SPANDIDOS PUBLICATIONS

ONCOLOGY REPORTS 49: 52, 2023

AZD6738 promotes the tumor suppressive effects of trifluridine in colorectal cancer cells

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Received October 18, 2022; Accepted December 6, 2022

DOI: 10.3892/or.2023.8489

Abstract. Ataxia telangiectasia and Rad3-related (ATR) is a kinase that repairs DNA damage. Although inhibitors that selectively target ATR have been developed, their effectiveness in colorectal cancer has not been widely reported. The present study hypothesized that anticancer agents that effectively act in the S phase before the G_2/M checkpoint may be ideal agents for concomitant use with ATR inhibitors, which act at the G_2/M checkpoint. Therefore, the present study examined the combined effects of AZD6738, an ATR inhibitor, and trifluridine (FTD), which acts in the S phase and has a high DNA uptake rate. *In vitro* cell viability assays, flow cytometry and western blotting were performed to evaluate cell viability, and changes in cell cycle localization and protein expression. The results revealed that in colorectal cancer cells, the combination of AZD6738 and FTD inhibited cell viability, cell cycle arrest at the G_2/M checkpoint and Chk1 phosphorylation, and increased apoptotic protein expression levels more than that when treated with FTD alone. HT29, a BRAF-mutant cell line known to be resistant to anticancer drugs, was used to induce tumors *in vivo*. Since FTD does not have sufficient efficacy when administered orally, it was mixed with tipiracil to prevent degradation; this mixture is known as TAS-102. TAS-102 alone exerted minimal tumor suppressive effects; however, when used in combination with AZD6738, tumor suppression was observed, suggesting that AZD6738 may increase the effectiveness of a weakly effective drug. Although ATR inhibitors are effective against p53 mutants, the present study demonstrated that these inhibitors were also effective against the p53 wild-type HCT116 colorectal cancer cell line. In conclusion, combination therapy with AZD6738 and FTD enhanced the inhibition of tumor proliferation *in vitro* and *in vivo*. In the future, we aim to investigate the potentiating effect of AZD6738 on 5-fluouracil-resistant cell lines that are difficult to treat.

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Abbreviations: 5-FU, 5-fluorouracil; DDR, DNA damage recognition; ATR, ataxia telangiectasia and Rad3-related; ATM, ataxia telangiectasia mutated; FBS, fetal bovine serum; FTD, trifluridine; HPMC, hydroxypropyl methylcellulose; HRP, horseradish peroxidase; PARP, poly(ADP-ribose) polymerase

Key words: ATR inhibitor, AZD6738, FTD, colorectal cancer

Introduction

Numerous types of anticancer drugs are available to treat colorectal cancer and combination therapies are often used for the treatment of this type of cancer. Drugs that directly damage DNA and drive cancer cells toward apoptosis have entered mainstream treatment and are often used in combination with molecular-targeted agents (1); however, studies focusing on therapies that inhibit cancer cell repair are limited. Our previous study focused on DNA damage recognition (DDR) during the cell cycle and suggested that tumor proliferation can be suppressed by preventing DNA-damaged cancer cells from repairing themselves (2). In particular, ataxia telangiectasia and Rad3-related (ATR) is an apical kinase that regulates the DNA damage response and organizes cellular processes, such as repair of arrested replication forks (replication stress), and associated DNA double-strand and single-strand breaks (3,4).

Recently, several potent and selective ATR inhibitors have been developed, including M6620 (Merck KGaA), AZD6738 (AstraZeneca) and BAY1895344 (United States Biological), which are in clinical development (5). Investigations using BAY1895344 and M6620 have provided clinical evidence of the antitumor activity of ATR inhibitors in patients with advanced cancer and ataxia telangiectasia mutated (ATM) abnormalities (loss of ATM protein expression and/or ATM adverse mutations) (6,7). In p53-mutant or ATM-deficient cells, AZD6738 treatment has been reported to cause replication fork arrest and result in the accumulation of unrepaired DNA damage; subsequently, when AZD6738-treated cells progress to mitosis, cell death by mitotic catastrophe occurs (8). However, the effects of AZD6738 in the absence of ATM abnormalities are unknown.

It is believed that in colorectal cancer, AZD6738 may enhance tumor suppression in combination with anticancer agents (9). The effects of AZD6738 have been reported in various types of cancer, including in pancreatic, gastric, hepatocellular and biliary tract cancer (10-13).

AZD6738 acts on different sites based on its concentration. It inhibits Chk1 phosphorylation at the G_2/M checkpoint, thereby inhibiting cell repair. Cancer cells that are not repaired may adapt to the cell cycle and subsequently undergo apoptosis (14,15). In the presence of ATR depletion, p53 mutations are lethal (16); however, little is known about the effects of DDR in p53 wild-type cells (17,18). Notably, ~50% of cases of colorectal cancer have p53 mutations (19), whereas the other 50% have wild-type p53, indicating that AZD6738 may be ineffective. Cell repair in p53-mutant colorectal cancer cells is dependent on the G_2/M checkpoint; therefore, a strategy to inhibit phosphorylation of Chk1 at the G_2/M checkpoint using AZD6738 is logical. However, we cannot conclude that AZD6738 is ineffective in p53 wild-type colorectal cancer cells; therefore, it is necessary to verify whether it is effective or not.

Our previous study demonstrated the treatment-enhancing effects of combination therapy with AZD6738 and 5-fluorouracil (5-FU) (20). 5-FU acts outside the S phase, and DNA-damaged cancer cells can be repaired at various cell cycle checkpoints. This fact complicates the evaluation of the effects of AZD6738. In cancer, loss of G_1 checkpoint regulation and activation of replication-promoting oncogenes increases the likelihood of cancer cells entering the S phase with increased replication stress (5). Trifluridine (FTD) replaces thymidine in DNA during the S phase, resulting in DNA dysfunction. However, the DNA is repaired soon thereafter by inducing the phosphorylation of Chk1 at the 345th residue at the G_2/M checkpoint (21). FTD also has a notably higher rate of incorporation into DNA than other drugs (22). The presence of the S phase just before the G_2/M checkpoint and the high rate of incorporation of FTD into the DNA are two reasons that led us to conclude that FTD may suppress tumors more efficiently than 5-FU. In a previous study, combination therapy with FTD and a Chk1 inhibitor significantly inhibited the proliferation of xenograft tumors derived from esophageal squamous cell carcinoma (23). Although FTD is ineffective at shrinking tumors (24), AZD6738 may enhance the effects of FTD. The combination of FTD and AZD6738, if effective, may be an alternative treatment for colorectal cancer, which has poor outcomes with the use of conventional drugs. The present study aimed to determine the *in vitro* and *in vivo* effects of combined treatment with AZD6738 and FTD on tumor proliferation and the expression of proteins involved in DNA damage checkpoints.

Materials and methods

Cell lines. The human colorectal cancer cell lines HT29, HCT116, DLD-1 and SW480 were purchased from the American Type Culture Collection. These cancer cell lines were certified by short tandem repeat profiling. HT29 and HCT116 cells were cultured in DMEM (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% fetal bovine serum [(FBS); Gibco; Thermo Fisher Scientific Inc.] and 1% penicillin/streptomycin. DLD-1 cells were cultured in RPMI (FUJIFILM Wako Pure Chemical Corp.) supplemented with 10% FBS and 1% penicillin/streptomycin. SW480 cells were cultured in Leibovitz's L-15 Medium (Gibco; Thermo Fisher Scientific Inc.) supplemented with 10% FBS and 1% penicillin/streptomycin. HT29, HCT116 and DLD-1 cells were cultured at 37˚C in 5% CO_2, whereas SW480 cells were cultured at 37˚C in 100% atmospheric air according to the datasheets for each cell line. Only the HT29 cell line was a BRAF mutant and only the HCT116 cell line was p53 wild-type.

WST-1 assays. HT29, HCT116, DLD-1 and SW480 cells were treated with FTD (Tokyo Kasei Kogyo Co. Ltd.) at concentrations of 0, 0.1, 1, 10, 100 and 1,000 μM with or without 0.5 μM AZD6738. Cell viability was assessed by WST-1 assay using the Premix WST-1 Cell Viability Assay System (Takara Bio, Inc.). The combination index scores were calculated to show the synergistic effects (25). The AZD6738 concentration of 0.5 μM was previously determined via cytotoxicity experiments in our laboratory (20). Cells (1.0×10^4 cells/well in 100 μl) were seeded in 96-well plates and allowed to adhere for 24 h at 37˚C. Thereafter, the cells were treated with different concentrations of FTD with or without AZD6738 for 72 h at 37˚C. WST-1 was diluted 10-fold and then added to the cells. After 1 h of incubation at 37˚C, the absorbance of cells in each well was measured at a wavelength of 450 nm using a microplate reader (SpectraMax ABC; Molecular Devices, LLC). In addition, the HT29 and HCT116 cells were divided into the following groups: Control (no drug added), AZD6738 (AZD6738 alone), FTD (FTD alone) and FTD + AZD6738 (FTD and AZD6738 combined). The concentrations of FTD used to treat HT29 and HCT116 cells were the IC_{50} values of 70 and 5 μM, respectively; for the concentration of AZD, 0.5 μM was adopted. Cell viability was assessed at 24, 48 and 72 h at 37˚C.

After oral administration of FTD, FTD is rapidly hydrolyzed by thymidine phosphorylase (TP) in the liver to an inactive form (26). The addition of tipiracil to FTD at a molar ratio of 1:0.5 or a weight ratio of 1:0.471 inhibits the degradation of FTD, and sufficient drug levels can be achieved in the blood (27). The mixture of FTD and tipiracil is called TAS-102. The present study investigated tipiracil (MedChemExpress) toxicity in HT29 cells. Cell viability was evaluated using the WST-1 assay at each of the following concentrations of FTD in TAS-102: 0, 0.1, 1, 10, 100 and 1,000 μM. The results were compared with the group treated with FTD alone. Cell viability was assessed after treatment for 72 h at 37˚C.

Flow cytometry. HT29 and HCT116 cells were divided into control, AZD6738, FTD and FTD + AZD6738 groups. After culturing the cells under their respective culturing conditions for 0, 24, 48 and 72 h, they were collected and stored in 70% ethanol at -20˚C. After 1 week, cell cycle progression was examined using flow cytometry. A cell suspension was prepared using the BD Cycletest Plus DNA Kit (BD Biosciences); cell concentration was adjusted to 1.0×10^6 cells/ml using Buffer Solution. The cell suspension was centrifuged at 400 x g for 5 min at room temperature (20-25˚C) and the supernatant was carefully decanted. For enzymatic degradation of solid tissue fragments, and digestion of the cell membrane and

cytoskeleton, cells were placed in a liquid in tetrahydrochlorospermine detergent buffer containing trypsin and mixed by hand with light tapping. The cells were incubated at room temperature (20-25˚C) for 10 min.

To inhibit the activity of trypsin and digest RNA, a solution containing trypsin inhibitor and ribonuclease A was added there and mixed with light tapping. The mixture was incubated at room temperature (20-25˚C) for 10 min. To bind PI to DNA, a solution containing PI and spermine tetrahydrochloride was further added at 2-8˚C and mixed with light tapping. The solution was incubated on ice 2-8˚C in the dark for 10 min. Cell cycle localization at each stage was measured using the FACSCanto II flow cytometer (BD Biosciences) at a wavelength of 488 nm and the data were analyzed using the BD FACSDiva Software version 8.0.2 (BD Biosciences).

Western blotting. HT29 and HCT116 cells were divided into the control, AZD6738, FTD and FTD + AZD6738 groups. After culturing for 48 and 72 h, the cells were collected and SDS sample buffer [2.75% SDS (pH 6.8), 9% glycerol, 87.5 mmol/l Tris-HCl, 150 mmol/l dithiothreitol, and 0.003% bromophenol blue] was added. Subsequently, $1x10^6$ cells were suspended in 150 μl SDS sample buffer. After heating at 98˚C for 5 min, 15 μl cell sample was loaded onto the gel (2,20,28). Proteins in the lysate were separated on 10% or 4-20% Mini-PROTEAN TGX Precast Gels (Bio-Rad Laboratories, Inc.) according to the molecular weight of the target protein. Separated proteins were transferred onto nitrocellulose membranes at 100 V for 1 h at 4˚C. The membranes were then blocked with 5% skim milk (BD Biosciences) in Tris-buffered saline-Tween 20 (10%) for 30 min at 20-25˚C and then incubated with the primary antibody diluted in 1% skim milk at 4˚C overnight while being rotated (Rotator RT-50; TAITEC Corporation). Subsequently, the membranes were incubated with the secondary antibodies diluted in 1% skim milk at 4˚C for 1 h while being rotated. When phosphorylated (p)-Chk1 was being detected, Western BLoT Immuno Booster Solution 1 and Western BLoT Immuno Booster Solution 2 (cat. no. T7111A; Takara Bio, Inc.) were used to dilute the primary and secondary antibodies, instead of skim milk, respectively. The following primary antibodies were used: p-Chk1 Ser345 (1:1,000; cat. no. 2348; Cell Signaling Technology, Inc.), Chk1 (1:1,000; cat. no. C9358; MilliporeSigma), β-actin (1:1,000; cat. no 3700; Cell Signaling Technology, Inc.), Apoptosis Western Blot Cocktail (1:250; cat. no. ab136812; Abcam) for cleaved caspase-3, procaspase-3 and PARP, H2A.X variant histone (H2A.X; 1:1,000; cat. no ab11175; Abcam) and p-H2A.X (γH2A.X; 1:2,000; cat. no. 05-636; Merck KGaA). The secondary antibodies used included horseradish peroxidase HRP-conjugated goat anti-mouse immunoglobulin (1:1,000; cat. no. P0447; Agilent Technologies, Inc.) for Chk1, γH2A.X and β-actin, and HRP-conjugated goat anti-rabbit immunoglobulin (1:1,000; cat. no. P0448; Agilent Technologies, Inc.) for p-Chk1 and H2A.X. For the primary antibody Apoptosis Western Blot Cocktail, the HRP-conjugated secondary antibody cocktail from this kit was used (1:100; cat. no. ab136812; Abcam) for cleaved caspase-3, procaspase-3, PARP. The following reagents were used as HRP chemiluminescent reagents: SuperSignal West Pico Chemiluminescent Substrate and SuperSignal West Femto Chemiluminescent Substrate were used to visualize p-Chk1, and Pierce ECL Western Blotting Substrate was used to visualize Chk1, γH2A.X, H2A.X, cleaved caspase-3, procaspase-3, PARP and β-actin (all reagents obtained from Thermo Fisher Scientific, Inc.). Immunoreactive protein bands were detected using the ImageQuant LAS-4000 mini control software (Cytiva). Each resulting band was semi-quantified via densitometric analysis using ImageJ software version 1.53 (National Institutes of Health).

Tumor generation and grouping. A total of 24 male BALB/c nu/nu mice (age, 4 weeks; mean weight, ~22.45 g) were purchased from Japan SLC Inc., and drug efficacy studies were performed using a xenotransplanted nude mouse model. The mice were acclimated to the environment, and water was orally administered using an oral sonde for 2 weeks. Mice were housed in standard Plexiglas cages (n=3 mice/cage) at a constant temperature (20-26˚C) and humidity (40-60%), under a 12-h light/dark cycle. Mice were given *ad libitum* access to normal autoclaved feed and drinking water. An individual identification number (cat. no. KN-295; Natsume Seisakusho Co., Ltd.) was attached to the right auricle using an ear punch method to enable the identification of individual animals. Seventh-generation HT29 cells (mycoplasma-free; mycoplasma detection kit supplied by Lonza Group, Ltd.) were injected subcutaneously to induce tumors. Subsequently, 2% isoflurane (product. no. 099-06571; FUJIFILM Wako Pure Chemical Corp.) was administered using a vaporizer (Rodent Circuit Controller; Vetequip, Inc.) as general anesthesia. HT29 cells ($5x10^6$ in 200 μl phosphate-buffered saline) were injected into the right lateral abdomen of each mouse. Subcutaneous swelling was observed in all mice. Mice were randomly divided into the following groups (n=6 mice/group): Control, AZD6738, TAS-102 and TAS-102 + AZD6738. The Control group received only FTD and tipiracil, and the AZD6738 solvent. Similarly, the AZD6738 group received the FTD and tipiracil solvent, and the FTD and tipiracil group received the AZD6738 solvent.

Drug administration. FTD and tipiracil were dissolved in 0.5 w/v% hydroxypropylmethyl cellulose (HPMC; Shin-Etsu Chemical Co., Ltd.; TC-5®; grade, R; indicated viscosity is 6 mPa·S). FTD and tipiracil were combined in a molar ratio of 1:0.5 and a weight ratio of 1:0.471, and were dissolved in 0.5 w/v% HPMC. The final FTD equivalent concentration was 7.5 mg/ml and the suspension was vortexed to dissolve the drug. AZD6738 was dissolved in 5% DMSO, 40% propylene glycol and ddH_2O according to the datasheet. TAS-102 was administered at a dosage of 200 mg/kg/day (100 mg/kg twice daily, 6-h interval), and body weights were measured daily to determine the drug dosage (29). The duration of the study was 3 weeks (5 days dosing, 2 days rest). AZD6738 was administered at a dose of 25 mg/kg based on previous studies (30,31). Syringes (cat. no. IC-1-4908-01; Terumo Corporation) and an 18-G oral sonde (cat. no. VS-493-18GS; Natsume Seisakakusho Co., Ltd.) were used for the oral administration of TAS-102 and AZD6738.

Tumor assessment and humane endpoints. The long and short diameters of the tumors were measured using calipers (Shinwa Co., Ltd.) and tumor volume was calculated using the

following equation: (longest tumor diameter) x (shortest tumor diameter)^2/2 (20). Drug administration was initiated when an average tumor volume of 100 mm^3 was achieved. Tumors and body weights were evaluated daily for 3 weeks. A total of 3 weeks after drug administration, all mice were euthanized by cervical dislocation under general anesthesia using 2% isoflurane and death was confirmed by cardiac arrest. Humane endpoints were defined as maximum tumor weight >10% of body weight, maximum tumor diameter >20 mm, tumor ulceration, necrosis, infection, gait disturbance, impaired water and food intake, maximum weight loss >20, and >25% at 7 days compared with the controls, and cachexia. No mice met these criteria during the study. All animal experiments were conducted with the approval of the Animal Welfare and Use Committee of the Nagoya City University Graduate School of Medicine (approval no. 22-006; Nagoya, Japan). The animal rooms and laboratories in the Center for Laboratory Animal Research and Education, Nagoya City University are equipped with P2A-level diffusion prevention measures and certified (certification no. FM3).

Immunohistochemistry. Tumors were fixed in 4% paraformaldehyde for 6 h at 4˚C and then embedded in paraffin. Paraffin-embedded tissues were cut into 3-μm sections and mounted on slides. Sections were deparaffinized twice with Hemo-De for 10 min, hydrated twice with 100, 90, 80 and 70% ethanol (5 min each time), and washed with running water. For antigen retrieval, the slides were immersed in 10 mM citrate buffer (pH 6.0) and heated in a 600-Watt microwave for 10 min. Endogenous peroxidase activity was blocked by immersion in a mixed solution of 0.3% hydrogen peroxide and 100% methanol at 20-25˚C for 30 min. Blocking was performed in a humidified box at 20-25˚C for 10 min with 4% Block Ace powder (cat. no. UKB80; DS Pharma Biomedical Co., Ltd.). Sections were stained overnight at 4˚C with γH2A.X primary antibody (1:500; cat. no. 05-636; Merck KGaA) and stained with anti-mouse EnVision+ HRP-conjugated polymer as the secondary antibody (1:1,500; cat. no. K4001; Dako; Agilent Technologies, Inc.) for 45 min at 20-25˚C. To detect antibody binding, 3,3-diaminobenzidine substrate (cat. no. K3467; Dako; Agilent Technologies, Inc.) was used as a chromogenic agent. The slides were incubated in the substrate solution for 10 min at 20-25˚C. After rinsing in running water, hematoxylin was used for contrast staining (30 sec at 20-25˚C). A total of 10 fields of view were examined for each tumor. The mean percentage of γH2A.X-positive cells per high magnification field of view ± SD was determined. Images of the slides were captured using a light microscope (BZ-X710; Keyence Corporation) and were analyzed using the BZ-X710 Analyzer software version 1.4.0.1 (Keyence Corporation).

Statistical analysis. *In vitro* experiments were generally performed at least three times; *in vivo* experiments were performed only once. Statistical analysis was performed using the EZR software (Easy R) version 1.41 (https://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmed.html; Saitama Medical Center, Jichi Medical University, Saitama, Japan). All data are presented as the mean ± SD. Two groups were compared using unpaired Student's t-test, whereas two or more groups were compared using one-way ANOVA followed by Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of combining AZD6738 with FTD. The viability of HT29, HCT116, DLD-1 and SW480 cells with or without 0.5 μM AZD6738 treatment was determined (Fig. 1A). The combined use of AZD6738 and FTD at multiple concentrations inhibited cell viability in all four cell lines compared with FTD alone. The combination index scores of HT29, HCT116, DLD-1 and SW480 were 0.775, 0.231, 0.134 and 0.718, indicating a synergistic effect (25). The point of action of AZD6738 varies with its concentration, as stated in the data sheet obtained from MedChemExpress. AZD6738 is a potent inhibitor of ATR kinase activity with an IC_{50} of 0.001 μM against the isolated enzyme and 0.074 μM against ATR kinase-dependent CHK1 phosphorylation in cells (Fig. 1B). Therefore, cell viability assays were performed after adding 1 nM AZD6738 to cells treated with each concentration of FTD to confirm that cell viability was not inhibited (Fig. S1). The IC_{50} values of AZD6738, calculated from 72-h MTT dose-response curves, are ≥1 μM for HCT116 and HT29 cells (32). The minimum concentration at which the drug acts adequately is unknown. Therefore, to determine the minimum concentration at which AZD6738 acts, the effects of each concentration of FTD and AZD6738 with an IC_{50} of 70 μM on HT29 cells were evaluated by flow cytometry (data not shown). The HT29 cell line was treated with 70 μM FTD combined with 0, 0.5, 5, 50, 100, 250, 500, 1,000 and 5,000 nM AZD6738. Flow cytometry was used to evaluate the localization of each cell cycle. The cell cycle localization did not change in response to 0.5, 5, 50, 100 and 250 nM AZD6738 at 48 h, but the number of cells in the G_2/M phase began to decrease at 500 nM (0.5 μM). Therefore, it was concluded that the lowest concentration at which AZD6738 begins to act on the HT29 cell line is 0.5 μM. Further toxicity experiments revealed that the optimal cell concentration at which AZD6738 acts is 0.5 μM. The IC_{50} for FTD was 70 μM in HT29 cells and 5 μM in HCT116 cells (33,34).

Inhibitory effects of combined FTD and AZD6738 treatment on cell cycle progression and cell viability. HT29 and HCT116 cells were divided into the control, AZD6738, FTD, and FTD + AZD6738 groups, and were cultured for 0, 24, 48 and 72 h. The cell cycle progression of each treatment group is shown in Fig. 2A. Cell cycle arrest was detected at the G_2/M checkpoint in the FTD group. By contrast, the FTD + AZD6738 group showed a decrease in the number of cells localized to the G2/M checkpoint. Cell viability was measured at each elapsed time, and a significant difference in cell viability was observed between the FTD + AZD6738 and FTD groups at 72 h for HT29 and HCT116 cells (Fig. 2B).

Combined FTD and AZD6738 treatment alters protein expression, leading to the accumulation of DNA damage and cell death. Chk1 phosphorylation in HT29 cells was suppressed in the FTD + AZD6738 group compared with that in the FTD group at 48 h (Fig. 3). Thus, AZD6738 was confirmed to suppress Chk1 phosphorylation. In HT29 and HCT116 cells,

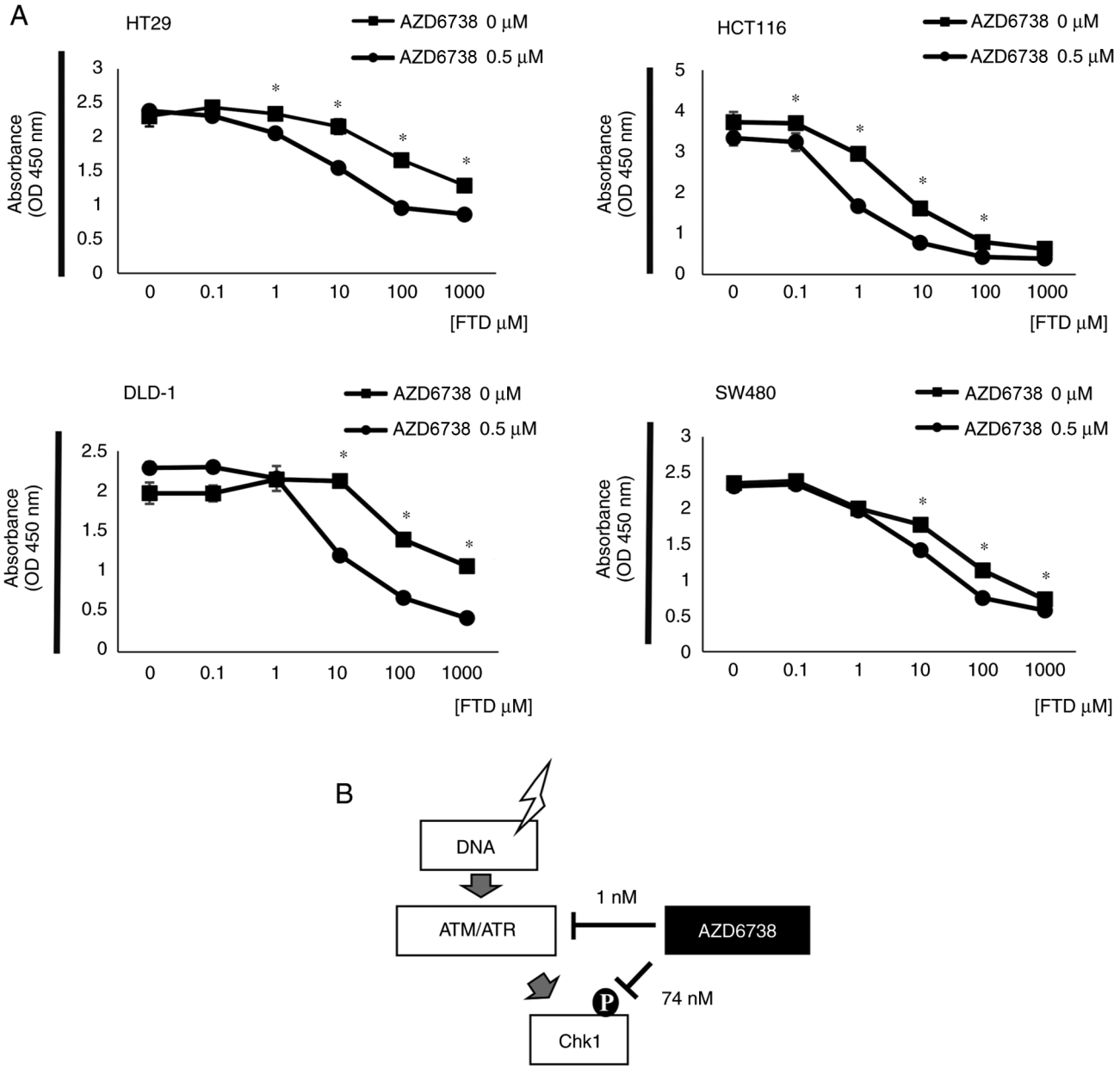

Figure 1. Addition of AZD6738 to FTD inhibits cell viability, despite the FTD concentration being <IC_{50}. (A) HT29, HCT116, DLD-1 and SW480 cells were treated with the indicated concentrations of FTD with or without AZD6738 (0.5 μM). Cells were collected at 72 h and cell viability was measured using a WST-1 assay. (B) AZD6738 has different effects at different concentrations. Data are presented as the mean ± SD (n=6). Statistical significance was determined using Student's t-test. *P<0.05 vs. AZD (0 μM). ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related; FTD, trifluridine.

DNA was more damaged in the FTD + AZD6738 group than that in the FTD group, as confirmed by the increased expression levels of γH2A.X (Fig. 4A and B) (35). Furthermore, the expression of apoptotic proteins in HT29 and HCT116 cells was higher in the FTD + AZD6738 group than that in the FTD group, as confirmed by the expression levels of cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase (Fig. 4C and D). These results indicated that DNA may be damaged in response to FTD + AZD6738 treatment, leading to apoptosis.

Synergistic effects of AZD6738 and FTD in vivo. The effects of FTD and TAS-102 on cell viability were comparable, thus indicating that cell viability was not affected by the addition of tipiracil to FTD (Fig. S2). In the TAS-102 + AZD6738 group, tumor volume was significantly smaller than that in the TAS-102 alone group on days 15, 19 and 22 (Fig. 5A). Images of the excised tumors in the TAS-102 and TAS-102 + AZD6738 groups are shown in Fig. 5B. Measurements of the excised tumors were consistent with those measured from the body surface. The TAS-102 + AZD6738 group exhibited slight weight loss compared with the other groups, but this was not significant (Fig. 5C).

No humane endpoints were reached in the mice. Furthermore, no significant differences in the weights of the liver, kidney and testes were detected between the groups (Fig. S3). As demonstrated by tumor immunostaining, γH2A.X protein levels were significantly higher in the TAS-102 + AZD6738 group than those in the TAS-102 group (Fig. 5D). The higher expression of γH2A.X in the TAS-102 + AZD6738

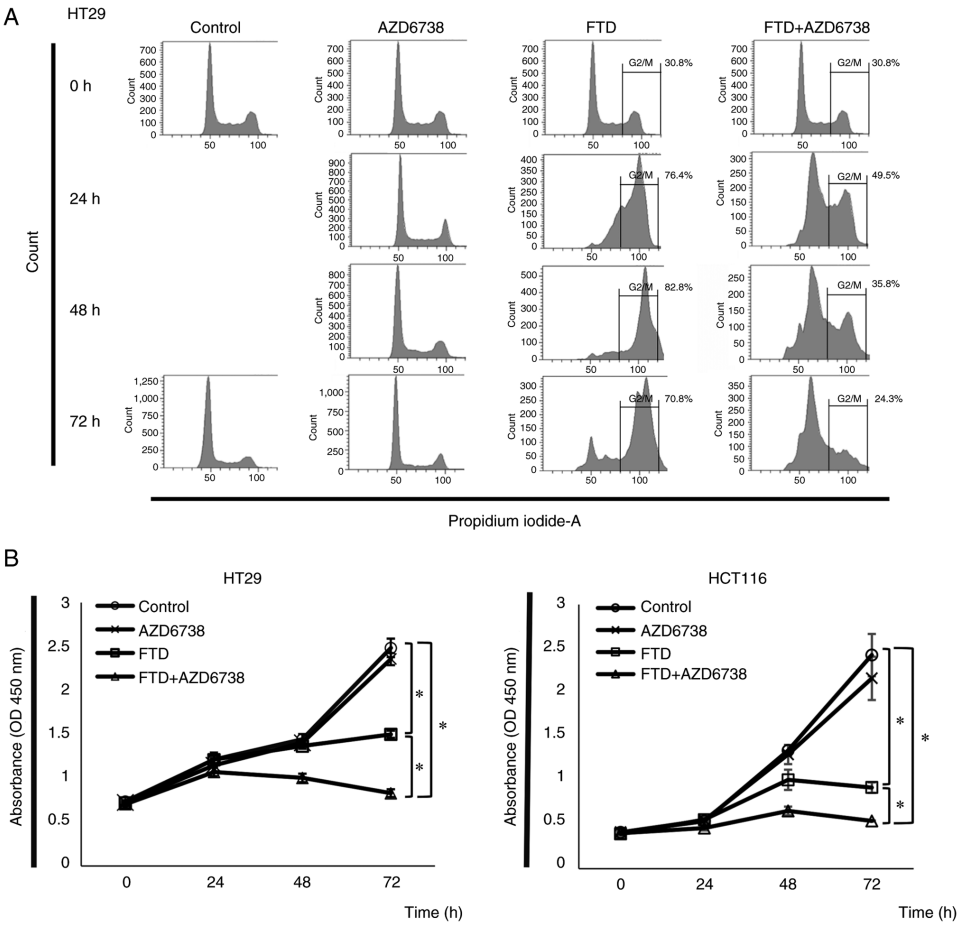

Figure 2. Cell cycle localization of HT29 cells, and changes in HT29 and HCT116 viability over time after treatment with FTD. Cells were collected at 0, 24, 48 and 72 h after no treatment (control), AZD6738 monotherapy (0.5 μM), FTD monotherapy (70 μM) or FTD (70 μM) + AZD6738 (0.5 μM) combination therapy. (A) Cell cycle localization at each time point in each group was evaluated via flow cytometry. The percentage of cells localized to the G2/M checkpoint is shown. (B) Changes in cell viability were evaluated using a WST-1 assay. Data are presented as the mean ± SD (n=6). Statistical significance was determined using one-way ANOVA and Tukey's multiple comparison tests. *P<0.05. FTD, trifluridine.

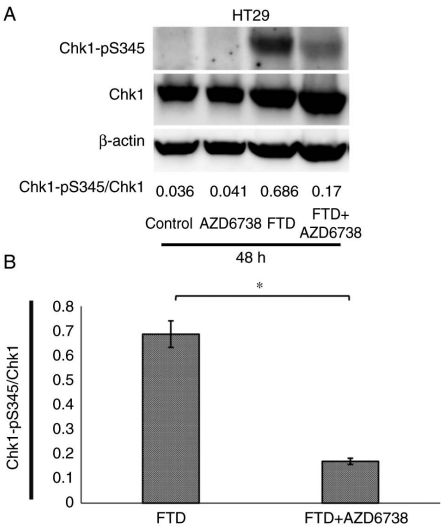

Figure 3. AZD6738 in combination with FTD decreases Chk1 phosphorylation. HT29 cells were separated into control, AZD6738 (0.5 μM), FTD (70 μM) and FTD (70 μM) + AZD6738 (0.5 μM) groups. (A) Cells were collected at 48 h and whole-cell extracts were subjected to western blotting with Chk1-pS345, Chk1 and β-actin antibodies. (B) Semi-quantification of Chk1-pS345/Chk1 48 h after drug administration. Data are presented as the mean ± SD (n=3). Statistical significance was determined using Student's t-test. *P<0.05. FTD, trifluridine; Chk1-pS345, phosphorylated form of Chk1.

group compared with that in the TAS-102 group indicated that DNA damage is accumulated without repair.

Discussion

AZD6738 has an IC_{50} of 0.074 μM for inhibiting Chk1 Ser345 phosphorylation (ATR substrate) in cells (30). AZD6738 exerts different effects depending on its concentration, including inhibition of Chk1 phosphorylation and, at the same concentration, inhibition of DNA damage repair. In the present study, AZD6738 suppressed cell viability when combined with FTD despite using a concentration of AZD6738 (0.5 μM) that did not kill cells in toxicity experiments (20). These findings indicated that combination therapy with FTD and AZD6738 inhibited the viability of colorectal cancer cells. In HT29 colorectal cancer cells, FTD-induced DNA damage arrested cells at the G_2/M checkpoint for repair, but the suppression of Chk1 phosphorylation by AZD6738 prevented the repair of cancer cells, resulting in the accumulation of DNA damage and, consequently, apoptosis. Furthermore, combination therapy with TAS-102 and AZD6738 suppressed tumor growth in a xenograft mouse model.

Based on the results of the present study and a previous study on 5-FU, the following may be inferred (20). In a HT29 xenograft mouse model, the volume of tumors in mice treated

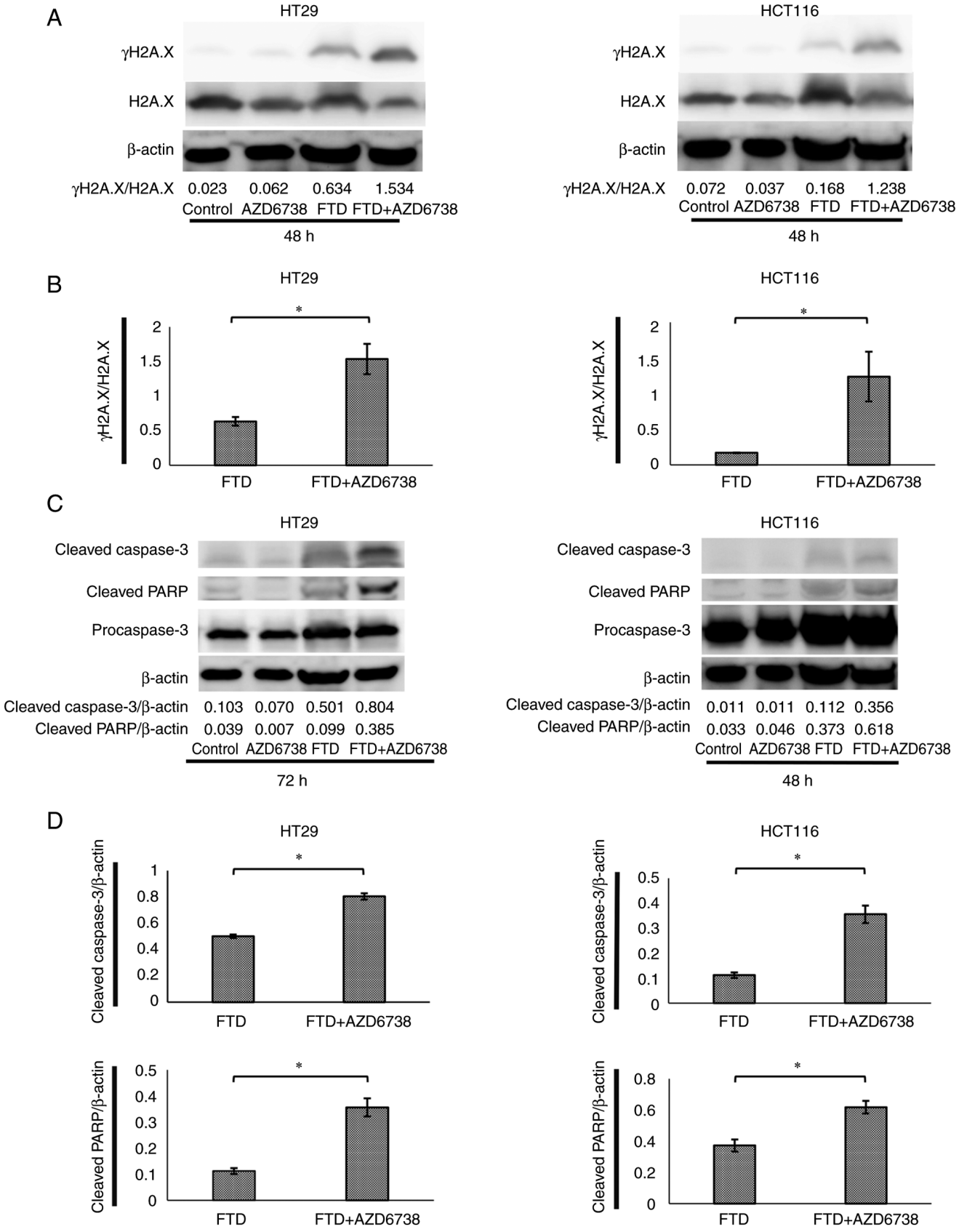

Figure 4. AZD6738 in combination with FTD results in the accumulation of more DNA damage and increased γH2A.X protein levels compared with FTD alone. AZD6738 + FTD also increased the expression of apoptotic proteins compared with FTD alone. HT29 and HCT116 cells were divided into control, AZD6738 (0.5 μM), FTD (70 μM or 5 μM) and FTD (70 μM or 5 μM) + AZD6738 (0.5 μM) groups and treated until the indicated time. Whole-cell extracts were subjected to western blotting with γH2A.X, H2A.X, cleaved caspase-3, cleaved PARP and β-actin antibodies. For FTD concentrations, the respective IC50 values were used: 70 μM for HT29 and 5 μM for HCT116. (A) γH2A.X expression in HT29 and HCT116 cells after 48 h. (B) γH2A.X/H2A.X in HT29 and HCT116 cells after 48 h. (C) Cleaved caspase-3 and cleaved PARP levels in HT29 and HCT116 cells at 48 and 72 h. (D) Cleaved caspase-3/β-actin and cleaved PARP/β-actin in HT29 and HCT116 cells at 48 and 72 h. Data are presented as the mean ± SD (n=3). Statistical significance was determined using Student's t-test. *P<0.05. γH2A.X, phosphorylated form of H2A.X; FTD, trifluridine; H2A.X, H2A.X variant histone; PARP, poly(ADP-ribose) polymerase.

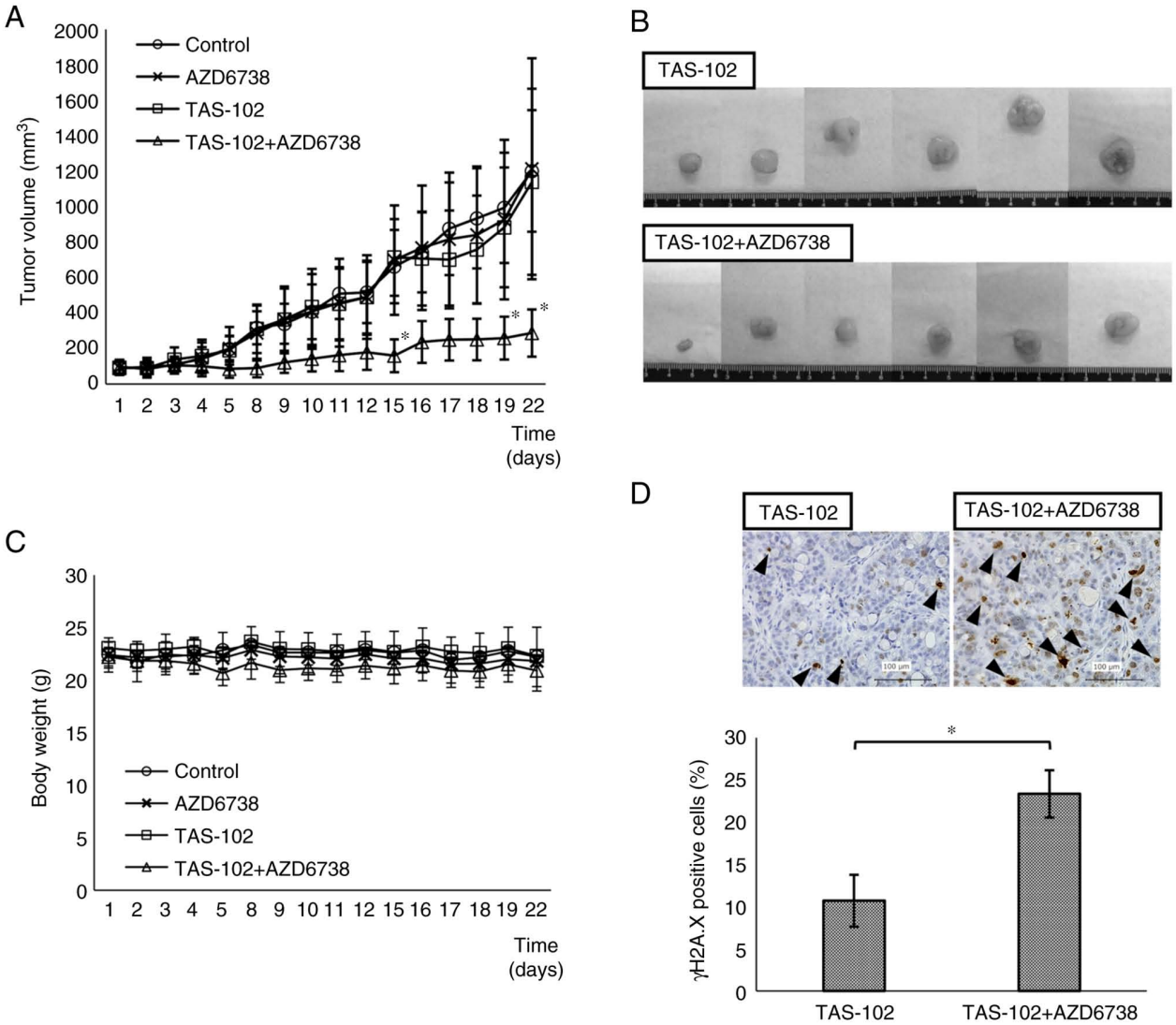

Figure 5. Combination therapy with TAS-102 and AZD6738 effectively inhibits tumor growth in the HT29 xenograft mouse model. (A) Tumor volume in the xenograft mouse model was measured daily. Statistical significance was determined using one-way ANOVA and Tukey's multiple comparison tests. *P<0.05 compared with all of the other groups. The combination group of TAS-102 + AZD6738 suppressed tumor volume significantly more than all of the other groups. (B) Images of tumors removed from the subcutis of mice in the TAS-102 and TAS-102 + AZD6738 groups. (C) Although there was a slight trend toward weight loss in the TAS-102 + AZD6738 group, no significant differences in weights were detected. No mice lost enough weight to reach the humane endpoint. (D) Tumors removed from the TAS-102 and TAS-102 + AZD6738 groups were immunostained for γH2A.X to verify the accumulation of DNA damage. More positive cells were found in the TAS-102 + AZD6738 group, and a representative image is shown (magnification, x200; scale bar, 100 μm). Arrowheads indicate γH2A.X-positive cells. Statistical significance was calculated using Student's t-test. *P<0.05. Data are presented as the mean ± SD (n=6). γH2A.X, phosphorylated form of H2A.X; H2A.X, H2A.X variant histone.

with 5-FU + AZD6738 was ~50% the volume of tumors in mice treated with 5-FU alone. In the present study, the volume of tumors in mice treated with TAS-102 + AZD6738 was ~25% of the volume of tumors in mice treated with TAS-102 alone. These results suggested that FTD was taken up more efficiently than 5-FU, resulting in a higher tumor suppressive effect. Cancer cells with p53 mutations at the G_1 checkpoint depend on the G_2/M checkpoint for repair (36). However, it could be hypothesized that HCT116 cells without p53 mutations have DNA damage in the S phase, which is often repaired at the G_2/M checkpoint, leading to cell death. Although ATR inhibitors were expected to be effective only in cells with p53 mutations, the present study demonstrated that ATR inhibitors were also effective in cells without p53 mutations.

Notably, the present results indicated that some anticancer drugs, including those that were considered ineffective, can achieve tumor suppression when combined with AZD6738. BRAF mutations occur in colorectal cancer with a low response rate to systemic chemotherapy. These mutations occur in ~10% of patients with metastatic colorectal cancer, and novel therapeutic agents are urgently needed for this type of cancer (37). The HT29 cells used in the present study are BRAF mutants. Notably, in the present study, FTD alone was unable to inhibit cell viability; however, the combination of FTD and AZD6738 inhibited the viability of HT29 cells, suggesting the acquisition of a chemotherapy response.

FTD exerts different effects on different cell lines. HCT116 cells are BRAF wild-type, and the IC_{50} of FTD in this cell line was 5 μM, which is sufficient for its efficacy.

Conversely, HT29 cells are BRAF mutant, and the IC_{50} of FTD in this cell line is ~100 μM; therefore, the effect of drug therapy is generally poor (33). In the present study, the IC_{50} of FTD alone was 70 μM in the HT29 cell line, which is a high value and is thus considered ineffective. However, when AZD6738 was used in combination with FTD, cell viability was significantly suppressed when FTD was administered at 1 μM or higher. Thus, the addition of AZD6738 was sufficient to suppress cell viability, indicating that drugs with poor inhibitory effects on cell viability can be converted into effective drugs. The combination therapy of FTD and AZD6738 in BRAF-mutant cell line was effective. In our previous report on the combination of 5-FU and AZD6738, 5-FU was administered intraperitoneally (20). By contrast, in the present study, FTD and AZD6738 were administered orally. The current animal experiments were able to demonstrate drug efficacy in a way similar to that performed in actual clinical practice.

The current findings suggested that combining AZD6738 with various drugs may increase the tumor suppressive effects of these drugs in colorectal cancer, which is usually resistant to anticancer drugs and difficult to treat in current clinical practice. The combination of FTD and AZD6738 was more effective than FTD alone *in vitro*. Furthermore, the combination of TAS-102 and AZD6738 was more effective than TAS-102 alone *in vivo*. In the future, In the future, if AZD6738 can be shown to be effective in cell lines resistant to 5-FU, the mainstay of anticancer drugs, it will make it easier to introduce AZD6738 into routine clinical practice.

The present study has several limitations. To accurately compare the effect of FTD with 5-FU on tumor suppression, these drugs need to be compared in the same cells. In addition, we cannot confirm that the wild-type p53 genotype of HCT116 cells contributed to the efficacy of FTD, as other genetic characteristics may be involved. Knock-in and knockout of the p53 gene in the same cell lines are warranted to confirm the role of p53. Moreover, only *in vitro* investigations were conducted on HCT116 cells; thus, the efficacy of AZD6738 on HCT116 cells, a wild-type p53 cell line, should be confirmed *in vivo*.

In conclusion, AZD6738 is effective for inhibiting colorectal cancer tumor proliferation when combined with FTD. Based on this finding, future clinical trials should be conducted to assess combination therapy with AZD6738 and FTD.

Acknowledgements

The authors would like to thank Ms.Seiko Inumaru, a laboratory assistant, for preparing the experimental reagents and handling the tumors, and Ms.Ryoko Hara, a laboratory assistant, for preparing the experimental reagents (both are affiliated with the Department of Gastroenterology, Nagoya City University Graduate School of Medicine).

Funding

This research was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (assignment no. 19K18158).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SH, TS, HT and TH contributed to the conception and design of this study, analysis and interpretation of the data, and writing and review of the manuscript. SH, TS, HT, TH, AK, KW, TY, HU, KS, RO, YM and ST designed the study. SH, TS, AK, KW, TY, HU, KS, AM and MK performed experiments and obtained data. SH, TS, KW, TY, HU, KS, RO, AM, MK, YM and ST confirm the authenticity of all the raw data. SH wrote the manuscript. TS and HT proofread the manuscript. TS, HT, TH, KS, RO, YM, AM, MK and ST supervised the study. All authors read and approved the final manuscript, and are equally responsible for all aspects of the study and guarantee its completeness and accuracy.

Ethics approval and consent to participate

In vivo mouse experiments were approved by the Animal Welfare and Use Committee of the Nagoya City University Graduate School of Medicine (approval no. 22-006, approved June 06, 2022).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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