5-FU resistance abrogates the amplified cytotoxic effects induced by inhibiting checkpoint kinase 1 in p53-mutated colon cancer cells

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Abstract. The emergence of chemoresistance is a major limitation of current cancer therapies, and checkpoint kinase (chk1) 1 positively correlates with resistance to chemo- or radio-therapy. Cancer cells lacking p53 pathways are completely dependent on the S and G2/M checkpoints via Chk1; therefore, Chk1 inhibition enhances the cytotoxicity of DNA-damaging agents only in p53-deficient cells. However, little is known about the synergistic effect of Chk1 inhibition with 5-FU, the most frequently used antimetabolite, in chemoresistant colorectal cells. In this study, we found that 5-FU induced S-phase arrest only in p53-deficient colorectal cancer cells. 5-FU treatment induced DNA damage and activation of ataxia telangiectasia mutated (ATM) and Chk1, leading to S-phase arrest, and Chk1 inhibition using SB218078 reduced S-phase arrest and increased apoptosis in the presence of 5-FU. In contrast, in p53-deficient, 5-FU-resistant (5FUR) colon cancer cells that we developed, 5-FU enhanced DNA damage but did not induce Chk1/ATM activation or cell cycle arrest. SB218078 in combination with 5-FU did not induce apoptosis. These results indicate that 5-FU-resistance abrogated the anticancer effect amplified by Chk1 inhibition, even in p53-deficient cancer cells.

Introduction

Many cancer therapies effectively kill proliferating tumor cells by causing DNA damage. However, a major limitation of current therapies is the emergence of resistance following the initial treatment. Various mechanisms, including, altered drug transport, increased expression of enzymes that the drugs target, enhanced catabolization of the drug by an enzyme, and/or increased tolerance of the cells to genotoxic stress via cell cycle checkpoints, DNA repair, and/or apoptosis, have been implicated in chemoresistance (1).

For the treatment of major solid tumors, particularly colorectal cancers, 5-FU has long been a mainstay chemotherapeutic antimetabolite drug. The response ratios of 5-FU monotherapy, combination therapy with irinotecan or oxaliplatin, and the newly developed combination therapy with bevacizumab and cetuximab are 15% (2), 40% (3), and 60‑70% (4), respectively. However, the current therapy causes severe side-effects because it is not tumor-specific and it injures normal organs. Therefore, the development of cancer-specific therapies is urgently required.

The major mechanism of the cytotoxicity of 5-FU is the inhibition of nucleotide synthesis. This drug rapidly enters tumor cells, and one of the principal intracellular derivatives of 5-FU, fluorodeoxyuridine monophosphate (FdUMP), forms a covalent complex with thymidylate synthetase (TS), thereby inhibiting the catalytic activity of TS (5), leading to depletion of the intracellular pools of deoxythymidine mono- and tri-phosphate (dTMP and dTTP) and an increase in the relative levels of the normal precursor dUMP and its anabolic derivative dUTP (6). In addition to the nucleotide pool perturbations, UTP andFdUTP incorporate into DNA, resulting in the induction of stalled replication forks and S-phase arrest in cells treated with 5-FU (6,7). FdUTP and dUTP misincorporation

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is potentially mutagenic and miscoding, but both can be excised through the action of base excision repair (BER) and mismatch repair (MMR). DNA strand breaks are generated as byproducts of the repair processes, and such DNA damage can induce apoptosis if left unrepair (8-10).

In response to DNA damage, cells activate a complex signaling network that mediates cell cycle arrest to allow time for DNA repair or, when the damage is extensive, to trigger apoptosis. The DNA damage response is initiated by activation of the ataxia telangietasia mutated (ATM) and ATM- and Rad-related (ATR) kinases and the DNA-dependent protein kinase catalytic subunit. These kinases recruit the repair machinery to sites of damaged DNA while halting cell cycle progression by activating the effector kinases checkpoint kinase (Chk) 1 and 2. The activation of checkpoints controlled by ATM/ATR-Chk1/Chk2 stalls cell cycle progression in G1, S, or G2 phase (11). G1 arrest is mediated by p53 through p21CIP1/WAF1 upregulation and, if the DNA damage is extensive, triggers apoptosis (12). However, many cancer cells show a loss of function of p53 or its regulatory pathways; therefore, chemotherapy-induced DNA damage fails to arrest the cancer cells in G1 phase and promote apoptosis. These cells are completely dependent on the S and G2/M checkpoints to arrest the cell cycle and facilitate DNA repair before entry into mitosis (M phase) after genotoxic exposure. The ATR/Chk1 kinases are involved in the intra-S and G2/M checkpoints (13), function in the regulation of cell cycle arrest following genotoxic stress, and prevent new replication origins from firing during S phase.

Checkpoint kinase 1 (Chk1) inhibition induces the premature entry of cells with DNA damage into M phase and leads to the promotion of abnormal mitotic spindles, altered chromosome segregation, abnormal cell division, the formation of multiple nuclei and apoptosis (14). This synergistic cytotoxicity of Chk1 inhibitors in combination with anticancer, DNA-damaging agents is especially effective against p53-deficient cancer cells compared with p53-proficient cells, including normal cells (15). These cancer-specific therapies have been considered examples of synthetic lethality, and many Chk1 inhibitors in combination with a variety of anticancer DNA-damaging agents are at various stages of preclinical and clinical development (16). In colorectal cancer, ATR, one of the regulators of Chk1 activation, is activated by DNA-damaging agents, and inhibition of ATR selectively sensitizes p53-deficient cells to cisplatin (17). It has also been reported that selective Chk1 inhibitors abrogate cell cycle checkpoints and potentiate the cytotoxicity of topoisomerase inhibitors in p53-deficient, but not in p53-proficient, colon cancer cells (18). 5-FU is the most frequently used chemotherapeutic agent for colorectal cancer. It has been reported that 5-FU activates Chk1 and that Chk1 downregulation abrogates S-phase arrest (19). Judging from these results, it is expected that the synergistic antitumor effects of Chk1 inhibition with 5-FU are more effective in p53-deficient cells than in p53-proficient colorectal cancer cells. However, these p53 status-dependent, synergistic antitumor effects of 5-FU and Chk inhibition in colorectal cancer are still unclear. Moreover, thus far, no therapy has reached the bedside, even though a highly selective Chk1 inhibitor would theoretically synergize with chemotherapy, suggesting the limitation of these therapies.

In this study, we investigated the effect of 5-FU treatment in p53-proficient and -deficient GI-tract cancer cells to develop tumor-specific anticancer therapy. In addition, we hypothesized that Chk1 inhibition might be effective in sensitizing 5-FU-resistant (5FUR) cancer cells to 5-FU because Chk1 activation is reported to be related to the resistance to chemotherapy (20). Therefore, we also investigated the synergistic cytotoxic potential of Chk1 inhibition on 5-FU treatment in p53-deficient colon cancer cells with/without 5-FU resistance. We observed that 5-FU specifically induced S-phase arrest in p53-deficient cancer cells, that 5-FU induced Chk1 activation and that Chk1 inhibition produced a synergistic effect on 5-FU cytotoxicity. We also found that in p53-deficient, 5FUR cancer, 5-FU did not induce S-phase arrest or Chk1 activation, although 5-FU induced significant DNA damage, and Chk1 inhibition did not sensitize the cells to 5-FU cytotoxicity.

Materials and methods

Cell lines and culture conditions. LS-174T and MKN45, which are wild-type p53 human colorectal and gastric cancer cell lines, respectively, and HT29, WiDr, and KATO III, which are p53-mutant human colorectal and gastric cancer cell lines, were obtained from the American Type Culture Collection (Rockville, MD, USA) and Riken Cell Bank (Ibaraki, Japan).

To prepare the 5FUR cancer cell line, HT29 was exposed to increasing doses of 5-FU, up to the clinically relevant plasma concentration of 2 µg/ml. The surviving resistant cells were named 5FUR cells. All cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum and 1% antibiotics and antimitotics at 37°C in a humidified atmosphere of 5% CO2.

Drugs and antibodies. 5-FU was purchased from Sigma-Aldrich (St. Louis, MO, USA). SB218078, a Chk1 inhibitor, was obtained from Calbiochem (San Diego, CA, USA). The antibodies used for western blotting were as follows: rabbit polyclonal antibodies to phospho-ATR (Ser428), phospho-Chk1 (Ser296), and β-actin; rabbit monoclonal antibodies to phospho-ATM (Ser1981) and phospho-Chk1 (Ser345); and mouse monoclonal antibodies to Chk1 and TS (Cell Signaling Technology, Inc., Danvers, MA, USA).

Cell cycle analysis. Cells were seeded at 2.0x104 cells/well in 6-well plates and treated with or without 5-FU (2 µg/ml) and SB218078 (1 µM) for the indicated time periods. The cell cycle profile was determined by the propidium iodide staining of nuclei isolated using the BD CycleTest Plus kit (BD Biosciences, San Jose, CA, USA) according to the supplier's directions. Fluorescence was quantitated using a FACScanto™ flow cytometer with FACSDiva 6.1.3 software (BD Biosciences).

Cell survival assay. The rates of drug resistance were assessed using the WST assay with the Cell Count Reagent SP (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol. Briefly, 8,000 cells of each cell line were plated in each well of a 96-well plate in 100 µl medium with or without 5-FU (2 µg/ml) for 48 h. After treatment, 10 µl WST reagent was added to each well, and the plate was incubated for 1 h.
Colorimetric analysis was then performed at a wavelength of 450 nm using a standard microplate reader. Cell survival was calculated by dividing the surviving cell number estimated by WST in the presence of the drug by the number in the absence of the drug.

**Alkaline comet assay.** The alkaline comet assay was performed according to the method described by Singh et al (21), with slight modifications (22). After staining with 20 µg/ml ethidium bromide for 1 min, we quantified the DNA damage of 100 randomly selected cells using the Comet Assay IV software (Perceptive Instruments Ltd., Suffolk, UK) on a computer attached to a fluorescence microscope (Olympus, Tokyo, Japan). We used the tail moment (the product of the relative intensity of the tail and the distance from the center of the nucleus to the center of gravity of the tail) to evaluate the degree of DNA damage.

**Intracellular concentrations of 5-FU.** The intracellular concentrations of 5-FU were measured by gas chromatography/mass spectrometry (GC/MS). Initially, cells were seeded at 1.2x10^6 cells/10-cm tissue culture dish and treated with 5-FU (2 µg/ml) for 48 h. The GC/MS system consisted of a Trace GC gas chromatograph separation module and a Trace MS mass spectrometer (both from Thermo Fisher Scientific, Inc., Waltham, MA, USA). A DB-5 column (length, 30 m; inside diameter, 0.32 mm; film thickness, 0.25 µm; J&W Scientific, Folsom, CA, USA) was used for the peak separation of 5-FU. The detector was used in SIM mode using the selected ion-monitoring procedure at m/z=309 for 5-FU and at m/z=311 for 5-FU-15N2. An internal standard solution (including 5-FU-15N2) was added to each sample, and the solution was then extracted using ethyl acetate. The reaction product was extracted using a solution of mixed ethyl acetate and n-hexane, which was then evaporated to dryness under a
stream of nitrogen. The residue was dissolved in ethyl acetate, and a 1-µl aliquot was injected into the GC/MS system.

**Western blotting.** For all western blotting, cells were seeded at 2.0x10⁶ cells/well in 6-well plates and treated with or without 5-FU (2 µg/ml) and SB218078 (1 µM) for 48 h. The cells were lysed in lysis buffer [1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1x protease inhibitor cocktail (Nacalai Tesque), and phosphate-buffered saline, pH 7.4]. Next, the lysates were cleared by centrifugation at 10,000 g at 4°C for 15 min. The protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Equal amounts (10 µg) of the protein lysates were then electrophoretically separated on SDS-polyacrylamide gels and transferred onto a polyvinylidene fluoride membrane. For immunodetection, the above-mentioned primary antibodies were used.

**Results**

**Cell cycle analysis of wild-type and mutant p53 human gastrointestinal cancer cells treated with 5-FU.** At first, we investigated the effect of 5-FU treatment on the cell cycle of human colorectal and gastric cancer cell lines expressing wild-type p53 or mutant p53. In neither LS-174T nor MKN45 cells, which express wild-type p53, treated with 5-FU, no change was observed in S- or G2/M-phase compared with control cells. In contrast, 5-FU treatment induced S-phase arrest in HT29, WiDr, and KATO III cells, which express mutant p53 (Fig. 1).

**Characteristics of 5FUR cells.** To prepare 5FUR HT29 cells, dubbed 5FUR cells, HT29 cells were treated with increasing concentrations of 5-FU for 1 year. Fig. 2A shows the 5-FU sensitivity assay of the parental and 5FUR cells.
The clinical 5-FU concentration in plasma is reported to be <1.5 µg/ml (23); the survival of the 5FUR cells was significantly higher than that of the parental cells in the presence of 2 µg/ml 5-FU (p<0.05). There was no significant difference in the cellular 5-FU concentrations of the 5-FU-treated parental HT29 cells and the 5-FU-treated 5FUR cells (Fig. 2B). In certain parts of the patient population, an elevated TS level is linked to 5-FU resistance, so we compared the amount of free TS between parental cells and 5FUR cells treated with or without 5-FU for 48 h. 5-FU treatment formed complexes ofFdUMP and TS, and the residual 5-FU in the 5FUR cells formed a small amount of the complex. However, the amounts of free TS in the 5-FU-treated parental HT29 cells and the 5-FU-treated 5FUR cells were not different (Fig. 2C). We then used alkaline comet assays to analyze the levels of damaged DNA in the HT29 and 5FUR cells 48 h after treatment with 2 µg/ml 5-FU. In the comet assays, both the parental and the 5FUR cells treated with 5-FU had longer tail moments than did the 5-FU-untreated parental and 5FUR cells (Fig. 2D). We also investigated the effect of 5-FU treatment on the cell cycle phase distribution of both cell types (Fig. 2E). The sub-G1 fraction was not detected in the parental HT29 or 5-FU-treated 5FUR cells, indicating that 5-FU did not induce apoptosis in either cell type. Additionally, 5-FU induced S-phase arrest in the parental HT29 cells, but in the 5FUR cells, 5-FU induced no change in the cell cycle compared with DMSO treatment, indicating that the acquisition of 5-FU resistance abrogated the 5-FU-induced S-phase arrest.

**Acquisition of 5-FU resistance abrogates 5-FU-induced Chk1 phosphorylation.** Chk1 regulates S-phase arrest; therefore, we determined whether 5-FU activated the Chk1 pathway in both parental and 5FUR cells (Fig. 3). Chk1 activation is associated with phosphorylation at Ser345 (the site of Chk1 phosphorylation by ATR/ATM) and autophosphorylation at Ser296. The assessment of Chk1 activation (using a DNA-damaging agent or antimetabolite) and Chk1 inhibition (by a Chk1 inhibitor) is most likely best accomplished by monitoring the phosphorylation of Ser296 (15). In particular, SB218078 inhibits Chk1 autophosphorylation (Ser296) and increases the phosphorylation of ATM (Ser1981) and Chk1 (Ser345) (24). In this study, the phosphorylation of Chk1 and ATM (Ser1981) was not enhanced in 5FUR cells, whereas the phosphorylation of Chk1 and ATM was enhanced in parental cells treated with 5-FU. As a previous study reported (24), SB218078 significantly reduced the phosphorylation of Chk1 (Ser296), whereas the phosphorylation of Chk1 (Ser345) and ATM (Ser1981) was actually enhanced in parental and 5FUR cells with or without 5-FU treatment. In contrast, the phosphorylation of ATR (Ser428) was not enhanced by 5-FU treatment in parental or 5FUR cells.

**Effect of the acquisition of 5-FU resistance on the synergistic effect of the Chk1 inhibitor SB218078 and 5-FU in HT29 cells.** The synergistic effect of combined 5-FU and SB218078 treatment was evaluated by analyzing the cell cycle phase distribution and cell death rates. SB218078 showed no effect on cell viability in the parent cell line during monotherapy, but when in combination with 5-FU, SB218078 reduced the early S-phase arrest induced by 5-FU treatment and increased the sub-G1 population, indicating induction of apoptotic cell death. In contrast, SB218078 exhibited no effect on cell death in 5FUR cell lines during monotherapy or when in combination with 5-FU (Fig. 4).

**Discussion**

In this study, we investigated the synergistic antitumor effect of a Chk1 inhibitor and 5-FU that was specific to p53-deficient cells, and we analyzed the potential of Chk1 inhibition to sensitize 5FUR cancer cells to 5-FU to develop a tumor-specific, effective therapy that overcame 5-FU resistance.

The enhanced excretion or degradation of 5-FU and the expression of TS are well-known mechanisms for 5-FU resistance. In this study, however, equal levels of intracellular 5-FU and free TS protein were detected in parental and 5FUR cells that were treated with 5-FU. Chk1 is overexpressed in a variety of human tumors, including breast, colon, liver, gastric, and nasopharyngeal carcinoma (25-31). Remarkably, Chk1 expression often positively correlates with tumor grade and disease recurrence and may contribute to therapy resistance (29,30,32). The enhanced activation of Chk1 is also related to the resistance of cancer cells, including cancer stem cells from brain glioblastoma, prostate, and lung NSCLC, to chemotherapy or radiotherapy (33-38). Therefore, we hypothesized that 5FUR cells might possess more Chk1 activity than control cells and that Chk1 inhibition might exert a synergistic, cytotoxic effect and sensitize chemo-resistant cells to 5-FU.

DNA repair systems promote the faithful transmission of genomes in dividing cells by reversing extrinsic and intrinsic DNA damage and are required for cell survival during replication. Cancer cells are frequently found to be deficient in certain aspects of DNA repair. The impairment of DNA repair systems...
contributes not only to the initial mechanism of carcinogenesis but also to its weakness, as these repair systems are required for the cancer cells to maintain their own survival (39).

Ovarian and breast cancer patients with BRCA mutations exhibit favorable responses to poly(ADP-ribose) polymerase (PARP) inhibitors compared with patients without BRCA mutations, as homologous recombination-deficient tumors can be effectively targeted by DNA double-strand break-inducing agents (40). This concept, that is, synthetic lethality, is attracting attention in the development of tumor-specific therapy. Synthetic lethal interactions are defined as two genetic alterations that cause cell death when they occur together, although neither mutation alone is lethal (41). The pharmacological inhibition of one gene product can be synthetically lethal when it occurs in combination with a pre-existing, cancer-related mutation, especially when the mutated cancer cells have become dependent on special pathways, leading to the ability to selectively target and kill the cancer cells while sparing the normal cells (42).

The development of anticancer regimens that take advantage of the molecular differences between normal and cancer cells is highly desirable. TP53 is one of the most frequently mutated genes in human cancers, so there is great interest in finding anticancer regimens that selectively target p53-deficient tumors (43). The cancer cells showing a loss of function of p53 or its regulatory pathways have a deficiency in the G1 checkpoint and are completely dependent on the S and G2/M checkpoints to arrest the cell cycle after genotoxic stress.
Chk1 is critical for S and G2/M arrest via downregulation of Cdc25A, cyclin A and CDK2 expression (44). Chk1 inhibition abolishes the S and G2 checkpoints induced by 5-FU, causes excessive accumulation of DNA damage, induces apoptosis, and ultimately selectively potentiates the efficacy of 5-FU in p53-deficient cells. In contrast, p53-dependent checkpoint(s) in p53-proficient cells allow DNA repair and thereby prevent sensitization to DNA damage (19).

In this study, we observed that 5-FU induced S-phase arrest only in p53-deficient cells and that 5-FU treatment induced Chk1 activation. We also found that Chk1 inhibition by SB218078 significantly increased the population of sub-G1 cells only in the presence of 5-FU. These results indicate that the development of tumor-specific anticancer therapy with 5-FU and Chk1 inhibitors for colorectal cancer, of which p53 is frequently mutated, is to be expected.

In the 5-FUR cell line, 5-FU treatment did not induce Chk1 activation or S-phase arrest. Moreover, SB218078 in combination with 5-FU did not induce a sub-G1 population. These results revealed that in 5FUR, 5-FU-induced DNA damage induced neither Chk1 activation nor S-phase arrest, and 5FUR proliferated in the presence of 5-FU.

To understand the mechanisms underlying the abrogation of Chk1 activation in 5FUR cells, we examined the activation status of a major upstream regulator, Chk1, in these cells. More specifically, Chk1 is a traditional target of ATR in the DNA damage response. We observed that 5-FU treatment did not induce the activation of ATR in 5FUR cells or parental cells, although this treatment activated Chk1 in the parental cells. ATM is also required for Chk1 activation under certain circumstances (45-47). In this study, we found activation of ATM by 5-FU in parental cells, which demonstrates another example of ATM-Chk1 signaling mediating early S-phase arrest. In contrast, in 5FUR, neither ATR nor ATM was activated by DNA damage induced by 5-FU treatment, leading to no induction of Chk1 activation, although there was no difference in the cellular 5-FU concentration or in the amount of free TS between the parental and 5FUR cells, indicating that 5-FU functioned in 5FUR cells as well as in parental cells. The precise mechanism of ATR, ATM, and Chk1 inactivation observed in 5FUR cells remains to be elucidated.

DNA-damaging agents combined with a Chk1 inhibitor cause tumor cells to undergo apoptosis in p53-deficient tumors (48). Mice with Chk1 disruption die during early development (49,50), and the conditional deletion of Chk1 in proliferating mouse mammary epithelial cells induces apoptosis and developmental defects. In contrast to these studies, we did not observe increased apoptosis in 5FUR cells, in which 5-FU treatment induced DNA damage but did not induce Chk1 activation, cell cycle arrest, or an increased sub-G1 fraction. These results indicated that the 5FUR cells acquired apoptotic resistance. It has been reported that the overexpression of Bcl-xL suppresses Chk1 inhibitory lethality (51-53). The precise mechanisms of the anti-apoptotic potentials remain to be elucidated; however, in this study, we observed enhanced expression of Bcl-w and c-FLIP (data not shown) in 5FUR cells.

This study re-evaluated the key role of Chk1 in regulating the 5-FU-induced DNA damage checkpoint and the utility of Chk1 inhibition in tumor-specific anticancer therapy by enhancing the anticancer efficacy of 5-FU only in p53-deficient cells. However, 5-FU resistance in p53-deficient colorectal cancer cells abrogated 5-FU-induced Chk1 phosphorylation, S-phase arrest, and sensitization of p53-deficient cancer cells to 5-FU by Chk1 inhibition. Therefore, Chk1 inhibition combined with 5-FU in p53-deficient cells does not appear to be a promising approach in the context of tolerance to 5-FU.

References


