

Bcl-xL-specific BH3 mimetic A-1331852 suppresses proliferation of fluorouracil-resistant colorectal cancer cells by inducing apoptosis

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Abstract. BH3 mimetics are small-molecule inhibitors of the antiapoptotic Bcl-2 family and have therapeutic efficacy against hematological malignancies. BH3 mimetic A-1331852 suppresses colorectal cancer cell proliferation. Progressive resistance to the widely used anticancer agent fluorouracil (5-FU) is a key reason for colorectal cancer recurrence; therefore, the present study tested if A-1331852 can suppress the proliferation of 5-FU-resistant colorectal cancer cells. A 5-FU-resistant colorectal cancer cell line was derived from HCT116 cells and compared with the parental line. Expression levels of the antiapoptotic Bcl-2 proteins Bcl-xL and myeloid cell leukemia 1 (Mcl-1) were determined via western blotting, proliferation in the presence of 5-FU and following small interfering (si)RNA-mediated Bcl-xL or Mcl-1 knockdown was assessed by WST-1 assay and sensitivity to A-1331852-induced apoptosis was assessed via western blotting and DNA fragmentation assay. In addition, a xenograft mouse model of 5-FU-resistant colorectal cancer was established via subcutaneous inoculation of 5-FU-resistant HCT116 cells to examine the *in vivo* antitumor efficacy of A-1331852. Compared with the parental line, 5-FU-resistant cells overexpressed Bcl-xL. Knockdown of Bcl-xL by siRNA and treatment with A-1331852 suppressed proliferation and induced the apoptosis of both 5-FU-resistant and parental HCT116 cells, but the potency of both effects was stronger in 5-FU-resistant than parental HCT116 cells. Furthermore, A-1331852 suppressed the growth

of xenograft tumors derived from 5-FU-resistant cells by inducing apoptosis. Overall, the present findings suggested that Bcl-xL upregulation contributes to 5-FU resistance of colorectal cancer and targeted inhibition by A-1331852 may be an effective treatment strategy.

Introduction

Colorectal cancer was the third most commonly diagnosed cancer worldwide in 2020 and the second most common cause of cancer-associated death (1,2). Although advances in diagnosis and surgical techniques have led to improvement in clinical outcomes, survival rate of patients with metastatic colorectal cancer remains poor (3). Median overall survival (OS) is 36 months, and a 5-year OS $\leq 20\%$ (4). For metastatic colorectal cancer, treatment options include systemic chemotherapy combined with targeted therapy or immunotherapy (4). Chemotherapy remains the standard treatment for metastatic colorectal cancer and 5-fluorouracil (5-FU) is a key component (5). However, prolonged use of 5-FU results in resistance and tumor recurrence; therefore, mechanisms underlying 5-FU resistance should be elucidated and alternative or adjunct therapies that sustain anticancer efficacy must be developed.

Dysregulation of apoptotic signaling pathways and evasion of apoptosis are strongly implicated in tumor progression and chemoresistance (6,7). Cellular apoptosis is induced by an extrinsic signaling pathway initiated by extracellular factors or by an intrinsic pathway initiated by intracellular injury and regulated by antiapoptotic and proapoptotic factors, including Bcl-2 family proteins (8,9). Antiapoptotic Bcl-2 family proteins are upregulated in numerous cancer cell types (including prostate, colorectal, lung, gastric, renal cancer, neuroblastoma, non-Hodgkin's lymphoma, and both acute and chronic leukaemia) (10) and associated with tumor progression or chemoresistance (11), suggesting they can serve as therapeutic targets (12). Levels of apoptosis are determined in part by the balance between proapoptotic and antiapoptotic Bcl-2 family proteins, as these antagonistic proteins can interact directly via various Bcl-2 homology (BH) protein motifs. BH3

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motif acts as a strong cell death induction signal, and Bcl-2 proteins containing only this motif, such as BH3-interacting domain death agonist (BID) and Bcl-2 antagonist of cell death (BAD), promote apoptosis by directly binding antiapoptotic Bcl-2 family protein. Therefore, BH3 motif may be a useful target for modulating apoptosis signaling.

Recently, small-molecule BH3 mimetics that can directly inhibit antiapoptotic Bcl-2 family proteins and induce cancer cell apoptosis have been developed (13). These anticancer effects have been studied primarily in hematological malignancy and the BH3 mimic ABT-199 (Venetoclax) has received United States Food and Drug Administration approval for treatment of primary and relapsed chronic lymphocytic leukemia/small lymphocytic leukemia, acute myeloma and several lymphoid malignancies (14). Several studies have reported that the upregulation of antiapoptotic Bcl-xL and Myeloid cell leukemia 1 (Mcl-1) promote colorectal tumor progression and confer chemoresistance (15-18), suggesting inhibition of these antiapoptotic proteins using BH3 mimetics may be a promising therapeutic strategy. To the best of our knowledge, however, only a few studies have examined the anticancer effects of Bcl-xL- or Mcl-1-specific BH3 mimetics on colorectal cancer cells (19-21). Given the poor prognosis following emergence of chemotherapy resistance in colorectal cancer, it is key to examine the effects of Bcl-xL- or Mcl-1-specific BH3 mimetics on 5-FU-resistant (FUR) colorectal cancer cells.

Wang *et al* (22) reported the development of A-1331852, an orally available and highly Bcl-xL-specific BH3 mimetic with notable efficacy in a xenograft colorectal cancer model. Our previous study demonstrated that stromal interactions enhance the expression of Bcl-xL and Mcl-1 in colorectal cancer cells (23), while inhibition of Bcl-xL and Mcl-1 by small interfering (si)RNA transfection or natural flavonoid treatment effectively induces apoptosis and suppresses proliferation of colorectal cancer and pancreatic cancer cells (24,25). Based on these findings, it was hypothesized that the Bcl-xL-specific BH3 mimetic A-1331852 may exert suppressive effects on proliferation and survival of 5FUR colorectal cancer cells.

Materials and methods

Cell culture. The human colorectal cancer cell line HCT116 (cat. no. CCL-247) was obtained from the American Type Culture Collection. HCT116 cell line is KRAS mutant; KRAS mutations are considered a poor prognostic factor (26). HCT116 cells were maintained in DMEM (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% FBS and 1% penicillin-streptomycin solution (both Gibco; Thermo Fisher Scientific, Inc.) at 37°C in 5% CO₂.

Isolation of 5FUR HCT116 (HCT116/5FUR) colorectal cancer cells. HCT116/5FUR was isolated by continuous passage over 100 passages, starting at 0.2 µM and gradually increasing the concentration of 5-FU in increments of 0.2-2.0 µM, as previously reported (5,27). A subline with 270-fold 5-FU resistance compared with the parental cells was isolated. HCT116/5FUR cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin for 2 weeks as aforementioned to eliminate the acute effects of 5-FU. The subline

was authenticated using short tandem repeat DNA analysis by Japanese Collection of Research Bioresources Cell Bank.

Reagents. FU was purchased from FUJIFILM Wako Pure Chemical Corporation; antibodies against Bcl-xL (cat. no. 54H6), Mcl-1 (cat. no. D35A5), PARP (cat. no. 46D11), cleaved PARP (cat. no. D64E10) and GAPDH (all 1:1,000, cat. no. 14C10) were purchased from Cell Signaling Technology, Inc.; horseradish peroxidase (HRP)-conjugated polyclonal goat antirabbit Ig (1:2,000, cat. no. P0448) was purchased from Dako (Agilent Technologies, Inc.) and BH3 mimics A-1331852 and S63845 were purchased from SelleckChem. For *in vitro* experiments, 20 mM stock solutions of A-1331852 and S63845 were prepared in DMSO and stored at -80°C until use.

Transfection of siRNA. Bcl-xL (cat. no. s1922; forward, 5'-GGA ACUCUAUGGGAACAAUTT-3' and reverse, 5'-AUUGU CC CAUAGAGU UCCAC-3'), Mcl-1 (cat. no. s8583; forward, 5'-CCAGUAUACUUCUAGAAATT-3' and reverse, 5'-UUU CUAAGAAGUAUACUGGGA-3') and negative control siRNA (all 5 nM, cat. no. 4390843, Silencer Select Negative Control #1) were purchased from Thermo Fisher Scientific, Inc. For proliferation assays, cells were seeded on 96-well culture plates at 5x10³/well and incubated overnight at 37°C without antibiotics, followed by transfection. For western blotting and DNA fragmentation assay, cells were seeded on 6-well culture plates at a density of 5x10⁵/well and incubated overnight at 37°C without antibiotics before transfection. In both cases, cells were transfected with siRNA (final concentration, 100 nM) for 24, 48 or 72 h at 37°C using Lipofectamine RNAiMAX in Opti-MEN medium (both Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Subsequent experiments were performed immediately following transfection.

Cell proliferation assay. The effects of BH3 mimetics (A-1331852 and S63845) and Bcl-2 protein knockdown (Bcl-xL or Mcl-1) on proliferation rates of HCT116 and HCT116/5FUR cells were evaluated using the Premix WST-1 Cell Proliferation Assay System (Takara Bio, Inc.) according to the manufacturer's protocol. Cells were seeded in 96-well plates at a density of 5x10³/well in 100 µl complete DMEM (FUJIFILM Wako Pure Chemical Corporation) and allowed to attach overnight at 37°C. 5FU, A-1331852 and S63845 were added to each well for 24, 48, or 72 h at 37°C. The concentration of 5-FU was 0-1,000 µM and the concentration of A-1331852 was 0-1,000 nM, the concentration of S63845 was 0-10,000 nM. The medium was removed and 10 µl Premix WST-1 was added to each well, followed by incubation for 1 h and measurement of absorbance at 450 nm using a microplate reader (SpectraMax ABC; Molecular Devices, LLC). IC₅₀ was calculated as follows: $10^{[\log 10(A/B) \times (50-D)/(C-D) + \log 10(B)]}$, where A and B are high and low concentration across 50% inhibition, respectively, and C and D are inhibition (%) at high and low concentration, respectively.

Western blotting. Cells were harvested and lysate samples were prepared in RIPA lysis buffer supplemented with Protease Inhibitor Single Use Cocktail and Phosphatase Inhibitor Cocktail (all Thermo Fisher Scientific, Inc.). Total lysate protein concentrations were measured using BCA protein

assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of lysate protein were denatured by boiling at 90°C for 5 min, separated on 10% Mini-PROTEAN TGX gels (20 µg/lane) and transferred to nitrocellulose membranes (both Bio-Rad Laboratories, Inc.). Membranes were treated with primary antibodies against Bcl-xL, Mcl-1, PARP, cleaved PARP and GAPDH and secondary antibody (HRP-conjugated polyclonal goat antirabbit Ig) using iBind Flex Western Device (Thermo Fisher Scientific, Inc.) and iBind Flex Solution Kit (Thermo Fisher Scientific, Inc.) at room temperature (4 h in total) following the manufacturer's protocol. Protein-antibody complexes were visualized using SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.). Immunoreactive protein bands were detected using the Amersham Imager 680 (GE Healthcare Life Sciences) and semi-quantified via densitometry using ImageJ version 1.54 (National Institutes of Health).

DNA fragmentation assay. Cells were seeded in 6-well culture plates and incubated at 37°C overnight. DMEM was replenished and cells were treated with 0, 1, 10, 100 nM of A-1331852 or transfected with Bcl-xL siRNA or Mcl-1 siRNA for 48 h as aforementioned. DNA fragmentation was analyzed using Cell Death Detection ELISA^{PLUS} (Roche Diagnostics) following the manufacturer's protocol. The rate of apoptosis is presented as fold-change relative to vehicle-treated or negative control siRNA-treated cells.

Animal experiments. All experiments were approved by the Animal Care and Use Committee of the Nagoya City University of Medical Science (approval no. IDO 23-049; Nagoya, Japan). A total of 10 male BALB/c nu-nu mice (age, 4 weeks; mean weight, 23.1 g) were purchased from Japan SLC and housed in standard Plexiglas cages at room temperature (20–26°C) and humidity (40–60%) under 12/12-h light/dark cycle with *ad libitum* access to autoclaved chow and water. HCT116/5FUR cells were suspended at a density of 5×10^6 cells in 200 µl PBS and injected subcutaneously into the right flank of each mouse. When average tumor volume surpassed ~100 mm³, mice were divided into A-1331852 and vehicle treatment groups and administered A-1331852 (25 mg/kg in 5% DMSO, 40% Polyethylene glycol 300, 5% Tween-80 and 50% ddH₂O) or vehicle (5% DMSO, 40% Polyethylene glycol 300, 5% Tween-80 and 50% ddH₂O) twice daily through oral gavage, respectively. The dosage of A-1331852 (25 mg/kg) was based on previous study (28). Tumor volume was calculated as follows: Longest tumor diameter x shortest tumor diameter²/2. The treatment duration was initially set as 21 consecutive days. Humane endpoints were defined as total tumor volume >10% of body weight, tumor diameter >20 mm, weight loss >20%, tumor ulceration, necrosis, gait disturbance and impaired water and food intake. All mice were euthanized on day 18 due to excessive weight loss via cervical dislocation under 2.0–2.5% isoflurane inhalation anesthesia and tumors were harvested for analysis.

Immunohistochemistry. Tumors were fixed with 4% paraformaldehyde for 6 h at 4°C, embedded in paraffin, cut into 3-µm-thick sections, and mounted on

3-aminopropyltriethoxysilane-coated slides. Sections were stained with hematoxylin and eosin. Automated immunohistochemistry was performed using the Bond RXm system (Leica Biosystems, Ltd., Newcastle, UK). The Compact Polymer detection system used BOND Polymer Refine Detection (cat no. DS9800, Leica Biosystems, Newcastle Upon Tyne, UK), which contains blocking reagent, polymer reagent (secondary antibody), DAB chromogen and hematoxylin. Primary antibodies were as follows: Bcl-xL (cat no. 54H6; 1:1,500), cleaved PARP (cat no. D64E10; 1:50; both Cell Signaling Technology, Inc.) and Ki-67 antibody (clone no. SP6; cat. no. 418071; 1:2; Nichirei Biosciences, Inc). All steps were performed according to manufacturer's protocol. Deparaffinization was performed using Bond Dewax Solution (cat no. AR9222, Leica Biosystems, Newcastle Upon Tyne, UK) at 72°C, followed by Alcohol and Bond Wash solution (cat no. AR9590, Leica Biosystems, Newcastle Upon Tyne, UK). Antigen retrieval was performed using Bond Epitope Retrieval Solution 1 (cat no. AR9961, Leica Biosystems, Newcastle Upon Tyne, UK) for 20 min at 100°C. Blocking was performed with peroxide block reagent for 5 min at ambient temperature. The primary antibody reaction was performed for 15 min at ambient temperature. Secondary antibody reaction was performed with Polymer reagent for 8 min at ambient temperature. Color development was performed with DAB chromogen for 10 min at ambient temperature. Hematoxylin was used for counterstaining for 5 min at ambient temperature. Images were captured using a fluorescence microscope (BZ-X710; Keyence Corporation, Osaka, Japan). A total of 10 high-magnification (x200) fields of view were acquired for each tumor to calculate the mean proportion of cleaved PARP- and Ki-67-positive cells using hybrid cell count software (BZ-X Analyzer software version 1.4.0.1; Keyence Corporation).

Statistical analysis. Statistical analyses were performed using EZR software (Easy R) version 4.2.2 (Saitama Medical Center, Jichi Medical University, Saitama, Japan). *In vitro* experiments were performed at least three times. Data are presented as the mean ± standard deviation. Treatment group means were compared using unpaired Student's t test or one-way ANOVA followed by Dunnett's post hoc test. P<0.05 (two-tailed) was considered to indicate a statistically significant difference.

Results

Emergence of 5-FU resistance is associated with overexpression of the antiapoptotic protein Bcl-xL. Long-term culture of HCT116 cells in 5-FU yielded HCT116/5FUR with an IC₅₀ 270-fold higher than that of the parental line (Fig. 1A and B). To investigate the mechanisms underlying acquired 5-FU resistance, the present study first compared the expression levels of antiapoptotic Bcl-2 family proteins Bcl-xL and Mcl-1 via western blotting and found significantly elevated Bcl-xL and significantly decreased Mcl-1 expression in HCT116/5FUR line compared with the parental line (Fig. 1C and 1D). This result suggested that Bcl-xL upregulation is a contributing factor to 5-FU resistance.

Inhibition of Bcl-xL via siRNA transfection suppresses the proliferation of 5FUR colorectal cancer cells. The present study

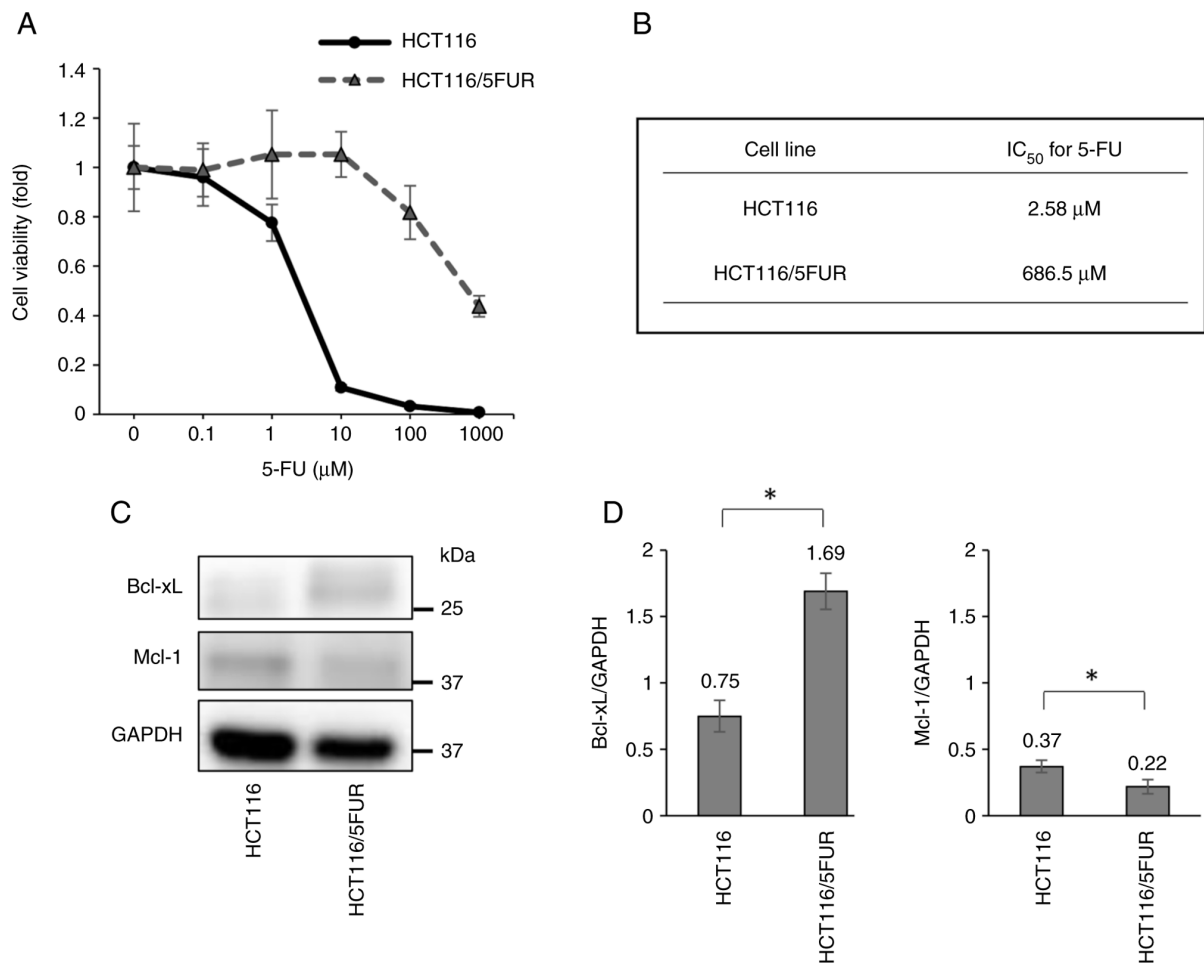


Figure 1. 5-FU resistance in HCT116 colorectal cancer cell line is associated with the upregulation of antiapoptotic Bcl-2 family protein Bcl-xL. (A) Enhanced viability of HCT116/5FUR compared with parental HCT116 cells following 72 h treatment with 5-FU was determined using WST-1 assay. (B) Marked elevation of 5-FU IC₅₀ in HCT116/5FUR compared with parental HCT116 cell line. (C) Upregulated expression of Bcl-xL and downregulated expression of Mcl-1 in HCT116/5FUR compared with parental line, as revealed via western blotting. (D) Densitometric analysis of western blots. * $P < 0.05$. IC₅₀, half-maximal inhibitory concentration; FUR, fluorouracil resistant.

examined the contribution of Bcl-xL overexpression to the pro-malignancy characteristics of colorectal cancer cells, as 5-FU resistance is a key determinant of poor clinical outcome (29). Transfection of HCT116 and HCT116/5FUR cells with siRNAs targeting Bcl-xL or Mcl-1 decreased expression of the corresponding protein (Fig. 2A). In addition, knockdown of Bcl-xL significantly inhibited proliferation of HCT116/5FUR and parental cells. This antiproliferative effect was significantly greater in the HCT116/5FUR subline than in the parental line after 48 h transfection (Fig. 2B). Knockdown of Mcl-1 significantly increased proliferation of HCT116/5FUR cells at 72 h (Fig. 2B).

Bcl-xL knockdown enhances apoptosis rate of 5FUR colorectal cancer cells. The present study investigated the effect of Bcl-xL knockdown on apoptosis of 5FUR and parental colorectal cancer cells using western blot and DNA fragmentation assays. Bcl-xL knockdown induced apoptosis (Fig. 3A and B). The effect was greater on HCT116/5FUR than on parental cells, as revealed via DNA fragmentation assay. This suggested that Bcl-xL serves an important role in the regulation of apoptosis in both 5FUR and parental colorectal cancer cells.

Bcl-xL-specific BH3 mimetic A-1331852 suppresses proliferation of 5FUR colorectal cancer cells via induction of apoptosis. As knockdown of Bcl-xL suppresses the proliferation of HCT116/5FUR cells and induces apoptosis, the present study evaluated the antiproliferative and proapoptotic effects of the Bcl-xL-specific BH3 mimetic A-1331852 (22). Treatment of HCT116/5FUR and parental HCT116 cells with A-1331852 for 72 h significantly decreased the viability of both lines, as evaluated using WST-1 assay (Fig. 4A); this effect was stronger on HCT116/5FUR than on parental cells. Consistent with Mcl-1 siRNA-mediated knockdown experiments, S63845 did not suppress the viability of either cell line (Fig. S1). Moreover, A-1331852 dose-dependently induced apoptosis of both cell lines and DNA fragmentation assay indicated that the proapoptotic effect was stronger on HCT116/5FUR than on parental cells (Fig. 4B and C). Western blot analysis revealed A-1331852 enhanced Bcl-xL expression in both cell lines (Fig. 4B). Therefore, inhibition of Bcl-xL by a specific small-molecule BH3 mimetic suppressed the proliferation of HCT116/5FUR cells and concomitantly enhanced apoptosis rate, suggesting that pharmacological Bcl-xL inhibition may be an effective strategy to prevent 5FUR colorectal tumor progression.

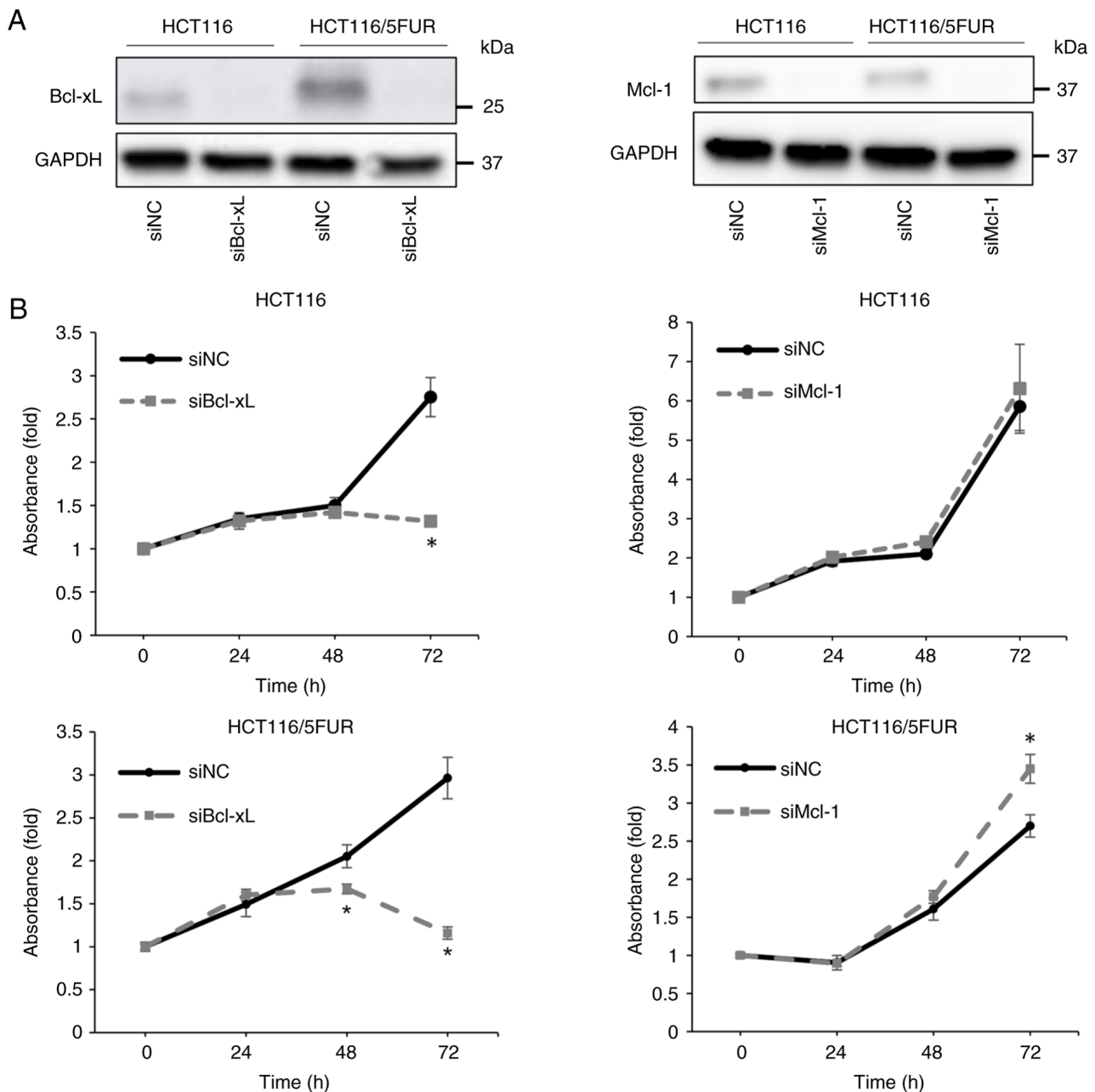


Figure 2. Knockdown of Bcl-xL suppresses proliferation of 5FUR colorectal cancer cells. (A) Confirmation of knockdown efficacy and specificity. HCT116/5FUR and parental HCT116 cells were transfected with Bcl-xL- or Mcl-1-targeted siRNA for 48 h and expression levels were evaluated via western blot. (B) Bcl-xL knockdown but not Mcl-1 knockdown decreased proliferation rates of HCT116/5FUR and parental HCT116 cells, as evaluated via WST-1 assay. * $P < 0.05$ vs. siNC. FUR, fluorouracil resistant; siNC, small interfering RNA negative control.

Oral A-1331852 suppresses the growth of 5FUR colorectal tumors *in vivo*. To examine if A-1331852 suppresses the growth of 5FUR colorectal tumors *in vivo*, the present study established a xenograft mouse model by inoculating HCT116/5FUR cells. While there was no significant difference in weight loss between the vehicle and treated groups by day 18 (Fig. S2), tumors were significantly smaller in the A-1331852 group than those in the vehicle group (Fig. 5A-C). Moreover, consistent with cell culture studies, immunohistochemistry for the apoptosis marker cleaved PARP revealed a significantly increased proportion of positively stained cells in tumors from mice receiving A-1331852. Furthermore, expression of Ki-67 was decreased in the A-1331852 group (Fig. 5D and E). These

results confirmed that the orally available Bcl-xL-specific BH3 mimetic A-1331852 suppressed the growth of 5FUR colorectal tumors in mice by promoting apoptosis.

Discussion

The emergence of 5-FU resistance in colorectal cancer cells was associated with overexpression of antiapoptotic protein Bcl-xL; inhibition of Bcl-xL activity by either siRNA-mediated knockdown or Bcl-xL-specific BH3 mimetic (A-1331852) decreased proliferation of colorectal cancer cells and enhanced apoptosis. Overall, these results indicated that Bcl-xL upregulation is an important mechanism conferring resistance to

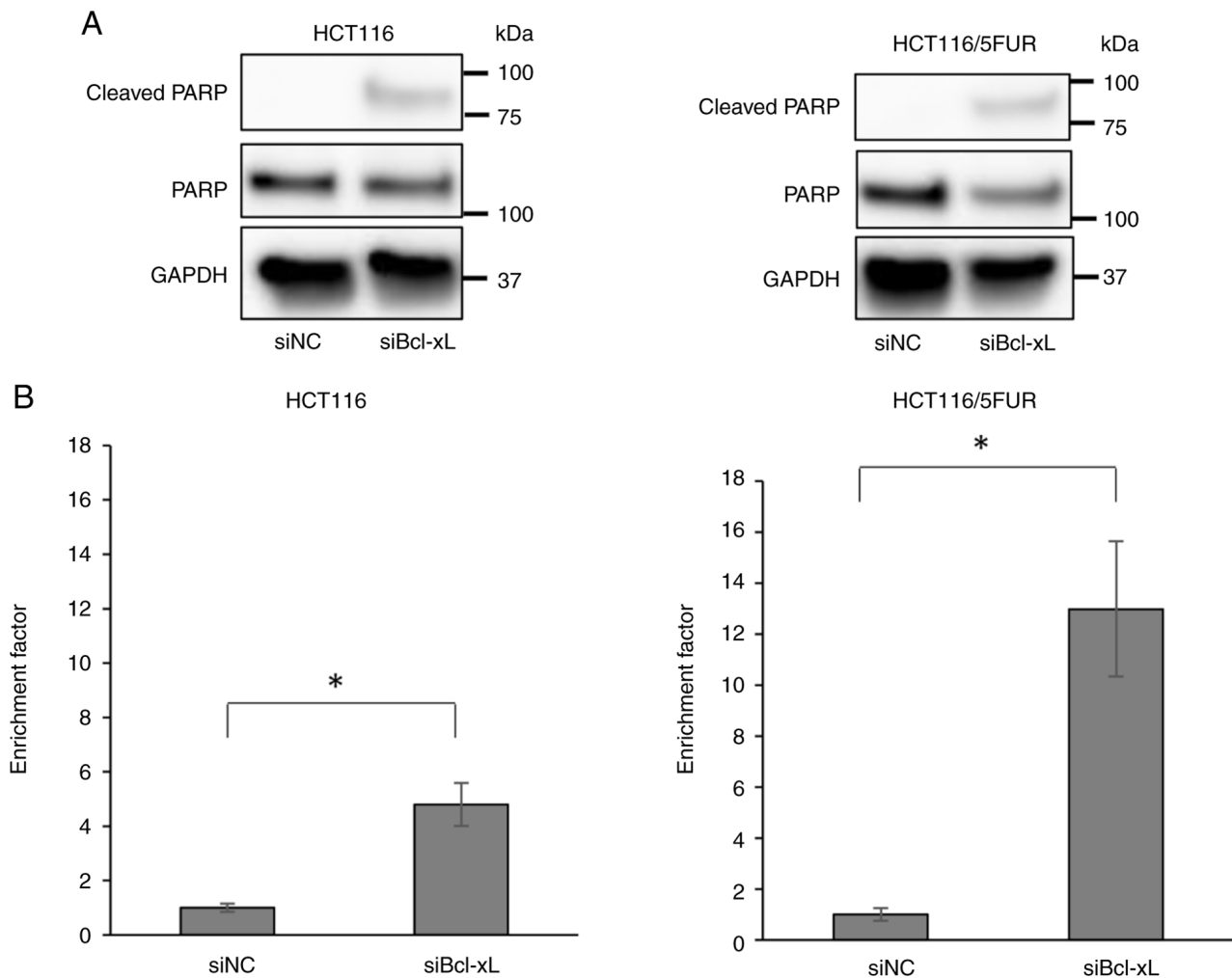


Figure 3. Knockdown of Bcl-xL induces apoptosis of 5FUR and parental colorectal cancer cells. (A) HCT116/5FUR and parental HCT116 cells were transfected with Bcl-xL siRNA for 48 h and expression of apoptosis marker cleaved PARP was evaluated via western blotting. (B) Evaluation of apoptosis using DNA fragmentation assay. * $P < 0.05$. FUR, fluorouracil resistant; siNC, small interfering RNA negative control.

5-FU and small-molecule Bcl-xL inhibitors may be effective for the treatment of colorectal cancer with 5-FU resistance. A-1331852 significantly suppressed the growth of tumors derived from 5FUR colorectal cancer cells in xenograft model mice by inducing apoptosis.

Multiple processes may contribute to the emergence of 5-FU resistance in colorectal cancer cells, such as upregulation of Bcl-xL. Evasion of apoptosis is a key mechanism that allows cancer cells to survive in the hostile tumor microenvironment and following treatment with cytotoxic chemotherapeutic agents, such as 5-FU (3). The intrinsic apoptosis pathway is regulated by interactions between multiple pro- and antiapoptotic Bcl-2 family proteins and overexpression of antiapoptotic Bcl-2 family proteins promotes tumor survival and chemoresistance (30,31). Thus, antiapoptotic Bcl-2 family proteins may be effective therapeutic targets for cancer treatment. Bcl-2 is upregulated in adenoma (32), while downregulation of Bcl-xL and Mcl-1 decrease the chemoresistance of colorectal cancer cells (15,17). In a previous study examining the function of antiapoptotic Bcl-2 family proteins in pancreatic cancer cells, Bcl-xL and Mcl-1, but not Bcl-2, were found to serve an important role in the regulation of apoptosis (24). Colorectal cancer cells are dependent on Bcl-xL for survival (19).

Moreover, Bcl-xL is overexpressed in human colorectal cancer specimens (33) and its overexpression is associated with poor prognosis (34). These findings suggest Bcl-xL as a potential therapeutic target for colorectal cancer and the present study suggested that Bcl-xL overexpression contributes to 5-FU resistance and that suppression of Bcl-xL can impede progression of 5FUR tumors. The present study did not explore the precise mechanism of Bcl-xL upregulation in acquiring 5-FU resistance. Activation of the NF- κ B/STAT3 signaling pathway is a key mechanism for 5-FU resistance and promotes antiapoptotic proteins (35); however, the upstream signaling pathways that regulate Bcl-xL overexpression in 5FUR cancer cells remain unknown. Our previous study demonstrated that IL-6 upregulates expression of Bcl-xL and Mcl-1 in colorectal cancer cells via phosphorylation of STAT3 (25) and interaction of cancer cells and cancer-associated fibroblasts enhance expression of Bcl-xL and Mcl-1 via the IL-6/JAK/STAT3 pathway (23); therefore, it was hypothesized that the IL-6/STAT3 signal pathway may also contribute to 5-FU resistance. Although the present study was conducted using only cancer cells and the upstream mechanism of overexpression of Bcl-xL was not assessed, future studies should investigate the role of the STAT3 pathway.

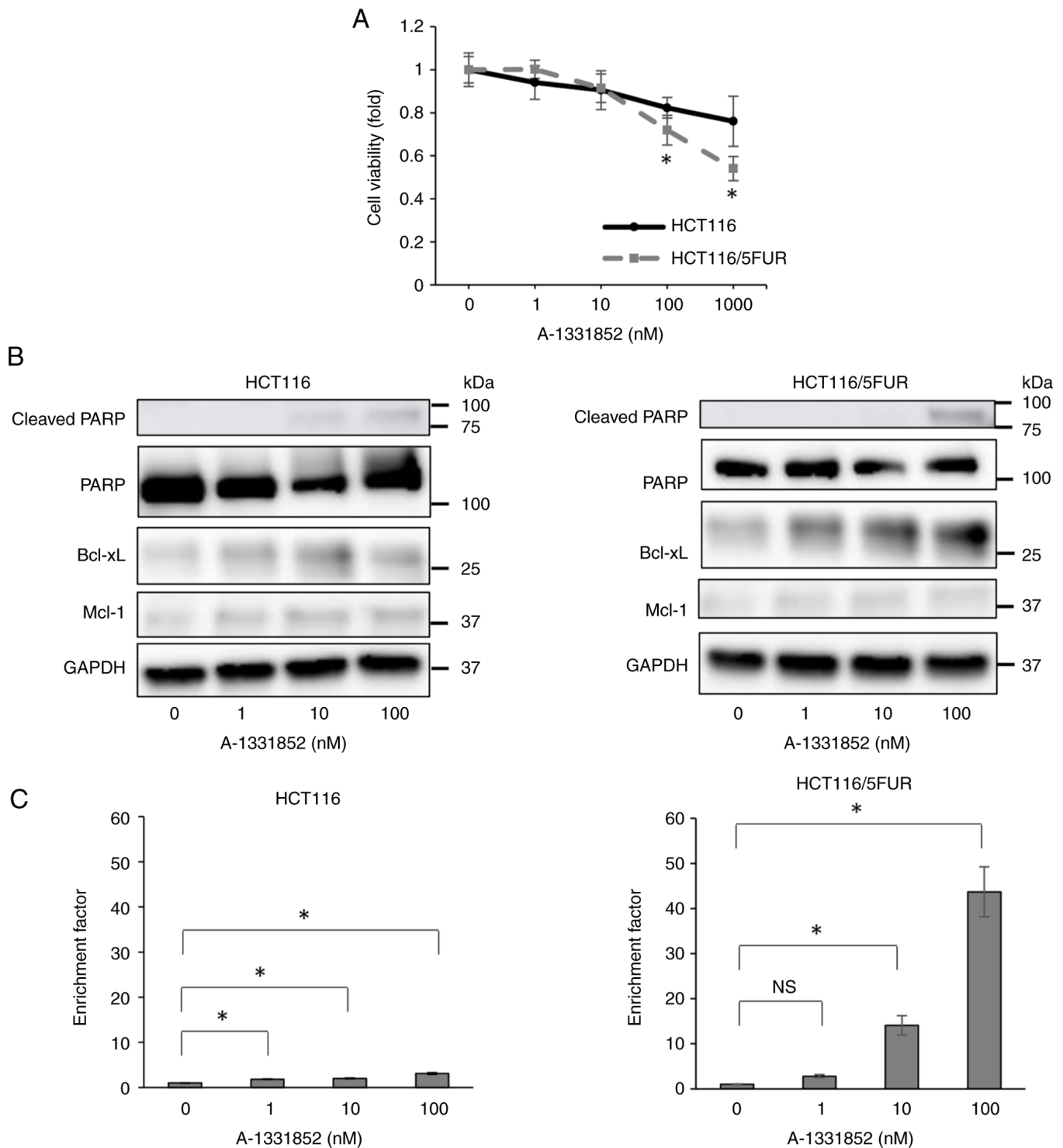


Figure 4. Small-molecule Bcl-xL-specific BH3 mimetic A-1331852 suppresses proliferation of 5FUR colorectal cancer cells by enhancing apoptosis. (A) HCT116/5FUR and parental HCT116 cells were treated with A-1331852 for 72 h and viability was evaluated via WST-1 assay. (B) HCT116/5FUR and parental HCT116 cells were treated with A-1331852 for 48 h and PARP cleavage and expression of antiapoptotic proteins (Bcl-xL and Mcl-1) were evaluated via western blot. (C) HCT116/5FUR and parental HCT116 cells were treated with A-1331852 for 48 h and apoptosis was evaluated via DNA fragmentation assay. *P<0.05 vs. HCT116. FUR, fluorouracil resistant; NS, not significant; Mcl, Myeloid cell leukemia.

Numerous BH3 mimetics have been developed to inhibit antiapoptotic Bcl-2 family proteins and prevent evasion of apoptosis by cancer cells under chemotherapy. The first BH3 mimetic developed, ABT-737, was reported to inhibit BCL-2, Bcl-xL and Bcl-W (32) and its orally available analog navitoclax has demonstrated anticancer efficacy against hematological

and solid malignancy both as monotherapy and in combination with chemotherapy. Overexpression of Bcl-xL is implicated in the survival and chemoresistance of solid tumors, including colorectal cancer (13,36); therefore, Bcl-xL-specific BH3 mimetics may be particularly effective for colorectal cancer treatment. To the best of our knowledge, however, only a few

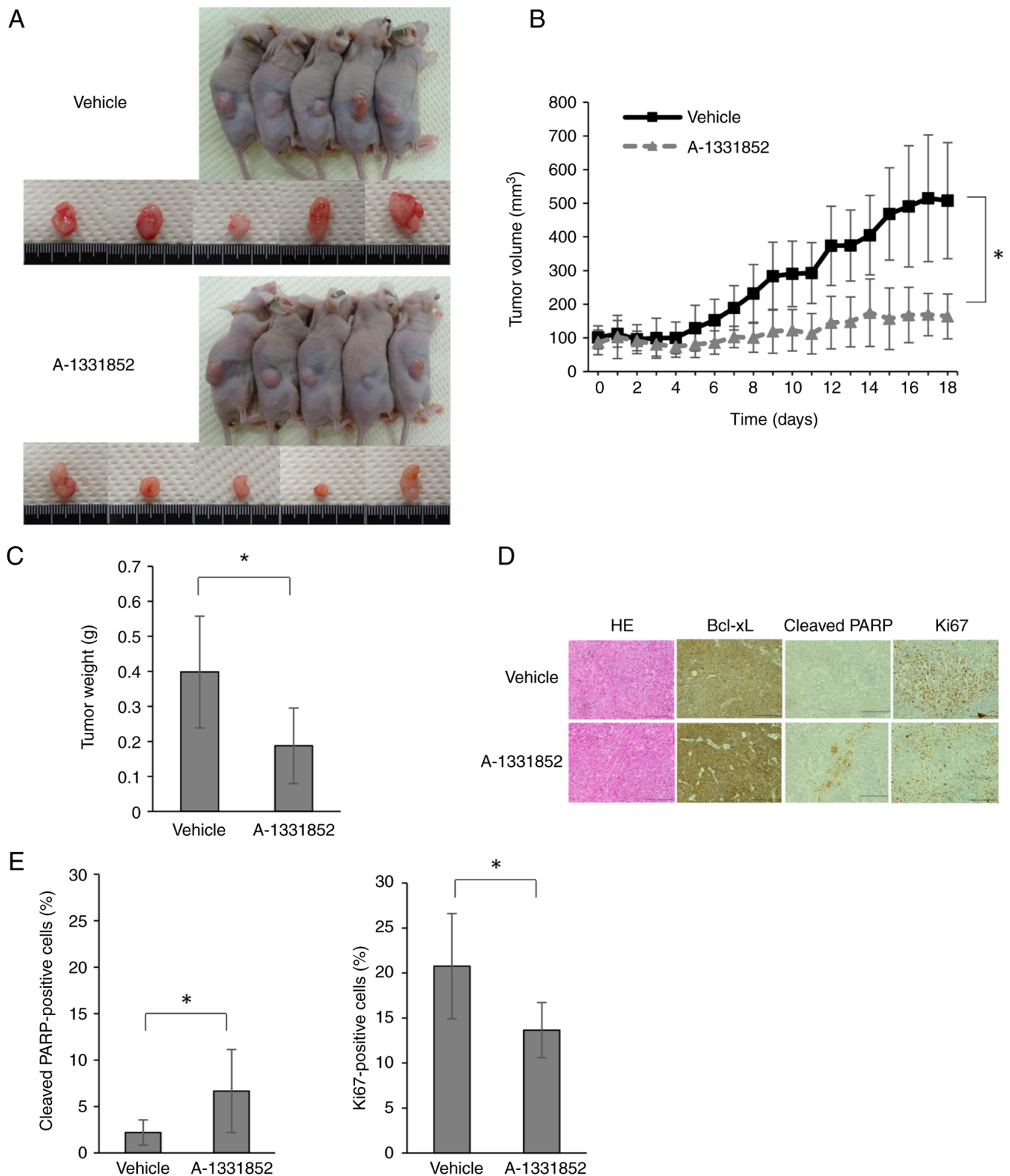


Figure 5. A-1331852 inhibits proliferation of subcutaneous tumors derived from 5FUR colorectal cancer cells in a mouse xenograft model. (A) Tumors from mice treated with A-1331852 or vehicle. (B) Tumors were significantly smaller following A-1331852 compared with vehicle treatment. (C) Tumor weight was significantly lower following 18 days of A-1331852 compared with vehicle treatment. (D) Representative images of tumors stained with HE and immunostained for Bcl-xL, Ki-67 and apoptosis marker cleaved PARP (magnification, x200). Scale bar, 200 μ m. (E) Cleaved PARP immunostaining intensity was significantly greater following A-1331852 compared with vehicle treatment. Ki-67 immunostaining intensity significantly decreased following A-1331852 compared with vehicle treatment. * $P < 0.05$. FUR, fluorouracil resistant; HE, hematoxylin and eosin.

studies have directly examined the efficacy of Bcl-xL-specific BH3 mimetics on colorectal cancer cells, including 5FUR

colorectal cancer cells (19,21). A-1331852 is the most recently developed Bcl-xL-specific BH3 mimetic (22). Greaves *et al* (20)

reported that A-1331852 induces apoptosis of colorectal cancer cells *in vitro* and Levenson *et al* (28) reported that A-1331852 inhibits tumor growth in a xenograft mouse model established by inoculating colorectal cancer cells. Here, A-1331852 was also effective against 5FUR tumors. Antiproliferative and proapoptotic effects of A-1331852 were stronger against 5FUR than parental cells. To the best of our knowledge, the present study is the first to report the efficacy of A-1331852 against 5FUR colorectal cancer in a xenograft mouse model. Bcl-xL serves an important role in platelet life span and inhibition of Bcl-xL is reported to be a cause of thrombocytopenia (32). Furthermore, A-1331852 has been reported to decrease platelet count in rats (28). Therefore, it may be necessary to decrease the dose of A-1331852 by combination with other therapeutic agents. Future studies should assess platelet count following A-1331852 treatment. Inhibition of Mcl-1 by the specific BH3 mimetic S63845 did not exert an antiproliferative effect on 5FUR and parental cells. Although inhibition of Mcl-1 by siRNA slightly increased proliferation of 5FUR cells, inhibition of Mcl-1 alone had no inhibitory effect on the proliferation of 5FUR and parental colorectal cancer cells. However, based on our previous report that simultaneous inhibition of Bcl-xL and Mcl-1 induces strong apoptosis in pancreatic (24) and colorectal cancer cells (25), Mcl-1 may serve a key role in the regulation of apoptosis in cancer cells in concert with Bcl-xL. To the best of our knowledge, the interaction between Bcl-xL and Mcl-1 during acquisition of 5-FU resistance in colorectal cancer cells has not been examined and should be assessed in future.

The stronger anticancer effect of A-1331852 against 5FUR colorectal cancer compared with parental cells may result from a greater dependence on Bcl-xL for survival, in accordance with a previous study reporting that 5FUR colorectal cancer cells are more sensitive to siRNA-mediated Bcl-xL knockdown than parental cells (30). However, the mechanisms by which 5FUR colorectal cancer cells depend on Bcl-xL for survival remain unknown. Furthermore, expression of antiapoptotic Bcl-2 proteins do not predict the efficacy of BH3 mimetics (21,37), suggesting that multiple mechanisms may be involved under different conditions or in distinct cancer cell types. Colorectal cancer is a heterogeneous disease, therefore the mechanisms of 5-FU resistance may differ among cell lines and tumor cell populations. Further investigation is needed to elucidate mechanisms underlying the efficacy of BH3 mimetics for apoptosis-mediated destruction of 5FUR colorectal cancer cells. It is also key to consider that colorectal cancer cells may acquire resistance to A-1331852. Resistance to BH3 mimetics has been reported in hematological malignancy (38), although the exact mechanism remains unknown. Elevated levels of antiapoptotic Bcl-2 family proteins, including Bcl-xL and Mcl-1, are a mechanism for resistance to BH3 mimetics in hematological malignancy (39). In the present study, A-1331852 enhanced Bcl-xL expression in both 5FUR and parental cells, which may contribute to resistance with long-term use.

The present study had limitations. All experiments were performed on a single 5FUR cell population. Given the aforementioned heterogeneity of colorectal cancer, it is necessary to establish multiple 5FUR cell lines and evaluate the contribution

of Bcl-xL overexpression to identify other potential resistance mechanisms. In addition, the animal experiments had a small number of samples. Furthermore, although the present study focused on the role of Bcl-xL in 5FUR colorectal cancer cells, abnormal apoptosis is only one factor in the acquisition of 5-FU resistance in colorectal cancer cells. There are various mechanisms of 5-FU resistance, including the alterations in drug transport, cell cycle, DNA-damage repair machinery, regulation of autophagy, epithelial-to-mesenchymal transition, cancer stem cell involvement, tumor microenvironment interactions, miRNA dysregulations, epigenetic alterations, redox imbalances (3).

In conclusion, HCT116/5FUR cells exhibited upregulation of Bcl-xL and Bcl-xL-specific BH3 mimetic A-1331852 suppressed proliferation and promote apoptosis *in vitro* and *in vivo*. Inhibition of Bcl-xL using specific BH3 mimetics may be an effective treatment strategy for 5FUR colorectal cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in the article.

Authors' contributions

AK and HT conceived and designed the study, analyzed and interpreted data and wrote the manuscript. HA, SU, SH, YF, KW, TY, TS, HU, KS, YY, RO, AM, YM and ST designed the study. AK, HT, SU, SH, KW, TS, and KS performed experiments. AK, HT, HA, SU, SH, YF, KW, TY, TS, HU, KS, YY and YM confirm the authenticity of all raw data. HT, AM, YM and ST supervised the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

In vivo mouse experiments were approved by the Animal Care and Use Committee of the Nagoya City University Graduate School of Medical Sciences (approval no. 23-049; Nagoya, Japan).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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