**ABSTRACT**

Esophageal squamous cell carcinoma (ESCC) is a disease characterized by a high mutation rate of the TP53 gene, which plays pivotal roles in the DNA damage response (DDR) and is regulated by checkpoint kinase (CHK) 2. CHK1 is another key DDR-related protein, and its selective inhibition is suggested to be particularly sensitive to TP53-mutated cancers, because a loss of both pathways (CHK1 and/or CHK2-p53) is lethal due to the serious impairment of DDR. Such a therapeutic strategy is termed synthetic lethality. Here, we propose a novel therapeutic strategy based on synthetic lethality combining trifluridine/tipiracil and prexasertib (CHK1 inhibitor) as a treatment for ESCC. Trifluridine is a key component of the antitumor drug combination with trifluridine/tipiracil (an inhibitor of trifluridine degradation), also known as TAS-102. In this study, we demonstrate that trifluridine increases CHK1 phosphorylation in ESCC cells combined with a reduction of the S-phase ratio as well as the induction of ssDNA damage. Because CHK1 phosphorylation is considered to be induced as DDR for trifluridine-mediated DNA damage, we examined the effects of CHK1 inhibition on trifluridine treatment. Consequently, CHK1 inhibition by short hairpin RNA or treatment with the CHK1 inhibitor, prexasertib, markedly enhanced trifluridine-mediated DNA damage, represented by an increase of γH2AX expression. Moreover, the combination of trifluridine/tipiracil and CHK1 inhibition significantly suppressed tumor growth of ESCC-derived xenograft tumors. Furthermore, the combination of trifluridine and prexasertib enhanced radiosensitivity both in vitro and in vivo. Thus, the combination of trifluridine/tipiracil and a CHK1 inhibitor exhibits effective antitumor effects, suggesting a novel therapeutic strategy for ESCC.

**Introduction**

Esophageal squamous cell carcinoma (ESCC) is the major histologic type of esophageal cancers (1), which are the seventh leading cause of cancer-related mortality and the eighth most common cancer worldwide (2). Despite recent progress in therapeutics, the prognosis of patients with ESCC remains poor (3–5). Specifically, the 5-year survival rates of patients with ESCC (stages II–IVB) receiving chemoradiotherapy or chemotherapy are 19.1% and 5.3%, respectively (5). Therefore, the development of novel chemotherapeutic strategies is required to improve the outcomes of patients with ESCC.

Recently, a novel therapeutic strategy targeting the DNA damage response (DDR) was reported (6, 7). DDR is a critical mechanism that maintains genome stability (8), and it is coordinated by two distinct kinase signaling cascades: ataxia telangiectasia Rad3-related (ATR)-checkpoint kinase 1 (CHK1; ATR-CHK1) and ataxia telangiectasia mutated (ATM)-checkpoint kinase 2 (CHK2; ATM-CHK2) pathways (9, 10). These pathways regulate specific cell-cycle checkpoints: the ATR–CHK1 pathway regulates the S-phase and G2-phase checkpoint (7, 11), whereas the ATM–CHK2 pathway regulates the G1-phase checkpoint (7).

The tumor suppressor protein p53 (encoded by the TP53 gene) is regulated by the ATM–CHK2 pathway (12), and, therefore, p53 plays a pivotal role in DDR (13). TP53 is frequently mutated in cancers (14) and is defective in >50% of human tumors (13). Accordingly, ATM-CHK2-p53-mediated DDR during the G1-phase checkpoint is compromised in many malignant p53-defective tumor cells (15) and, alternatively, those tumor cells are dependent on the ATR-CHK1-mediated S and G2-M-phase checkpoints for DNA damage repair (15). Thus, ATR-CHK1-mediated DDR is suggested to play an important role in the survival of p53-defective cancer cells.

The selective inhibition of CHK1 has been predicted to be particularly sensitive in those p53-defective cells because the loss of both pathways (CHK1 and/or CHK2–p53 pathway) is lethal (13, 16). Such a therapeutic strategy has been termed synthetic lethality (13, 16, 17). Indeed, treatment for p53-defective tumors with CHK1 inhibitors is potentiated by combining it with other anticancer drugs that induce DNA damage (17). Because the TP53 mutation frequency in ESCC is as high as approximately 79.8%–93% (18–22), we consider that ESCC treatment with CHK1 inhibitors may effectively potentiate the antitumor effect by promoting synthetic lethality.

TAS-102 (C10H11F3N4O5⋅C8H24ClN2O2⋅HCl; CAS Number: 733030-01-8) is an orally administered combination drug of a trifluridine (C11H11F3N4O5) as a thymidine-based nucleic acid analog, and a tipiracil hydrochloride (C9H11ClN4O2⋅HCl; ref. 23) as a...
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Trifluridine phosphate inhibitor (TPI). TPI prevents the degradation of trifluridine, allowing the maintenance of adequate plasma trifluridine levels (24). Trifluridine is the active cytotoxic component of TAS-102 (trifluridine/TPI); its triphosphorylated form is incorporated into the DNA, leading to antitumor effects (25). Trifluridine/TPI is approved for metastatic colorectal cancers previously treated with chemotherapy such as fluoropyrimidine, oxaliplatin, and irinotecan (26). It has also been applied for metastatic gastric cancers that have progressed after treatment with other chemotherapy regimens (27).

In this study, we propose a novel therapy combining trifluridine/TPI and a CHK1 inhibitor for ESCC treatment. We found that trifluridine markedly increased CHK1 phosphorylation in ESCC cells. CHK1 phosphorylation is considered to be induced by trifluridine-mediated DNA damage, and the combination of trifluridine and CHK1 inhibition resulted in the induction of severe DNA damage as well as antitumor effects, indicating the efficient induction of synthetic lethality. Furthermore, we show that the combination therapy of trifluridine and a CHK1 inhibitor enhances the radiosensitivity of ESCC cells.

Materials and Methods

Cells and reagents

We used human ESCC cell lines TE-1, TE-10, and TE-11, which were obtained from the Riken BioResource Center. TE-11R cells are 5-fluorouracil-resistant ESCC cells established with TE-11 cells, as described previously (28). We used TE-11R cells in this study because they generate tumors in BALB/cAJcl-nu/nu mice, while other cells (TE-1, TE-10, and TE-11) did not result in xenograft tumors in our previous study (28). These cells were cultured in RPMI1640 medium (Life Technologies Corp.), supplemented with 10% FBS (Life Technologies Corp.), 100 μg/mL of streptomycin, and 100 units/mL of penicillin (Life Technologies Corp.). All cells were cultured at 37°C in a 5% CO2 incubator.

Chemical compounds

Prexasertib (C18H19N7O2: CAS Number: 1234015-52-1) was purchased as the CHK1 inhibitor from Selleck Chemicals. Trifluridine was purchased from Tokyo Chemical Industry, Co., Ltd. Trifluridine/TPI (TAS-102) was provided by Taiho Pharmaceutical Co., Ltd.

EdU flow cytometry assay

A Click-IT EdU Flow Cytometry Assay Kit (Thermo Fisher Scientific, Corp.) was used to assess the effects of trifluridine on the cell cycle. After the cells were cultured in the presence or absence of trifluridine, they were incubated with Click-IT EdU, harvested, and treated according to the manufacturer’s instructions. Cells were analyzed by flow cytometry (LSRFortessa Flow Cytometer; BD Biosciences), and the data were analyzed using BD FACSDiva Software (BD Biosciences). The percentages of cells in the S-phase were determined; cells in a proliferating population (S-phase) show high fluorescence intensity, whereas cells in nonproliferating populations show low fluorescence intensity.

Cell viability assay

Cell viability was determined using the WST-1 Assay (Roche Applied Science) according to the manufacturer’s instructions. Cells (1–5 × 10^3 cells/well) were seeded in 96-well plates and exposed to the indicated concentrations of trifluridine with or without prexasertib (10 mmol/L) for 72 hours. All data were obtained with a Multwell Plate Reader (Infinite 200 Pro, Tecan Group Ltd.) at wavelengths of 450 and 630 nm.

Immunofluorescence

Trifluridine-mediated ssDNA damage was detected using immunofluorescence. Cell pellets were fixed in methanol, and heated in formamide, according to the manufacturer’s instructions (Enzo Life Sciences, Inc.). After the heating, they were blocked with 1% nonfat dry milk in PBS for 15 minutes at room temperature. Then, they were incubated with mouse anti-single-stranded DNA mAb (F7-26; 10 μg/mL: Enzo Life Sciences, Inc.) for 15 minutes at room temperature followed by a fluorescein-conjugated goat anti-mouse IgM (Sigma, catalog no. F-9295) for 15 minutes at room temperature. Nuclei were counterstained by 4′,6-diamidino-2-phenylindole using VECTASHIELD Mounting Medium (Vector Laboratories, Inc.). Stained objects were examined with an EVOS FL Cell Imaging System (Thermo Fisher Scientific, Corp.).

RNA isolation, cDNA synthesis, and real-time reverse transcriptase-PCR

RNasey Midi Kit (Qiagen, Inc.) was used to isolate total RNA from cultured cells. RNA was treated with DNase I (Invitrogen). cDNA synthesis was carried out with the SuperScript First-Strand Synthesis System (Invitrogen) using 3.3 μg of total RNA as a template. The cDNA synthesis reactions without reverse transcriptase yielded no ampiclons in the PCRs. Real-time reverse transcriptase-PCR (RT-PCR) was performed with the LightCycler 480 Instrument II Real-Time PCR System (Roche Diagnostics Ltd.). The relative expression of each mRNA was normalized to β-Actin as an internal control. The primers used in this study were as follows: CHK1: forward 5′-CGGTATAATAATGTTAGGGG-3′; reverse 5′-TTCCAAGGTGTTGAGGTATGT-3′; and ACTB: forward 5′-TTGTTACAGGAAGTCCCCCTGCC-3′; reverse: 5′-ATGCTATCACCCTCCCTGTGTG-3′.

Western blotting

Whole-cell lysates were prepared as follows. Cells were washed twice with ice-cold PBS and lysed with a RIPA Buffer (Nacalai Tesque). After 30 minutes on ice, the cell lysates were centrifuged at 14,000 rpm at 4°C for 30 minutes. Protein concentrations were determined by BCA Protein Assay (Pierce Biotechnology). Protein (10–15 μg) was heat-denatured in a sample buffer solution with reducing reagent (6×) for SDS-PAGE (Nacalai Tesque) at 70°C for 10 minutes. The protein samples were separated on Mini-PROTEAN TGX Gels (Bio-Rad Laboratories, Inc.) and transferred to a polyvinylidene difluoride membrane (Trans-Blot Turbo Transfer Pack, Bio-Rad Laboratories, Inc.). The membrane was blocked in 5% nonfat milk and 1% BSA, pH 5.2, Fraction V (Wako Pure Chemical Industries) in TBS-T (10 mmol/L Tris, 150 mmol/L NaCl, pH 8.0, and 0.1% Tween 20) for 1 hour at room temperature. Membranes were probed with the primary antibody diluted in 5% nonfat milk and 1% BSA in TBS-T overnight at 4°C, washed three times in TBS-T, incubated with secondary antibody in 5% nonfat milk and 1% BSA in TBS-T for 1 hour at room temperature, and, finally, washed three times in TBS-T. The signal was visualized with an enhanced chemiluminescence solution (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Fisher Scientific or Immobilon Western Chemiluminescent HRP Substrate, Merck Millipore) and exposed to a ChemiDoc Touch Imaging System (Bio-Rad).
Laboratories, Inc.). Densitometric analyses of Western blot bands were performed using Image Lab Software (Bio-Rad Laboratories, Inc.). Data were calibrated with β-Actin as a loading control in arbitrary units.

Primary antibodies and the titers used in this study were as follows: mouse monoclonal anti-CHK1 antibody (2G1D5, #2360, Cell Signaling Technology, Inc.; 1:1,000), rabbit monoclonal anti-phospho-CHK1 (Ser 345) antibody (133D3, #2348, Cell Signaling Technology, Inc.; 1:1,000), rabbit polyclonal anti-phospho-CHK1 (Ser 296) antibody (#2349, Cell Signaling Technology, Inc.; 1:1,000), rabbit monoclonal anti-phospho-Histone H2A.X (Ser 139) antibody (20E3, #9718, Cell Signaling Technology, Inc.; 1:1,000), and rabbit monoclonal anti-β-Actin antibody (HRP conjugate, 1:2,000, Cell Signaling Technology, Inc.).

Short hairpin RNA

The following lentiviral vectors were used in this study: GIPZ non-silencing Lentiviral shRNA control (control for CHK1 knockdown), V3LHS_637957 (shCHK1-1), and V3LHS_637959 (shCHK1-2). They were transfected into HEK293T cells to produce viral particles using the Trans-Lentiviral shRNA Packaging Kit (GE Healthcare, Inc.). The plates including medium with tri-methylcellulose (10 mL/kg) and/or 20% captisol (CyDex Pharmaceuticals, Inc.) was used as a control for tri-methylcellulose (10 mL/kg) and/or 20% captisol (CyDex Pharmaceuticals, Inc.) was used as a control for tri-methylcellulose (10 mL/kg) and/or 20% captisol (CyDex Pharmaceuticals, Inc.).

In vivo experiments

ESCC cell-derived xenograft tumors and/or ESCC patient-derived xenograft tumors were utilized to assess the therapeutic effects of trifluridine/TPI and/or prexasertib in vivo. Moreover, we also examined the synergistic therapeutic effects of trifluridine/TPI and prexasertib with radiotherapy. All animal experiments conformed to the relevant regulatory standards and were approved by the Institutional Animal Care and Use Committee of Kyoto University (Kyoto, Japan, Med Kyo 18284, 18285) and the Ethics Committee of Kyoto University (Kyoto, Japan, G0770-2) or Institutional Animal Care and Use Committee of Taiho Pharmaceutical Co., Ltd. (18PB01, 18PB13).

To establish that xenograft tumors derived from ESCC cells, TE-11R cells (5 × 106 cells) were suspended in 50% Matrigel (BD Biosciences), followed by subcutaneous implantation in the left flank of 6-week-old BALB/cA-Icnu/c-Prkdcscid (Crlj: SHO-Prkdc scidHrhr) mice (Charles River Laboratories Japan, Inc.), which was then closed by suturing. Those xenografted tumors were used for the following experiments when they reached a volume of about 150–300 mm3. The mice were randomly assigned to groups (n = 5 or 6, each) and they were treated with trifluridine/TPI (200 mg/kg/day, orally) and/or prexasertib (20 mg/kg/day, subcutaneously). 0.5% hydroxypropyl methylcellulose (10 mL/kg) and/or 20% capitol (CyDex Pharmaceuticals, Inc.) was used as a control for trifluridine/TPI and prexasertib, respectively.

Radiation treatment [RT, 4 grays (Gy)] was conducted in a single fraction to tumors, as follows. Mice were positioned in a modified 50 mL conical plastic tube to allow irradiation of the tumor area while keeping the rest of the body outside the RT field using a collimator. The tumors were locally irradiated with 4 Gy of 137Cs γ-rays using a Gammacell 40 Exactor (MDS Nordion International).

Tumor growth was evaluated every 2 or 3 days until 10–14 days after final treatment. The tumor diameters were measured with a caliper, and the tumor volume (mm3) was calculated using the following formula: (length) × (width)2 × 0.5.

Histologic and IHC staining

Tissue samples were fixed in 4% paraformaldehyde phosphate buffer solution (Wako Pure Chemical Industries, Ltd.) overnight at 4°C, embedded in paraffin, and cut into 4-μm sections for standard hematoxylin and eosin (H&E) staining and IHC. Incubation and washing procedures were carried out at room temperature. Deparaffinization and antigen retrieval by incubation in protease solution (Nichirei Biosciences) were carried out for 5 minutes. The glass slides were washed in PBS (2 times, 2 minutes each) and mounted with 3% BSA in PBS for 30 minutes. The primary antibody, a rabbit monoclonal anti-γ-H2AX (20E3, #9718, Cell Signaling Technology, Inc.) at 1:100 dilution was subsequently applied for 60 minutes and followed by PBS washes (3 times, 5 minutes each). Slides were incubated with a secondary antibody solution in a HistoSimple Stain MAX PO (R) Kit (Nichirei Biosciences) for 30 minutes and followed by PBS washes (3 times, 5 minutes each). A coloring reaction was carried out with diaminobenzidine, and nuclei were counterstained with hematoxylin.

Immunostained tissues were assessed using a BIOREVO BZ-9000 Microscope (Keyence), for γH2AX staining. Positive cells were scored by counting at least 300 cells per high-power field under light microscopy.

Clonogenic survival assay

TE-11 cells were plated at 0.1–1.5 × 103 cells per well in 6-well plates and incubated for 24 hours. Trifluridine (4 μmol/L) and/or prexasertib (3 nmol/L) was added to the plates and incubated for 10 minutes, and then the plates were irradiated with 8, 6, 4, 2, or 0 Gy. The plates including medium with trifluridine and/or prexasertib were incubated for 24 days, and then the medium was changed to the normal medium, and the plates were incubated for 9 days. Cells were fixed with 20% glutaraldehyde, stained with 0.05% crystal violet, and counted. Plating efficiency (PE; number of colonies formed/number of cells seeded) was determined and used to calculate toxicity. Assays were performed in triplicate, and independent experiments were carried out three times. Radiation dose modification factors (DMF) were calculated by taking the ratios of radiation doses at the 10% survival level (90% toxic irradiation dose; DMF = 90% toxic control irradiation dose/each 90% toxic irradiation dose in the trifluridine and/or prexasertib treatment groups; ref. 29). The 90% toxic irradiation dose was calculated using XLiit software. DMF values greater than 1.0 indicate enhanced radiosensitivity (29).
Statistical analysis

Data are presented as the means ± SD of triplicate experiments, unless otherwise stated. The two-tailed Student t test was selected for data analysis of two groups. The interaction between trifluridine/TPI and prexasertib treatments for TE-11R-derived xenograft tumors as well as ESCC PDX tumors, or trifluridine/tipiracil + prexasertib treatment and RT for TE-11R-derived tumors was assessed using two-way ANOVA. When significant interactions were noted, analysis of more than two groups was conducted with Tukey Honest Significant Difference test. A P < 0.05 was considered significant. All statistical analyses were performed using SPSS 21 for Windows (SPSS Inc., IBM Corp.).

Results

Effects of trifluridine on cell proliferation and CHK1 phosphorylation (Serine 317 and Serine 345) in ESCC cells

To examine the effects of trifluridine on ESCC cells, we assessed the proliferation of ESCC cells after treatment with trifluridine. When we treated ESCC cells (TE-1, TE-10, and TE-11) with trifluridine, the ratio of the S-phase was sharply reduced (Fig. 1A). Moreover, ssDNA damage was induced by treatment with trifluridine (Fig. 1B). Next, we examined whether trifluridine influenced the phosphorylation of CHK1 because the cell cycle in the S-phase is regulated by CHK1 (11) and induced by ssDNA damage (30, 31). As shown in Fig. 1C, trifluridine increased CHK1 phosphorylation (Serine 317 and Serine 345).
phosphorylation (Serine 317 and Serine 345) dose dependently in all ESCC cells.

**Effects of CHK1 inhibitors on trifluridine-mediated cytotoxicity and DNA damage in ESCC cells**

Next, we examined the effects of the CHK1 inhibitor on trifluridine-mediated cytotoxicity and DNA damage. The inhibitory effect on CHK1 was evaluated by expression levels of CHK1 phosphorylation (Serine 296) because CHK1 is autophosphorylated at S296 following phosphorylation of S317 and S345 (32), and S296 phosphorylation is involved in subsequent DDR (33). As shown, prexasertib, a CHK1 inhibitor, successfully suppressed CHK1 phosphorylation (S296) in a dose-dependent manner (Fig. 2A). Next, we examined the cytotoxic effects of trifluridine and/or prexasertib on ESCC cells (Fig. 2B and C). As shown in Fig. 2B, prexasertib enhanced trifluridine-mediated cytotoxicity, and the IC_{50} value for trifluridine in ESCC cells was markedly reduced. In addition, combination treatment with trifluridine and prexasertib strongly induced γH2AX compared with the treatment with trifluridine or prexasertib alone (Fig. 2D).

**Effects of CHK1 knockdown on trifluridine-mediated DNA damage and/or tumor growth**

We then examined the effects of CHK1 knockdown on trifluridine-mediated DNA damage and/or tumor growth. We created CHK1-knockdown ESCC cells (TE-11R cells) using two types of short hairpin RNAs (shRNA). As shown in Fig. 3A and B, CHK1 expression levels were reduced by both shRNAs at mRNA as well as protein levels.
Induction of trifluridine-mediated DNA damage was increased by CHK1 knockdown (Fig. 3C). When xenograft tumors derived from CHK1-knockdown or control TE-11R cells were treated with trifluridine/TPI, the tumor growth inhibitory effect of trifluridine/TPI treatment in CHK1-knockdown cells was significantly stronger than in control cells (mean tumor growth inhibitory rate due to trifluridine/TPI treatment in each group: scramble control: 46% ± 0%, shCHK1 #1 group: 16% ± 12%, and shCHK1 #2 group: 12% ± 4%; Fig. 3D and E).

Antitumor effects of trifluridine/TPI and CHK1 inhibitors on TE-11R-derived xenograft and/or ESCC PDX tumors

Furthermore, we examined whether the combination therapy of trifluridine/TPI and a CHK1 inhibitor exhibits actual antitumor effects in ESCC cell (TE-11R)-derived xenograft and/or ESCC PDX tumors. As shown in Fig. 4A, the tumor growth rates (on day 28) in the groups receiving trifluridine/TPI monotherapy, prexasertib monotherapy, and/or combination therapy with trifluridine/TPI and prexasertib compared with the control group were 60.3, 67.7, and 25.9%, respectively.
Figure 4. Antitumor effects of trifluoruridine (FTD)/tipiracil and/or prexasertib on ESCC cell-derived xenograft tumors. A, Time course of tumor volumes in TE-11R-derived xenograft tumors treated with trifluoruridine/TPI and/or prexasertib. TE-11R cell-derived xenograft tumors were treated with trifluoruridine/TPI (200 mg/kg, orally) and/or prexasertib (20 mg/kg, subcutaneously) for 3 weeks (on days 1–5, 8–12, and 15–19 for trifluoruridine/TPI, on days 1–3, 8–10, and 15–17 for prexasertib). Tumor growth was evaluated every 2–3 days until day 29. n = 7 in each group. B, H&E images of xenografted tumor tissues in each treatment group on day 29, and IHC staining images for γH2AX. Scale bar, 50 μm. C, A γH2AX positively stained nuclei rate observed in six random fields (**, P < 0.01 between the indicated groups). Two-way ANOVA analysis revealed that trifluoruridine/TPI and/or prexasertib treatment significantly suppressed tumor growth of TE-11R-derived xenograft tumors, compared with control groups for TE-11R-derived xenograft tumors (P < 0.05, trifluoruridine/TPI treatment vs. control; P < 0.05, prexasertib treatment vs. control), without significant interaction between the trifluoruridine/TPI treatment and the prexasertib treatment (P = 0.9027). Here, we assessed γH2AX expression in tumors treated with trifluoruridine/TPI and/or prexasertib. Consequently, the combination treatment of trifluoruridine/TPI and prexasertib showed a significant induction of γH2AX in the xenograft tumors compared with the trifluoruridine/TPI monotherapy, prexasertib monotherapy, or vehicle control (Fig. 4B and C).

In ESCC PDX tumors, the tumor growth rates (on day 22) in the monotherapy group with trifluoruridine/TPI or prexasertib and/or combination therapy with trifluoruridine/TPI and prexasertib group compared with the control group were 41 ± 10, 37 ± 12, and 10 ± 5, respectively. Two-way ANOVA analysis revealed that there was a significant positive interaction between trifluoruridine/TPI treatment and/or prexasertib treatment for ESCC PDX tumors (P = 0.000156), so a multiple comparison of all groups was conducted. As a result, we found that the tumor growth rate in each treatment group was significantly suppressed compared with the control group (P < 0.01, trifluoruridine/tipiracil alone vs. control; P < 0.01, prexasertib alone vs. control; and P < 0.01, trifluoruridine/TPI and prexasertib vs. control). Moreover, tumor growth in the group receiving combination therapy with trifluoruridine/TPI and prexasertib was also significantly suppressed compared with the group receiving monotherapy with trifluoruridine/TPI or prexasertib (P < 0.01, trifluoruridine/TPI and prexasertib vs. trifluoruridine/TPI alone or prexasertib alone; Fig. 5A).

No mice died and apparent ill effects were not observed in these experiments. No significant weight loss was observed in groups on day 29 of the experiment using TE-11R-derived xenograft tumors (Supplementary Fig. S1) or day 22 of the experiment using ESCC PDX tumors (Fig. 5B).

Figure 5. Effects of treatment with trifluoruridine (FTD)/tipiracil and/or prexasertib on ESCC PDX tumors. A, The time course of tumor volumes in ESCC PDX tumors treated with trifluoruridine/TPI and/or prexasertib is shown. ESCC PDX tumors were treated with trifluoruridine/TPI (200 mg/kg, orally) and/or prexasertib (20 mg/kg, subcutaneously) for 2 weeks (on days 1–5 and 8–10 for prexasertib). Tumor growth was evaluated every 2–3 days until day 22. **, P < 0.01 versus vehicle control; #, P < 0.01, trifluoruridine/TPI + prexasertib versus trifluoruridine/TPI alone; $, P < 0.01, trifluoruridine/TPI + prexasertib versus prexasertib alone; n = 7 in each group. B, The time course of body weight changes in mice treated with trifluoruridine/TPI and/or prexasertib is shown. n = 7 in each group. Animal weights on day 22 were not significantly different between the treatment groups.
Radiosensitizing effects of trifluridine and CHK1 inhibitor in in vitro and in vivo experimental models

To assess the radiosensitizing effect of trifluridine and/or prexasertib in vitro, we conducted a clonogenic assay. The average PE in each treatment group with or without irradiation (0, 2, 4, 6, and 8 Gy) is shown in Fig. 6A. To determine DMF, we calculated the irradiation doses required to suppress the colony formation rate to 90% (90% toxic irradiation dose). The irradiation doses required to suppress the colony formation rate to 90% were 9.67 Gy in the control group, 6.74 Gy in the trifluridine group, 9.04 Gy in the prexasertib group, and 3.92 Gy in the trifluridine + prexasertib group. Accordingly, DMFs in the monotherapy group with trifluridine or prexasertib and/or the combination therapy of trifluridine and prexasertib were 1.4, 1.1, and 2.5, respectively. Because DMF values greater than 1.0 indicate an enhancement of radiosensitivity, a radiosensitizing effect of trifluridine/TPI or prexasertib alone or in combination was observed in ESCC cells (Fig. 6A).

We also examined the antitumor effects of the combination of trifluridine/TPI and prexasertib in the presence or absence of RT on TE-11R-derived xenograft tumors. As shown in Fig. 6B, the tumor growth rates (on day 22) in the groups receiving chemotherapy alone (trifluridine/TPI and prexasertib), RT alone, and/or combination therapy of trifluridine/TPI, prexasertib, and RT compared with the control group were 51% ± 7.5%, 47% ± 10%, and 19% ± 11%, respectively. Two-way ANOVA analysis revealed a significant positive interaction between RT and/or trifluridine/TPI + prexasertib treatment (P = 0.0182), so that we conducted a multiple comparison of all groups. The tumor growth rate in each treatment group with trifluridine versus RT was significantly suppressed compared with the control group [P < 0.01, chemotherapy (trifluridine/TPI and prexasertib) alone vs. control; P < 0.01, RT alone vs. control; and P < 0.01, trifluridine/TPI, prexasertib, and RT vs. control]. Moreover, tumor growth in the group receiving combination chemoradiotherapy of trifluridine/TPI, prexasertib, and RT was also significantly suppressed compared with that in the chemotherapy alone group [trifluridine/TPI and prexasertib, P < 0.01, trifluridine/TPI, prexasertib, and RT vs. chemotherapy (trifluridine/TPI and prexasertib) alone] or RT alone (P < 0.01, trifluridine/TPI, prexasertib, and RT vs. RT alone; Fig. 6B). No mice died and apparent ill effects were not observed in this experiment, and no significant body weight loss was observed on day 22 in groups (Fig. 6C).

Discussion

In this study, we showed that trifluridine markedly increased CHK1 phosphorylation (S317 and S345) in ESCC cells. Because CHK1 is phosphorylated at S317 and S345 by ATR (34, 35), which is activated by DNA damage (i.e., ssDNA break; refs. 36, 37), CHK1 phosphorylation is suggested to be caused by trifluridine-mediated DNA damage. Consequently, CHK1 phosphorylation leads to the activation of CHK1 signaling through autophosphorylation of CHK1 at Serine 296 (32), as well as subsequent induction of DDR (33, 38). Our data suggest that trifluridine treatment reduces the relative number of cells in the S-phase via DDR induction through CHK1 signal activation.

This is the first reported preclinical study that CHK1 inhibition enhances trifluridine/TPI-mediated antitumor effects in ESCC cells. Because CHK1 is considered to be activated to repair trifluridine-induced DNA damage, we examined the effects of CHK1 inhibition on trifluridine-mediated DNA damage and/or its antitumor effects. As a result, the combination of trifluridine and CHK1 inhibition via CHK1 knockdown or a CHK1 inhibitor, prexasertib, resulted in potent cytotoxic effects as well as antitumor effects with marked DNA damage. Our data suggest that CHK1 inhibition suppresses the repair of trifluridine-induced DNA damage and that the accumulation of DNA damage has antitumor effects. Therefore, we believe that increasing trifluridine-mediated cell death by suppressing physiologic DDR activation via CHK1 inhibition is reasonable.

In this study, trifluridine was considered to work as a trigger to induce DNA damage. Consequently, trifluridine-mediated DNA
damage is not successfully repaired when TP53-mutated tumor cells are treated with triluridine and CHK1 inhibitors. Thus, synthetic lethality is caused by the impairment of DDR due to the inhibition of both ATM–CHK1 and the ATM–CHK2–p53 pathway (17). In ESCC, the TP53 mutation frequency is as high as approximately 79.8%–93% (18–22, 39), whereas other DDR-related genes such as ATR, ATM, CHEK1, and CHEK2 are not frequently mutated (refs. 18, 39–41; Supplementary Fig. S2A and S2B). Therefore, inhibition of these DDR-related proteins is considered to be reasonable in most cases of ESCC with TP53 mutations.

In this study, we examined the TP53 mutation status in ESCC cells (TE-1, TE-10, and TE-11) by TP53 target sequence, and confirmed TP53 mutation presence in all cells as follows: TE-1: V272M, TE-10: C242Y, and TE-11: R110L. Because C242Y and R110L show a loss of p53 function (42, 43), TE-11, and TE-11R cells are p53-defective cells. TP53 mutations (V272M) in TE-1 cells were described as variants of uncertain significance in the NCBI ClinVar variation report (44); however, a recent report on TP53

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References

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Shinya Ohashi, Osamu Kikuchi, Yukie Nakai, et al.


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