A Novel Bioavailable BH3 Mimetic Efficiently Inhibits Colon Cancer via Cascade Effects of Mitochondria

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Abstract

Purpose: Gossypol and its analogs, through their ability to bind to and inactivate BH3 domain-containing antiapoptotic proteins, have been shown to inhibit the growth of various human cancer cells in culture and xenograft models. Here, we evaluated the antitumor efficacy of a novel gossypol derivative and BH3 mimetic ch282-5 (2-aminoethanesulfonic acid-sodium-gossypolone) in colon cancer models. Several innovative combination strategies were also explored and elaborated.

Experimental Design: Ch282-5 was synthesized by modifying the active aldehyde groups and R groups of gossypol according to a computer-aided drug design program. The stability of ch282-5 was examined by high-performance liquid chromatography, and cytotoxic effects of ch282-5 on colon cancer cells were assessed by MTS assay. Activation of mitochondrial apoptotic pathway by ch282-5 was evidenced with a series of molecular biology techniques. In vivo antitumor activity of ch282-5 and its combination with chloroquine, rapamycin, oxaliplatin, and ABT-263 was also evaluated in colon cancer xenograft models and experimental liver metastasis models.

Results: Ch282-5 showed antiproliferative and pro-cell death activity against colon cancer cells both in vitro and in vivo, and the response to the drug correlated with inhibition of antiapoptotic Bcl-2 proteins, induction of mitochondria-dependent apoptotic pathway, and disruption of mitophagy and mTOR pathway. Ch282-5 also suppressed liver metastasis produced by intrasplenic injection of colon cancer cells. Furthermore, ch282-5 could potentiate the effectiveness of oxaliplatin and rescue ABT-263 efficacy by downregulation of Mcl-1 and elevation of platelet number.

Conclusions: These findings provide a rational basis for clinical investigation of this highly promising BH3 mimetic in colon cancer.

Introduction

Bcl-2 family prosurvival proteins are abundantly expressed in a wide variety of human cancers and associated with oncogenesis, tumor proliferation, metastasis, and chemoresistance (1). Therefore, targeting Bcl-2 draws a great deal of attention in cancer drug development. In recent years, Bcl-2 small-molecule inhibitors such as ABT-737, ABT-263, ABT-199, and GX-15-070 have entered clinical trials. Gossypol, a polyphenol derived from cottonseed oil, was the first natural compound discovered that demonstrated inhibition of Bcl-2 family proteins (2). NMR and fluorescence polarization competitive binding experiments demonstrated that gossypol can bind to the BH3 domain of the Bcl-2 family anti-apoptotic proteins in the tumor cells thus initiating caspase-dependent apoptosis events (3). However, the clinical use of gossypol is limited by its toxicity problem most likely due to two reactive aldehyde groups and its high hydrophobicity (4). As a result, many derivatives of gossypol have been generated including AT-101 and ApoG2 that have entered clinical trials (4).

We previously reported a gossypol derivative named “compound-7” (6-APA-Na-gossypoline) as a Bcl-2 inhibitor that demonstrated competitive binding to the BH3 domain of Bcl-xL (Kᵢ = 1.76 µmol/L) causing release of proapoptotic proteins Bim and Bax, and ultimately leading to apoptosis (5). Compound-7 not only inhibited the growth of several cancer cell lines in vitro but also suppressed tumor growth in vivo with less toxicity when compared with natural gossypol. More importantly, compound-7 could potentiate the antitumor effects of 5-fluorouracil (5-FU) both in vitro and in vivo in colon cancer models. However, the log P of compound-7 is 8.33 indicating its poor absorption and permeation (6), which limits a possible clinical application. The stability of compound-7 is also not appreciable.
Mitochondria is an important place of biologic oxidation and energy conversion for eukaryotic cells, involving cell homeostasis, proliferation, motility, aging, death, and other biologic processes. Bcl-2 family inhibitors could damage mitochondria and change permeability of mitochondrial membrane, uncoupling oxidative phosphorylation. ATP excessive consumption induces necrosis or swelling mitochondria triggers apoptosis by released cytochrome c. Timely removal of damaged mitochondria is essential for the normal growth and metabolism of cells. Mitochondrial autophagy (mitophagy) is a specific phenomenon of autophagy by selectively removing damaged mitochondria (7). Autophagy is generally regarded as a prosurvival factor under cellular stress, and autophagy inhibition could enhance killing effects in tumor cells both in vivo and in vitro (8). Therefore, the strategy to inhibit autophagy to enhance the effectiveness of chemotherapy is constantly attempted.

As of 2015, colorectal cancer has been the third most common cancer in United States (9). More than half of the patients with colorectal cancer are expected to develop metastatic disease, a quarter of whom would have distant metastatic lesions at diagnosis, often in the liver (10). Therefore, antimitastastic property becomes a compulsory requirement for a colon cancer drug candidate. mTOR is a Ser/Thr kinase that plays a key role in regulation of a wide range of biologic processes including cell growth, proliferation, survival, autophagy, metabolism, and cytoskeletal organization. The frequent hyperactivation of mTOR signaling in cancer cells has made it an attractive target for therapeutic intervention and has stimulated the development of a number of mTOR inhibitors, some of which have already undergone clinical trials (11). mTOR is also positioned upstream and downstream of multiple oncogenic pathways such as AKT, ERK, and STAT signaling that may contribute to some of the resistance that can occur with mTOR-targeted therapies (12).

Combination therapy is a common strategy for cancer clinical treatment. Combination of two or more chemotherapeutics not only augments the cytotoxicity but also reduces the drug resistance and side effects. The current clinical therapy of colon cancer employs a combination of oxaliplatin and 5-fluorouracil (5-FU), both of which are DNA-damaging agents (13). Thus, discovery and development of new targeted drugs for colon cancer treatment is urgently needed. Accumulating evidence also indicates that a single BH3 mimic may not be sufficient as a monotherapy to treat cancer patients and the best clinical outcome may be achieved by appropriate drug combinations. Combination of ABT-737 or ABT-263 with autophagy inhibitors and other targeted agents has proved beneficial in preclinical and clinical cancer treatment (14).

In this study, we identified a new gossypol derivative ch282-5 by changing the active aldehyde groups of gossypol into keto groups to reduce side effects, and replacing the R group of gossypol with sodium taurate to enhance hydrophilicity. We found that ch282-5 selectively induced colon cancer cell death by specific and competitive binding to the BH3 domain of Bcl-2 family apoptotic proteins. Inhibition of mitophagy and disruption of mTOR signaling enhanced the antitumor effects of ch282-5. Ch282-5 significantly inhibited experimental liver metastasis and rescued ABT-263 efficacy by downregulation of Mcl-1 and increasing the platelet count. Taken together, these findings provide new understanding of the mechanisms by which the BH3 mimetic mediates its antitumor effects and facilitates its clinical development.

Materials and Methods

Ch282-5 synthesis

Sodium2,2’-(1E,1’E)-(6,6’,7,7’-tetrahydroxy-5,5’-diisopropyl-3,3’-dimethyl-1,1’,4,4’-tetrahydro-1,1’-binaphthyl-8,8’-dil) bis (methyl-1-yl-1-ylidene) bis (azan-1-yl-1-ylidene) diethanesulfonate. Taurine (250 mg, 2 mmol) was added drop-wise to a solution of NaOH (88 mg, 2.2 mmol) in dry ethanol (20 mL) at 40°C under Ar to obtain taurate. Gossypolone (546 mg, 1 mmol) was then added and drop-wise to the reaction mixture at room temperature. The solution turned red wine immediately and the reaction mixture was stirred at room temperature for 1.5 hours then warmed to 40°C for another 0.5 hour. The resulting reaction mixture was filtered under reduced pressure to give a red solid powder, which was then washed with dry ethanol (2 × 10 mL) and dry ether (3 × 10 mL) to give a Schiff base product (604 mg) with a yield of 75%.1H NMR (500 MHz, DMSO) δ 14.19 [s, 2H], 9.25 [s, 4H], 3.97 [s, 4H], 3.79-3.86 [m, 2H], 2.82-2.84 [m, 4H], 1.90 [s, 6H], 1.36 [t, J = 6.2 Hz, 12H], 13C NMR(126 MHz, DMSO) δ 186.70, 184.92, 168.65, 165.21, 153.25, 144.89, 137.38, 130.96, 126.22, 123.70, 106.70, 106.06, 56.00, 49.96, 48.48, 27.52, 20.10, 19.94, 18.53, 14.02, LR-ESI: [M-H]− 375.0; [M-Na]− 359.1; [M-K]+ 341.9; [M-2Na−H]+ 176.5; [M-Na−2H]+ 160.4; [M-Na−3H]+ 144.3 (Supplementary Figs. S1 and S2).

Cell culture

Human colon cancer cell lines HCT116 and HT29 (ATCC) were cultured in McCoy 5A medium, SW620 in L-15 medium, and mouse colon cell line CT26 in 1640 medium in a 5% CO2 humidified atmosphere at 37°C. All media were supplemented with 10% FBS (Gibco), 1% penicillin and streptomycin (Invitrogen). Other cell lines were cultured in growth medium as per ATCC recommendations.

Cell viability assay

Three thousand or 5,000 cells were treated with ch282-5 or other combinations for different time in 96-well plates in a final volume of 100 μL, and viability was assessed by CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS assay).
Flow cytometry
For apoptosis, ch282-5-treated cells were incubated with Annexin V-FITC and propidium iodide (PI) for 15 minutes at room temperature in the dark. For cell cycle, ch282-5–treated cells were fixed in 70% ethanol at 4°C for 30 minutes, and labeled with PI (5 mg/mL) in the presence of RNase A at 37°C in darkness for 30 minutes. Then, the samples were analyzed by FACSscan flow cytometer (Becton Dickinson). Analysis of the results was carried out using FlowJo 7.6.1 software.

Immunoblot assay
Coimmunoprecipitation (Co-IP) experiments were completed using a Pierce Direct IP Kit. Cell lysates were equally loaded to 10%–12% SDS-polyacrylamide gels, electrophoresed, and transferred to an Immobilon-P PVDF Transfer Membrane (0.45 μm, Millipore). Membranes were blocked for 1 hour in TBS-Tween-20 containing 5% nonfat milk and then incubated with primary antibodies at 4°C overnight. After being washed 3 times, the membranes were incubated with horseradish peroxidase (HRP)-linked secondary antibodies at room temperature for 1 hour. Membranes were incubated with horseradish peroxidase (HRP)-linked secondary antibodies at room temperature for 1 hour. The membranes were visualized by a SuperSignal West Pico Chemiluminescent Substrate according to the manufacturer's instructions. All experiments were repeated three times.

Electron microscopy
Ch282–5–treated HCT116 cells were fixed in ice-cold 2% glutaraldehyde, rinsed with PBS, postfixed in 1% osmium tetroxide with 0.1% potassium ferricyanide, dehydrated through a graded series of ethanol (30%–100%), and embedded in Epon. Ultrathin sections (65 nm) were stained with 2% uranyl acetate and Reynold lead citrate and examined at 80 kV using a PHILIP CM-120 transmission electron microscope.

Fluorescence microscope
For TUNEL assay, ch282-5–treated cells were fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. After washing with PBS, cells were processed in permeabilization solution for 2 minutes at 4°C, washed with PBS, and incubated in TUNEL reaction mixture for 1 hour at 37°C in the dark. Nuclei were stained with Hoechst33342. For JC-1 assay and Mito-tracker/Lyso-tracker staining, the treated cells were incubated with JC-1, Mito-tracker, or Lyso-tracker for 20 to 30 minutes at 37°C. After washing with PBS, cells were processed in permeabilization solution for 2 minutes at 4°C, washed with PBS, and incubated in TUNEL reaction mixture for 1 hour at 37°C in the dark. Nuclei were stained with Hoechst33342. For JC-1 assay and Mito-tracker/Lyso-tracker staining, the treated cells were incubated with JC-1, Mito-tracker, or Lyso-tracker for 20 to 30 minutes at 37°C, and then washed twice with PBS. Samples were observed under a fluorescence microscope. Three random fields were recorded for statistical analysis.

Invasion and migration assay
For invasion assay, the transwell system (24 wells, 8 mm pore size with polycarbonate membrane; Corning Costar) was coated with 3 mg/mL Matrigel (BD Biosciences). Three hours later, 5 × 10⁵ CT26 cells in 100 μL serum-free 1640 medium were seeded into the top chambers and 1,000 μL 1640 medium containing 20% FBS and 10 μmol/L ch282-5 was added into the bottom chamber. Twenty-four hours later, cells that did not invade through the pores of the transwell inserts were removed with a cotton swab and the inserts were fixed in cold methanol for 10 minutes and then stained with 0.1% crystal violet. For migration assay, the experiments were performed as above except that cells were plated on top of uncoated (Matrigel-free) inserts. The migrated or invaded cells were photographed under a light microscope.

In vitro scratch assay
CT26-MSCV and CT26-MSCV-AEP cells were seeded in 24-well plates. When the cells grew to 90% confluence, a scratch was introduced through the cell monolayer using a 200 μL pipette tip. The cells were washed with PBS and immediately incubated with 10 μmol/L ch282-5. The scratch area was photographed at 0 and 24 hours after the drug exposure.

In vivo studies
Five- to 6-week-old male Nu/Nu mice and BALB/c mice were purchased from Shanghai SLAC Laboratory Animal Center and care of the mice was consistent with the guidelines of Shanghai Model Organisms Research Center. In toxicity study, Nu/Nu mice were given 10% ethanol as vehicle control, 120 μmol/kg/day of ch282-5 or gossypol for 12 consecutive days. After treatment, small intestine and colon were fixed in 4% paraformaldehyde (PFA) and stained with hematoxylin and eosin (H&E). In efficacy studies, Nu/Nu mice or BALB/c mice received subcutaneous injection of 5 × 10⁵ CT26 cells, 1 × 10⁵ HCT116 or HT29 cells in the right flank area. Once tumors grew up to 60 mm³, mice were randomly divided into various groups with six mice per group at least. The mice were treated with 120 μmol/kg ch282-5 (intragastrically; vehicle was 10% ethanol, 60% Phosal 50 PG/30% PEG 400/10% ethanol or 82% PBS/5% Tween-80/5% PEG 400/8% ethanol), 120 μmol/kg CQ (intraperitoneally; vehicle as described above), 20 mg/kg rappamycin (intraperitoneally; vehicle as described above), 3 mg/kg oxaliplatin (intraperitoneally; vehicle was 5% glucose solution), 50 mg/kg ABT-263 (intragastrically; vehicle was 60% Phosal 50 PG/30% PEG 400/10% ethanol) or their given combination every other day. The tumors were measured by length and width every 3 days, and tumor volume was calculated by the formula: (length × width²)/2. In experimental liver metastasis study, 1 × 10⁵ CT26 cells were injected into spleen of Balbc mice. Mice were treated with 120 μmol/kg/ch282-5 every other day, until any one of mice died first. The livers of dead mice were fixed by 4% PFA, and the survival dates were recorded.

Statistical analysis
All experiments were performed at least thrice, and the results were expressed as mean ± SD wherever applicable. GraphPad Prism 5 software was used for statistical analysis. Student t-test was used to compare the differences between variables. P value < 0.05 was considered statistically significant (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

Results
Ch282-5 is an efficacious Bcl-2 family inhibitor without obvious side effects
To augment the bioavailability of the gossypol derivative compound-7 that we have previously reported (5), we introduced 2-aminothanesulfonic acid sodium into R groups to develop a novel gossypol derivative ch282-5 (MW 804) based on the formation of a gossypol Schiff base (Fig. 1A–C and Supplementary Fig. S3). In light of Lipinski Rule of Five, ideal drugs should exhibit log P values in the range from −0.4 to +5.6. We applied a forecasting system that we established to calculate the hydrophobic constant of ch282-5 (log P = 4.63) which is much lower than that of compound-7 (log P = 8.33), indicating an increase of hydrophilicity (Supplementary Table S1). As expected, over 12
mmol ch282-5 can be dissolved in 100 mL H2O at 37°C, compared with 9.3813 mmol compound-7 and 0.0019 mmol gossypol. For a stability test, ch282-5 was dissolved in PBS buffer (pH 7.4) for 180 minutes and above 70% identifiable ch282-5 could be detected by high-performance liquid chromatography, which indicated a better stability of ch282-5 at a similar condition of physiology (Supplementary Fig. S4). In addition, the predicted acute toxicity of ch282-5 was below compound-7 (Supplementary Table S1). Therefore, ch282-5 might be more amenable to absorption, distribution, and metabolism than compound-7.

Molecular models of ch282-5 binding with Bcl-2, Bcl-xL, and Mcl-1 (PDB code: 4AQ3, 1YSI, and 4HW2) were simulated by the FlexX software, which disclosed a high affinity of ch282-5 to the target proteins. A fluorescence polarization-based assay further confirmed that His-Bcl-2, His-Bcl-xL, and His-Mcl-1 bound to the FAM-Bim-BH3 peptide with a Kd value of 400 nmol/L, 100 nmol/L, and 250 nmol/L, respectively (Supplementary Fig. S5A–S5C), and ch282-5 competitively disrupted the interaction of Bcl-2 (Ki 3.0 μmol/L), Bcl-xL (Ki 1.6 μmol/L), Mcl-1 (Ki 0.254 μmol/L), and proapoptotic proteins (Supplementary Fig. S5D–S5F).

Figure 1. Ch282-5 is an efficacious Bcl-2 family proteins inhibitor without obvious side effects. Chemical structure of gossypol (A), 6-APA-Na-gossypolone: 6-aminopenicillanic acid sodium- gossypolone (compound 7; B), and ch282-5: 2-aminoethanesulfonic acid sodium-gossypolone (C). Molecular model studies by the FlexX software, molecular model of Bcl-2 (D), Bcl-xL (E), and Mcl-1 (F) binding with ch282-5. Toxicity test in colon cancer xenograft model. G, the body weight of Nu/Nu mice was measured every day. H, tumor volume at day 15 after commence of gossypol or ch282-5 treatment every other day. I, percentage survival curves of Nu/Nu mice with different treatments; Results of biochemical indexes (J) and routine blood test (K). L, ch282-5-treated mice have no obvious damage in H&E-stained small intestine (b) and colon (e) compared with the control (a and d), while gossypol lead to small intestine basement membrane hyperplasia (c) and colon expansion (f).
To assess the antitumor activity and possible side effects of ch282-5 in vivo, we measured the mice body weight, tumor volume, survival rate, biochemical indexes, and routine blood test as well as performed pathologic examinations on mice organs. Body weight and survival of ch282-5–treated mice had no significant reduction compared with the control, whereas the loss of body weight and a decrease of survival in gossypol-treated mice were significant (Fig. 1G and I). In contrast, treatment with ch282-5 at the same dose significantly inhibited the tumor growth as compared with either gossypol- or vehicle-treated group (Fig. 1H). We compared biochemical indexes (Fig. 1I) and routine blood tests (Fig. 1K) between vehicle control, 120 μmol/kg gossypol, and 120 μmol/kg ch282-5 treated mice. An increase in high-density lipoprotein (LDL-C) indicating liver damage was seen in gossypol-treated mice not in ch282-5–treated group. A decrease in alkaline phosphatase (ALP), creatinine (CRE), glucose (GLU), and inorganic phosphate may indicate gossypol breakdown and absorption. Ch282-5 raised inorganic phosphate level in contrast to the control and gossypol group, so we had to confirm or exclude the nephrotoxicity by other indexes. As ch282-5 decreased urea acid (UA) level and caused no significant changes in urea nitrogen (BUN) and potassium (K+) levels, we may exclude the possibility of renal toxicity. Ch282-5 doubled the level of white blood cell (WBC). Both gossypol and ch282-5 treatment markedly increased blood platelet count with a larger effect for gossypol, which implicated a possible risk of thrombosis. In addition, ch282-5 caused a most noticeable increase of neutrophilic granulocyte. A decrease in lymphocyte, monocyte, and eosinophil granulocyte was observed in both groups. Histologic examinations revealed that Ch282-5–treated mice had no damage in the small intestine and colon (Fig. 1L, b and e) compared with the controls (Fig. 1L, a and d), whereas gossypol caused small intestine basement membrane hyperplasia (Fig. 1L, c) and colon expansion (Fig. 1L, f).

Ch282-5 inhibits cell proliferation by cell-cycle arrest and the mitochondria-dependent apoptosis

To assess the growth inhibitory effect of ch282-5 on colon cancer cells, 40 different cancer cell lines were treated with ch282-5 and tested for cell viability by MTS assay. The results showed that ch282-5 significantly suppressed the growth of multiple cancer cell lines including colon, breast, prostate, and gastric cancer (Supplementary Table S2). Human colon cancer cells were sensitive to ch282-5 with the EC50 between 5 and 20 μmol/L (72-hour continuous exposure). Treatment of HCT116, HT29, SW620, or C126 cells with ch282-5 reduced cell viability in a dose- and time-dependent manner (Fig. 2A and Supplementary Fig. S6A). Ch282-5–induced growth inhibition was further confirmed by a colony formation assay (Supplementary Fig. S6B).

To determine the factors responsible for the reduction in cell viability, we considered three aspects: apoptosis, necrosis, and cell-cycle suppression. Necrosis is characterized by cell swelling, plasma membrane permeabilization, mitochondria dysfunction, and release of mitochondrial content to cytoplasm (15). All characteristics of necrosis are similar to apoptosis except no DNA fragmentation. We found that ch282-5–treated cells died of apoptosis with DNA fragmentation (Fig. 2B and C). Flow cytometric analysis showed that 35% to 40% of HCT116 and HT29 cells treated with 20 μmol/L ch282-5 were at the early and late stages of apoptosis (Fig. 2D). Moreover, ch282-5 induced apoptosis in a time- and dose-dependent fashion. Immunoblots showed that PARP were activated by ch282-5 (Fig. 2E), which confirmed apoptotic cell death induced by ch282-5. Meanwhile, in both HCT116 and HT29 cells, ch282-5 induced Sub-G1 phase arrest in a dose- and time-dependent manner (Fig. 2F). This phenomenon was confirmed by decreased Cyclin D1 and increased CHOP expression (Fig. 2G). CHOP was identified as a C/EBP-homologous protein, which suppresses cell-cycle progression from G1–S phase.

Mitochondrial damage is a principal incentive of apoptosis. To further interrogate the mechanism of ch282-5–induced apoptosis in colon cancer cells, transmission electron microscopy, and fluorescence microscopy were employed. We observed pyknosis nucleus, apoptotic vesicles, damaged spinal, mitochondria vacuoles in ch282-5–treated cells, whereas normal nuclei and mitochondria were observed in control cells (Fig. 2H). Changes of mitochondrial membrane potential (ΔΨm) were assessed in a fluorescence microscope with the mitochondrial-specific dual fluorescence probe JC-1. We observed green fluorescence in the ch282-5–treated HCT116 cells and HT29 cells but red fluorescence in the untreated control (Fig. 2I), suggesting that ch282-5 significantly damaged mitochondria.

Ch282-5 regulates Bcl-2 and IAP family proteins and induces caspase-dependent apoptosis pathway

Coimmunoprecipitation was used to detect ch282-5–induced competitive disruption of the connection between Mcl-1 and Bim, Noxa in ch282-5–treated HCT116 cells (Fig. 3A) as dissociated proapoptotic proteins would oligomerize to mitochondrial membrane and formed pores. As a result, mitochondria were damaged, and mitochondria contents were released to cytoplasm in ch282-5–treated cells (Fig. 3B). The data further demonstrated that ch282-5 induced a cytochrome c–initiated apoptosis pathway. Meanwhile, an increase in phosphorylated-JNK (Fig. 3C) might promote Bak and Bax dissociating from the 14-3-3 protein, and encourages the progression of Bak and Bax oligomer into and permeabilizes the mitochondria membrane (16). Hence, a certain degree of Bak and Bax reduction occurred in the cytoplasm (Supplementary Fig. S7A). Dissociated Bcl-2 family antiapoptosis proteins were significantly decreased (Fig. 3D). Real-time quantitative PCR excluded mRNA regulation and proteasome inhibitor MG132 could restrain this reduction (3D and Supplementary Fig. S7B and S7C), that is to say, partial dissociated Bcl-2, Bcl-xl, and Mcl-1 were ubiquitinated and degraded (17). IAP family proteins inhibit caspase function to impede apoptosis. Ch282-5 induced Smac/Diablo and AIF release from the mitochondria, which can disrupt connection of IAP proteins and caspase proteins. It is known that dissociated IAP family proteins could be degraded by autoubiquitination (18). Therefore, decreases of c-IAP1, c-IAP2, XIAP, and survivin were observed and MG132 could restrain these decreases to a certain extent (Fig. 3E).

Damaged mitochondria would inevitably cause a drop in ATP level. The AMPK is a sensor of cellular energy status, which is activated by an elevated AMP/ATP ratio (19). We detected an increased phosphorylation of AMPKα at the site of Thr172 (Fig. 3F), which would inhibit mTOR signaling. Recent studies show that survivin is regulated by PI3K/Akt/p70s6K1 pathway (20), and some Raf kinase inhibitors could induce mTOR-dependent survivin downregulation (21). Therefore, we went further to investigate the effects of ch282-5 on mTOR, Akt, and p70s6k pathway. As expected, ch282-5 significantly reduced phosphorylation of mTOR at Ser2448 and Ser2481 site (Fig. 3F), mTOR was...
Figure 2.
Ch282-5 inhibits cell proliferation by cell-cycle arrest and the mitochondria-dependent apoptosis. A, cells were treated with 0, 5, 10, 20 μmol/L ch282-5 for 48 hours and subjected to the MTS assay. B, cells were treated with ch282-5 for 24 hours and then stained with Hoechst33342 and TUNEL. C, record and analysis of positive tunel puncta by GraphPad Prism 5 software based on three random fields. Cells were treated with 10 μmol/L or 20 μmol/L ch282-5 for 12 or 24 hours, respectively, and apoptosis (D) and cell cycle (F) were determined by Annexin V–FITC/PI labeling using flow cytometry. Cells were treated with ch282-5 for 24 hours, and PARP, PARP-1 (E), Cyclin D1 and CHOP (G) were detected by Western blot analysis. H, representative electron micrographs of HCT116 cells (a–f) and SW620 cells (g–i) treated with or without 20 μmol/L ch282-5 for 24 hours. Contrasted normal nucleus (a), pyknosis nucleus (b), apoptotic bodies (c), normal mitochondria (d and g), damaged mitochondria ridge (e and h), mitochondria vacuolization (f and i). I, HCT116 and HT29 cells were treated without 10 μmol/L ch282-5 for 6 hours, and then imaging technique was used to assess the change of mitochondrial membrane potential of cells treated with ch282-5 by the JC-1 method.
phosphorylated at Ser2448 via the PI3 kinase/Akt signaling pathway and autophosphorylated at Ser2481 (22, 23). Akt and Erk promote mTORC1 signaling through phosphorylation of a GTPase activator protein (GAP), referred to as tuberous sclerosis complex 2 (TSC2; ref. 24), and the tuberin/hamartin (TSC2/TSC1) complex inhibits mTOR activity indirectly by inhibiting Rheb (25). In accord with these previous observations, we found that the level of P-Akt (Ser473), P-Erk, and Rheb proteins was all reduced (Fig. 3F). The decrease of phosphorylated mTOR led to the reduction of P-Akt (Ser473), P-Erk, and Rheb proteins was all reduced (Fig. 3F). The decrease of phosphorylated mTOR led to the reduction of phosphorylated mTOR and inhibition of p70s6k (an activator of translation) and activation of p65 (transcription factors of the NF-κB; Fig. 3G). Inhibition of p70s6k and p65 could directly cause a reduction in the synthesis of some proteins such as Bcl-2 and IAP family antiapoptotic proteins.

Decrease in antiapoptotic proteins and activation of proapoptotic proteins (Fig. 3D, E, and H and Supplementary Fig. S7A) could directly cause a reduction in the synthesis of some proteins such as Bcl-2 and IAP family antiapoptotic proteins. Ch282-5 induced competitive disrupting the connection of Mcl-1 and Bim, Noxa in HCT116 cells treated with 20 μmol/L. Ch282-5 for 6 hours. B, cells were treated with ch282-5 for 30 minutes, and then the distributions of Cyto-c, Smac, and AIF in both cytoplasm and mitochondrion were analyzed by Western blot analysis. HCT116 and HT29 cells were treated with 0, 10, 20 μmol/L ch282-5 for 6 hours. B, cells were treated with ch282-5 for 30 minutes, and then the distributions of Cyto-c, Smac, and AIF in both cytoplasm and mitochondrion were analyzed by Western blot analysis. HCT116 and HT29 cells were treated with 0, 10, 20 μmol/L ch282-5 for 24 hours, and then JNK, P-JNK (C), mTOR, P-mTOR, AMPKα (Thr172), AMPKα (Ser2481), Akt, and Erk 1/2, P-Akt, P-Erk 1/2, P-JNK, and P-mTOR were detected by Western blot analysis (I).
Ch282-5 antitumor effect is augmented by disturbing mitophagy and mTOR pathway

Given that Bcl-2 inhibitors simultaneously induce mitochondrial damage in tumor cells and activate mitochondrial quality control, and mitophagy has emerged as a key mechanism in this quality control for the elimination of superfluous or damaged mitochondria (26), we sought to determine whether ch282-5 triggers mitophagy in colon cancer cells. Electron microscopy ultrastructural analyses revealed a series of characteristic phenomena of mitophagy in ch282-5-treated HCT116 and SW620 cells that included an increase of primary lysosome (Fig. 4A, a, white arrow), secondary lysosome (Fig. 4A, a, black arrow), damaged mitophagy in the autophagosome (Fig. 4A, b, d, and e), secondary lysosome with a monolayer membrane structure fused with the autophagosome to become autolysosome (Fig. 4A, c, black arrow), and digested autolysosome (Fig. 4A, c, white arrow, and 4A, f). Colocalization staining of Mito-tracker and Lyso-tracker can be used to track mitophagy (27). We observed an increase of colocalization (yellow) staining in ch282-5-treated CT26 cells in an early phase (Fig. 4B), and the similar phenomena were all found in HCT116, HT29, and SW620 cells (Supplementary Fig. S8A–S8C), clearly indicating that ch282-5 induced a typical mitophagy. Ch282-5-induced mitophagy was also confirmed in HCT116-GFP-LC3b and HT29-GFP-LC3b cell lines as evidenced by GFP-LC3b punctate aggregation (Fig. 4C and D). Moreover, decreased Beclin-1, Lamp-2, and p62, and increased LC3I/II were all unfolded in a late phase of mitophagy (Fig. 4E). These results suggest that ch282-5-induced dose-dependent mitophagy in colon cancer cells. Coimmunoprecipitation experiment revealed that ch282-5 competitive dissociated Beclin-1 from Bcl-2, Bcl-xl, and Mcl-1 service to mitophagy (Supplementary Fig. S8D–S8F).

It is conceivable that combining ch282-5 with a mitophagy inhibitor may augment inhibitory effects of ch282-5 on colon cancer cells. Indeed, 5 μmol/L or 10 μmol/L CQ can significantly potentiate the growth inhibitory effect of ch282-5 on HCT116 and HT29 cells (Fig. 4F and G). Mitophagy inhibitors 3-MA and Wortmannin could also sensitize these cells to ch282-5 (Supplementary Fig. S8G and S8H). Colocalization of lyso-tracker–labeled lysosome and GFP–labeled autophagosome suggested that CQ plays a role in blocking autophagosome fusion with lysosome in ch282-5–treated cells (Supplementary Fig. S8I). The JC-1 method, DNA ladder assay, and Western blot analysis all showed that CQ could enhance ch282-5–induced mitochondrial-dependent apoptotic cell death (Supplementary Fig. S8J–S8L). The assumption that disruption of mitophagy could augment ch282-5–induced inhibition on colon cancer cells were validated in HCT116 and HT29 xenograft models. We obtained the results that the antitumor effect of ch282-5 or CQ alone at the same dose of 120 μmol/kg/2 days was similar, whereas combined treatment significantly enhanced the effect (Fig. 4I and L).

A triple combination of CQ, rapamycin, and ch282-5 was further investigated in the colon cancer models. To our surprise, this triple combination therapy remarkably inhibited CT26 cells both in vitro (Fig. 4H) and in vivo (Fig. 4K) compared with single use or double combination. A similar result was also obtained with HCT116 cells (Supplementary Fig. S8M). We next investigated the effects of triple combination on IAP family proteins by Western blot analysis. The results showed that phosphorylation of mTOR was reduced to the maximum extent by triple combination (Fig. 4L), and P-p70S6k was completely disappeared under this treatment (Fig. 4M). Accordingly, downstream proteins of 4E-BP1 and p65 were also suppressed and the greatest degree of reduction was seen in IAP family proteins (Fig. 4M and N). In the same time, we observed the autophagy flux by detecting p62, LC3I/II, Lamp1, and Lamp2 (Supplementary Fig. S8N).

Ch282-5 suppresses liver colonization of colon cancer cells

The intrasplenic injection of human cancer cells into the nude mice produces experimental metastases in the liver, and cells injected into the spleen reach the liver parenchyma within minutes (28). Thus, the intrasplenic implantation of cells measures their ability to grow in the liver parenchyma and the effect of drug treatment represents its efficacy in suppression of established metastasis. As shown in Fig. 5A, treatment with 120 μmol/kg ch282-5 every other day substantially increased survival of the treated mice. We observed that the liver volume of ch282-5–treated mice was slightly smaller than that of vehicle-treated mice, and ch282-5–treated mice had less metastasis (Supplementary Fig. S9A). To further clarify that antimetastatic effect of ch282-5 was mediated through suppression of established metastasis, we set up two groups of experimental liver metastasis models, and then treated one group at day 1 and the other at day 8 with the same dose of ch282-5 after the intrasplenic injection of CT26 cells. There was no significant difference in the inhibition of liver metastasis between the two groups (Fig. 5B and Supplementary Fig. S9B) implying that ch282-5 did not inhibit the process of CT26 cells migrated from the spleen to the liver but rather suppressed the proliferation of metastasized cells in the liver.

In vitro experiment demonstrated that ch282-5 could significantly reduce migration and invasion of CT26 cells (Fig. 5C) although moderate reduction in cell viability by 10 μmol/L ch282-5 treatment (data not shown) may partially contribute to the less invasive capabilities of the cells. Tumor metastasis may be associated with STAT, AKT, ERK, and mTOR pathway (12, 29). We found STAT3, STAT5b, STAT6, and their phosphorylation forms were reduced by ch282-5 (Fig. 5D), and phosphorylated AKT, ERK, and mTOR were all decreased (Fig. 5E). Ch282-5 also reduced the expression of metastasis-related proteins MMP-2, MMP-9, AEP, Cathepsin-B, and Cathepsin-L (Fig. 5F and I). We validated that AEP also increased metastatic potential of colon cancer by scratch assay. AEP-overexpressing CT26 cells migrated faster than AEP-WT CT26 cells, and ch282-5 slowed down the cell migration in both cell types (Fig. 5G and H).

Ch282-5 potentiates effectiveness of oxaliplatin and rescues ABT-263 efficacy

A more rational approach to utilize Bcl-2 inhibitors in clinical applications is the combination with other targeted agents. In this regard, the anticancer property of ch282-5 in combination with clinical and preclinical anticancer drugs against colon cancer was examined in vitro and in vivo. Oxaliplatin, widely used for colon cancer chemotherapy, and another BH3 mimic compound ABT-263 were selected for our drug combination study. As shown in Fig. 6A and D, the combination of ch282-5 and oxaliplatin or ABT-263 had a better growth inhibitory effect than used alone. In CT26 xenograft models, the combination of ch282-5 at 120 μmol/kg/2 days with oxaliplatin at 3 mg/kg/2 days or ABT-263 at 50 mg/kg/2 days was considerably more effective than ch282-5, oxaliplatin, or ABT-263 alone (P < 0.05) without enhanced toxicity (Fig. 6B, C, and E). ABT-263 is a potent Bcl-2 family inhibitor as it can bind to Bcl-1, Bcl-xl, Bcl-w, but not Mcl-1 (30), while ch282-5 is sensitive to Mcl-1. We assumed that
combination of these two may be beneficial in terms of targeting different Bcl-2 family antiapoptotic proteins and possible reduction of ABT-263 side effects. ABT-263 had entered phase II clinical trial but its use is limited by the adverse effect of platelet reduction (31). We found that ch282-5 alone increased the platelet count and could rescue more than 50% of platelet drop induced by ABT-263 (Fig. 6F). These observations indicate that ch282-5 not only augments ABT-263–curative effect, but also possibly reduces a risk of thrombocytopenia.

**Discussion**

The American Cancer Society provides an estimate that 45,890 men and 47,200 women will be diagnosed with colorectal cancer and 26,100 men and 23,600 women will die of this disease in 2015 (9). Although some styles of colorectal cancer can be effectively treated with combination chemotherapy, many cancer patients die of metastasis and multidrug resistance. In recent years, a series of excellent Bcl-2 inhibitors have been developed to solve these problems. A natural Bcl-2 inhibitor gossypol has drawn a great deal of attention from cancer researchers in the field, but is limited in clinical applications for its low hydrophilicity and negative side effects (4, 32). We previously reported a series of gossypol derivatives, one of which is referred as “compound-7” that demonstrated a relatively positive effect on colon cancer treatment (5). Compound-7 competitively binds to Bcl-xl. inducing apoptosis in cells and inhibiting tumor growth in the CT26 xenograft model. However, stability and hydrophobicity still limit its value in clinical application.

Ch282-5 was conceived on the basis of the compound-7 synthesis process by changing the active aldehyde group of gossypol into keto group in an attempt to reduce side effects. As expected, ch282-5 caused less severe liver injury than gossypol but almost no damage to the small intestine or colon and did not affect the digestion and absorption. Ch282-5 significantly improved the immune system in tumor bearing mice compared with gossypol. Furthermore, the additional step of replacing the R group of gossypol with sodium taurate to enhance hydrophilicity was also taken. Ch282-5 may adapt to kill the cancer cells that overexpress Mcl-1 due to its high affinity to Mcl-1. Possibly, ch282-5 is complementary to the small-molecule Bcl-2 inhibitors that have a low affinity binding to Mcl-1, such that the combination of ch282-5 and ABT-263 obtained a favorable result. In addition, ch282-5 has appropriate hydrophilicity constant and stability for medicinal applications.

Multiple cancer cell lines were sensitive to ch282-5, such as breast cancer, colon cancer, gastric cancer, glioma, lung cancer, melanoma, pancreatic cancer, prostate cancer, etc. (Supplementary Table S2), which indicates that ch282-5 has a broad-spectrum antitumor property. Just like any other Bcl-2 inhibitors, ch282-5 inhibits tumor cells in both time- and dose-dependent manner and induces typical mitochondrion-dependent apoptotic cell death. As proposed in Supplementary Fig. S10B for ch282-5 induced mitochondria cascade effects, ch282-5 competitively binds to the BH3 domain of Mcl-1 and releases proapoptotic proteins Bim and Noxa with decreased Bcl-2 prosurvival proteins in HCT116 cells (Fig. 3E and I). Ch282-5 phosphorylates SAPK/JNK then promotes Bax/Bak dissociation from the 14-3-3 protein and oligomersize into the mitochondrial membrane, resulting in release of mitochondrial contents though membrane channels (16). Cytoplasmic cytochrome c combined with caspase-9 formed activated apoptosis that activated caspase-3, 6, and 7. Released AIF from mitochondria directly enters the nucleus and participates in DNA damage event. IAPs regulate ubiquitin-dependent signaling events that participate in activation of NF-kB and MAPK pathways that control expression of critical genes related to inflammation, immunity, cell migration, and cell survival (33). AIF and Smac/Diablo can relieve the inhibition of IAP family proteins to caspase-3, 7, and 9. Furthermore, dissociated IAP family proteins were degraded by autoubiquitination (18). We have excluded the possibility that a decrease in antiapoptotic Bcl-2 family protein level results from change in RNA level. In addition, both lysisosomal-dependent autophagy degradation and lysosomal-independent ubiquitin degradation can reduce proteins level. Ubiquitin degradation can be inhibited by MG132, a proteasome inhibitor (17). We confirmed that dissociated Bcl-2, Bcl-xl, and Mcl-1 underwent ubiquitination degradation by MG132. Dissociated Bim is alternatively spliced into three major forms BimL, BimS, and BimI. BimL is the most cytoxic and is transiently expressed during apoptosis. BimL, BimS, and BimI may be sequestered to the dynamic motor complex and released from this complex during apoptosis (34). Apoptosis is closely related to a G0–G1 cell-cycle arrest (35). Under ch282-5 stress, the cell cycles of HCT116 and HT29 were blocked at the Sub-G1 phase. Correspondingly, changes in key protein markers of the G1 phase transition to S-phase, decreased Cyclin D1, and accumulated CHOP were observed (36, 37).

Autophagy and apoptosis often occur in the same cell, mostly in a sequence in which autophagy precedes apoptosis (38). Nonetheless, if the cell commences apoptosis, autophagy can be inactivated, in part owing to the caspase-8–mediated cleavage of...
Beclin-1 which is essential to autophagy (8). Inactivating mutation of caspase-8 has been identified in various kinds of colorectal cancers, which prevents the recruitment of the wild-type form of caspase-8 to active death receptors, thereby inhibiting apoptosis (39, 40). SEM, fluorescence microscopy, coimmunoprecipitation, and Western blot analysis confirmed ch282-5–induced autophagy. Hence, we had better prevent autophagy at occurrence stage by inhibitors. Ch282–5 competitively binds to the BH3 domain of Bcl-2, Bcl-xl, and Mcl-1 and then releases Beclin-1. Combination of released Beclin-1 with PI3K, p150, and hVPS34 was involved in the formation of autophagosome membranes (41). CQ inhibits autophagy by disrupting the fusion of autophagosome and lysosome (42). Both wortmannin and 3-MA inhibit autophagy by disrupting the fusion of autophagosome and lysosome (41). CQ inhibits the formation of autophagosome membranes (41). CQ inhibits autophagy by disrupting the fusion of autophagosome and lysosome (42). Both Wortmannin and 3-MA inhibit autophagy by inactivating PI3K (43). As expected, CQ, wortmannin, and 3-MA enhanced ch282-5–induced apoptotic cell death to some extent in HCT116 and HT29 cells. In combination treatment, CQ also plays a role in inhibiting autophagy and enhances ch282-5–induced mitochondria-dependent apoptosis, resulting in increased DNA fragmentation and the release of mitochondrial contents. Numerous preclinical studies have found that inhibition of autophagy by CQ restores chemosensitivity and promotes tumor cell death by diverse anticancer therapies (42).

Although there is a dispute between autophagy inhibition and induction as a cancer therapeutic strategy, preclinical and clinical trials of these modalities were underway (44). Autophagy inhibition is a common combined strategy in Bcl-2–targeting therapy (45), while autophagy induction is also occasionally attempted in this targeting therapy (46). This may seem contradictory, because we often fix the protein in one kind of signal pathway, thinking that regulation of this protein would only affect “its pathway” thus ignoring functions of the protein beyond “its pathway.” Autophagy inhibitor CQ is currently under clinical stage I/II/III investigation in combination with standard treatment in multiple tumor types (44, 47). Autophagy-inducer rapamycin inhibits mTOR signals and promotes autophagy, while decreasing phosphorylation of mTOR also inhibits antiapoptotic protein

![Image](https://www.aacrjournals.org)
Ch282-5 could potentiate effectiveness of oxaliplatin and rescue ABT-263 efficacy. A, SW620 cells and CT26 cells were treated with ch282-5, oxaliplatin, and their combination for 72 hours, and then the cell ability was assessed by MTS assay. CT26 xenograft mice were treated with ch282-5, oxaliplatin, or their combination, and tumor volume (B) and body weight (C) were analyzed. D, HCT116 cells and CT26 cells were treated with ch282-5, ABT-263, and their combination for 72 hours, and then the cell ability was assessed by MTS assay. CT26 xenograft mice were treated with ch282-5, ABT-263, or their combination, and tumor volume (E) and routine blood were tested (F).

Ch282-5 not only effectively inhibited the growth of colon cancer cells xenografted in a nude mice but also suppressed colon cancer cell migration, invasion, and experimental liver metastasis. We have previously reported that overexpression of AEP is significant for breast cancer invasion/metastasis (48), and ABT-263 had been a new functional target for tumoricidal prodrug development and therapy. AEP localized in the front of invasive tumor cells, forming a complex with integrins, and the combined AEP can activate MMP-2 and Cathepsin-L for metastasis (49).

Combination of ch282-5 and oxaliplatin or ABT-263 seems to be a feasible strategy against colon cancer. Ch282-5 and oxaliplatin do not share the same target, so they might synergize each other at a lower dose. Although ABT-737 and ABT-263 reduced palates in vitro, they are still potent Bcl-2 family inhibitors useful for several cancer treatments. While ABT-199 alleviated the inhibition of platelet to a large extent (31), ABT-737 and ABT-263 combination studies are always popular and meaningful. Rapamycin rescued ABT-737 induced platelet decrease by unknown mechanisms (46), but rapamycin and ch282-5 have similar effects on mTOR activation and IAP family protein regulation, which might explain ch282-5–induced platelet reversion.

In summary, the novel gossypol derivate ch282-5 overcomes the undesirable side effects and hydrophobicity of natural gossypol, and improves the stability and bioavailability over compound-7. Ch282-5 antitumor effectiveness can be amplified through combination with CQ and rapamycin, thus making it an excellent candidate as an anticancer therapeutics. Furthermore, anti-metastasis is a crucial feature of ch282-5. Clarification of the molecular mechanisms responsible for the potent antitumor activity of ch282-5 in colon and other cancer models merits further study. Because ch282-5 is a relatively safe and inexpensive agent, further clinical studies are warranted to confirm the experimental observations obtained from this study.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: X. Wang, C. Zhang, B. Lan, J. Wang, C. Wei, B. Jiang, X. Lin, F. Guo
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A Novel Bioavailable BH3 Mimetic Efficiently Inhibits Colon Cancer via Cascade Effects of Mitochondria

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