 and caspase-3 were observed after YLT205 treatment, and these results are consistent with our initial hypothesis that YLT205 induces apoptosis *via* the mitochondrial intrinsic pathway. In order to further investigate whether YLT205-induced apoptosis is caspase-dependent or not, the

irreversible inhibitor, Z-VAD-FMK (caspase inhibitor) was used. We observed that Z-VAD-FMK significantly decreased the apoptosis cells of the YLT205-induced in HCT116 cells, suggesting that caspase plays a role in YLT205-induced apoptosis.

In the intrinsic apoptotic pathway, many important events are closely related with the mitochondria, including the disruption of the mitochondrial transmembrane potential and the induction release of cytochrome c [10, 11]. In general, cytochrome c is localized in the mitochondrial membrane and would be released into the cytosol when intrinsic apoptosis was induced [11]. Then it would lead to cleavage of caspase-9, which would finally activate downstream caspase-3 to initiate apoptosis [24]. In this study, we observed substantial reduction in $\Delta\Psi m$ by FCM and an increase in the release of cytochrome c from mitochondria into the cytosol after YLT205 treatment.

Bcl-2 family is the center regulator in mitochondrial apoptosis pathway, and this family includes the anti-apoptotic Bcl-2 and pro-apoptotic Bax [12], increasing in pro-apoptotic protein but decreasing in anti-apoptotic protein would stimulate the release of cytochrome c [25]. In our study, anti-apoptotic protein Bcl-2 was down-regulated while pro-apoptotic protein Bax was up-regulated in HCT116 cells after treated with YLT205. We assume that the change of the Bcl-2 family proteins is involved in the release of cytochrome c and may provide a selective growth advantage and confer resistance to YLT205.

In addition, other signaling proteins, such as phosphatidylinositol 3-kinase (PI3K)/Akt and MAPK pathways, are involved in apoptosis pathways [26]. Both Akt and MAPK pathways play important roles in promoting cell proliferation and modulating cancer prevention and treatment through apoptosis. Our results showed that phosphorylation of p44/42 MAPK was decreased after YLT205 treatment while phosphorylation of Akt was not affected. But, we observed that the percentage of apoptotic cells only had a slight effect on YLT205-induced apoptosis after adding the MER/ERK inhibitor PD98059. Combined this results and the significant decrease in apoptosis cells after Z-VAD-FMK addition, we conclude that caspases play an important role in YLT205-induced apoptosis.

Furthermore, tumor growth inhibition *in vivo* was evaluated in tumor-bearing nude mice. The dose of 150 mg/kg/day treatment by YLT205 was able to inhibit tumor growth at the inhibitory rate of 68.8% and 51.3% in human colon cancer SW620 and HCT116 models, respectively. Besides, the results of immunohistochemistry and TUNEL assay revealed that YLT205 could induce apoptosis in the tumor sections, as shown by the TUNEL-positive and cleaved caspase-3-positive cells. YLT205 also showed inhibition of cell proliferation demonstrated by a decrease in Ki67-positive cells. Furthermore, YLT205 was well tolerated in mice in the study. In conclusion, YLT205 not only inhibited cancer cell proliferation *in vitro*, but also had an effective inhibition of tumor growth *in vivo* without causing obvious toxicities.

Taken together, we demonstrated, for the first time, that YLT205 could inhibit human colon cancer growth both *in vitro* and *in vivo,* and induce human colon cancer cells apoptosis *via* mitochondrial intrinsic pathway. The mechanism of apoptosis was related to down regulation of Bcl-2 and conversely up regulation of Bax, along with the disruption of mitochondrial membrane, followed by release of cytochrome c from mitochondria to cytosol, which eventually leads to cleavage of executor caspase-3 and the apoptosis. YLT205 also showed antitumor activities in the colon cancer xenograft *in vivo* with no obvious toxicity, suggesting that it may be a relatively nontoxic adjuvant treatment drug for cancer therapy while its anti-tumor activities in other cancer types and the more precise mechanisms are still needed in future study.

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