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TREATMENT COMBINING X IRRADIATION AND A RIBONUCLEOSIDE ANTICANCER DRUG, TAS106, EFFECTIVELY SUPPRESSES THE GROWTH OF TUMOR CELLS TRANSPLANTED IN MICE

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Running Title- Treatment of transplanted tumors with X irradiation and anticancer drug TAS106

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ABSTRACT

Purpose: This research was carried out to examine the in vivo antitumor efficacy of X irradiation combined with administration of a ribonucleoside anticancer drug, 1-(3-C-ethynyl-β-D-ribo-pentofuranosyl)cytosine (TAS106, ECyd), to tumor cell-transplanted mice.

Methods and Materials: Colon26 murine rectum adenocarcinoma cells and MKN45 human gastric adenocarcinoma cells were inoculated into the footpad in BALB/c mice and severe combined immunodeficient (SCID) mice, respectively. They were treated with a relatively low dose of X irradiation (2 Gy) and low amounts of TAS106 (0.1 mg/kg and 0.5 mg/kg). The tumor growth was monitored by measuring the tumor volume from day 5 to day 16 for Colon26 and from day 7 to day 20 for MKN45. Histological analyses for proliferative and apoptotic cells in the tumors were performed using Ki-67 immunohistochemical- and TUNEL-staining. The expression of survivin, a key molecule related to tumor survival, was assessed using quantitative PCR and immunohistochemical analysis.

Results: When X irradiation and TAS106 treatment were combined, significant inhibition of tumor growth was observed in both types of tumors compared to mice treated with X irradiation or TAS106 alone. Marked inhibition of tumor growth was observed in half of the mice that received the combined treatment three times at two-day intervals. Parallel to these phenomena, the suppression of survivin expression and appearance of Ki-67-negative and apoptotic cells were observed.

Conclusions: X irradiation and TAS106 effectively suppress tumor growth in mice. The inhibition of survivin expression by TAS106 is thought to mainly contribute to the suppression of the tumor growth.

Key words: Transplanted tumor, Anticancer drug, X irradiation, Survivin
INTRODUCTION

Anticancer drugs targeting DNA synthesis in tumors have been widely used in clinical studies. In particular, nucleoside analogs such as 1-β-D-arabinofuranosyleytosine (araC), 2-chloro-2’-deoxyadenosine (CldA), 2’,2’-difluorocytidine (gemcitabine) and 5-fluorouracil (5-FU), have been reported to be effective for cancer therapy (1-3). However, some drugs have adverse effects such as nausea, vomiting and diarrhea, inducing severe toxicities (2). To exclude these effects, a method combining low-dose drug administration and exposure to a low dose of radiation has been a potent strategy for cancer therapy. In fact, gemcitabine and 5-FU have been shown to have radiosensitive effects on tumor cell lines in vitro and in vivo (4-6).

Genetic changes in cells that impair their ability to control cell cycling and/or to regulate apoptosis, which are generally part of the tumorigenetic process, can affect the sensitivity of tumor cells to radiation therapy and chemotherapy (7). Resistance of cells to radiation therapy and chemotherapy has been found in some cancer cell lines with a low propensity for apoptosis (8). Differences in the length and magnitude of radiation-induced G2/M delay may also be critical determinants of cellular radiosensitivity (9). In previous studies, we reported that apoptosis was induced through the abrogation of arrest at the G2/M phase in X-irradiated human (MKN28 [TP53 mutated] and MKN45 [TP53 wild]) and murine (colon26) tumor cells, as well as Chinese hamster V79 cells, when X irradiation was combined with a ribonucleoside anticancer drug, 1-(3-C-ethynyl-β-D-ribo-pentofuranosyl)cytosine (TAS106, ECyd). This was coupled to the enhancement of the loss of clonogenic survival (10, 11). In those cases, down-regulation of X-ray-induced expression of survivin, a key molecule related to tumor survival, and the abrogation of the G2/M checkpoint due to the attenuation of CDC2-cyclin B1 kinase activity...
by TAS106 were responsible for this combined effect. Therefore, it is of interest to investigate whether changing the sensitivity of X-irradiated tumor cells to apoptosis by TAS106 administration significantly contributes to antitumor efficacy, though it has been shown in several studies with cells in vitro and tissues in vivo that changing the sensitivity to apoptosis produces little or no change in the overall sensitivity of the cells or tissues to radiation or anticancer drugs (12).

The ribonucleoside analogue TAS106, shown in Fig. 1, was first synthesized in 1995. It has recently been developed as a novel anticancer drug (13). This compound is effectively phosphorylated by uridine/cytidine kinase 2 (UCK2), which has higher activity in tumor cells than in normal cells (14, 15). The resultant metabolite, ECTP, a 5’-triphosphate form of TAS106, inhibits RNA synthesis by competing with cytidine 5’-triphosphate and results in a compound cytotoxic to tumor cells (3, 13-18). Since survivin has been reported to attenuate the activities of caspases 3, 7, and/or 9 (19-21) and to regulate proliferation of tumor cells in association with the mitotic apparatus (19), its down-regulation by TAS106 is expected to induce significant enhancement of radiation-induced cell death not only in vitro but also in vivo.

The purpose of this study was to investigate the in vivo antitumor efficacy of radiation treatment combined with TAS106 administration to tumor-cell-transplanted mice with special emphasis on the down-regulation of survivin expression.
METHODS AND MATERIALS

Animals and tumor models

Female BALB/c mice aged 8 to 10 weeks and C.B-17/Icr SCID Jcl mice aged 6 weeks were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and Japan CLEA (Tokyo, Japan), respectively.

Murine rectum adenocarcinoma Colon26 and human gastric adenocarcinoma MKN45 cells were grown in RPMI 1640 medium (Gibco-BRL/Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Filtron, Brooklyn, Australia). \(5 \times 10^6\) cells were subcutaneously injected into the footpad of the right hind leg. Tumor growth was monitored every day by measuring the footpad diameter with calipers. The measurements of tumor volumes were started from day 5 (Colon26, approximately 40~60 mm\(^3\)) or day 7 (MKN45, approximately 60~80 mm\(^3\)) after the inoculation of cells. The volume (V) was calculated according to the following formula: \(V = length \times width^2 \times \pi/6\) (22).

Animal Welfare

This study was conducted according to the established guidelines of “The Guidelines Concerning Animal Experiments, Hokkaido University” and the “Law for The Care and Welfare of Animals in Japan.” and approved by the Animal Experiment Committee of the Graduate School of Veterinary Medicine, Hokkaido University. The observations of tumor size were stopped when tumor volumes of the untreated group became excessive. This endpoint was in accord with “The Guidelines for Endpoints in Animal Study Proposals, The Office of Animal Care and Use, The National Institutes of Health.”

Drug treatment and X irradiation
1-(3-C-ethynyl-β-D-ribo-pentofuranosyl)cytosine (TAS106) was synthesized as described elsewhere (13). Five days after the initial measurement, animals were randomized into four groups of 5~7 animals each: (1) no treatment; (2) X irradiation with 2 Gy alone; (3) TAS106 administration alone; (4) combined treatment with X irradiation and TAS106 administration. To determine the optimal time sequencing between X irradiation and TAS106 administration, five combination experiments were performed. Three groups were administered TAS106 3, 6 and 9 h before X irradiation. Two groups were administered TAS106 1 and 6 h after X irradiation. TAS106 was intraperitoneally injected into mice at the amounts of 0.1 mg/kg and 0.5 mg/kg. Transplanted tumors were irradiated using a Shimadzu PANTAK HF-350 X-ray generator at a dose rate of 0.8 Gy/min.

In the case of repetitive treatments, Colon26 tumor-bearing mice were treated with the combination of 0.1 mg/kg TAS106 and 2 Gy of X rays on days 10, 12 and 14 after inoculation.

Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining

Tumor tissues were excised from mice at 2~3 mm under the surface and fixed in 4% buffered formaldehyde. Tumors were embedded in paraffin and sectioned 5-μm thick.

After being deparaffinized and rehydrated, sections were incubated with 20 μg/ml proteinase K (Sigma Chemical, St. Louis, MO) for 15 min. Endogenous peroxidase was quenched by 1% hydrogen peroxide for 5 min. The sections were incubated with 0.15 U/μl of TdT (Invitrogen, Carlsbad, CA) and 10 μM Biotin-dUTP (Roche Diagnostics, Tokyo, Japan) for 1 h at 37°C. The sections were covered with a 3.3 μg/ml active conjugate of horseradish peroxidase and streptavidin at the molar ratio of 1.5:1.0 (Horseradish Peroxidase Streptavidin, Vector Laboratories, Burlingame, CA) for 30 min. Nuclei of apoptotic cells were detected
using diaminobenzidine (DAB) (Dojindo Laboratories, Kumamoto, Japan).

*Electron microscopic analysis*

Tumors from the control and treated groups were perfused with 4% paraformaldehyde and immersed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at 4°C for 4 h. After being postfixied with 2% osmium tetraoxide at 4°C for 2 h, samples were dehydrated and embedded in epoxy resin. After ultrathin sections (80 nm) were stained with uranyl acetate and lead citrate, they were subjected to electron microscopic observation.

*Immunohistochemical analysis of Ki-67, survivin, phospho-ERK1/2 and phospho-Akt*

Antigen retrieval was performed with a 0.01 M citrate buffer for 10 min at 121°C. After quenching endogenous peroxidase activity and blocking nonspecific binding sites, the sections were incubated with primary antibodies overnight at 4°C. The following dilutions were found to produce optical staining: anti-mouse Ki-67 1:100 (Dako Cytomation, Kyoto, Japan), anti-human Ki-67 1:100 (Lab Vision Corp., Fremont, CA), survivin 1:400 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-ERK1/2 1:100 and phospho-Akt 1:200 (Cell Signaling Technology, Inc., Beverly, MA). The sections were reacted with a biotinylated secondary antibody at 1:600 for 1 h at 37°C and then incubated with avidin-biotin complex (VECTASTAIN Elite ABC Kit; Vector Laboratories, Burlingame, CA). Immunoreactivity was visualized via incubation with DAB.

Each stained slide was lightly counterstained with hematoxylin. The sections were analyzed under an Olympus BX41 microscope.

*Scoring of stained sections*
All immunohistochemical analyses were assessed by two independent observers and quantified by calculating the ratio of positively stained cells vs. all tumor cells in five random fields at ×400 magnification.

Quantitative real-time RT-PCR analysis of survivin

Total RNA was isolated from MKN45 tumors at 3, 12 or 24 h after X irradiation and/or TAS106 administration. Real-time PCR were performed with a SuperScript™ III Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The following primers were used in real-time PCR: for SURVIVIN: sense, 5’-AGAACTGGCCCTTCTTG-3’, antisense 5’-TTCCTCTATGGGGTCGTC-3’; and for GAPDH: sense, 5’-GAAGGTGAAGGTCGGAGTC-3’, antisense, 5’-GAAGATGGTGATGGGATTTC- 3’.

Real-time PCR reactions were performed in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Expression of SURVIVIN measured in triplicate was normalized to the GAPDH expression level and presented as a ratio relative to the untreated tumor at each time.

Statistical analysis

All results were expressed as the mean±SE. The variance ratio was estimated by the F-test and differences in means of groups were determined by Student’s t-test or Welch’s t-test. The minimum level of significance was set at P < 0.05.
RESULTS

In initial experiments, the effects of X irradiation alone and TAS106 alone on tumor growth were examined in transplanted tumors. 2 Gy of X irradiation exerted no statistically significant effect on tumor growth either in Colon26 or MKN45 tumors (Fig. 2). Thus, the dose of 2 Gy of X rays was chosen to evaluate the antitumor effects of the combined treatment with TAS106. No significant retardation of tumor growth was observed in tumor-bearing mice treated with 0.1 and 0.5 mg/kg TAS106 alone for Colon26 (Figs. 2A and B) and 0.1 mg/kg TAS106 alone for MKN45 (Fig. 2C). It exerted some effect on MKN45 tumor growth at 0.5 mg/kg, but this effect was not significant except for those on days 16, 18 and 19 (Fig. 2D). No statistically significant loss of body weight, behavioral disorder or atrophy of parenchymatous organs was found in any mice treated with 0.5 mg/kg TAS106, although 10 mg/kg TAS106 caused significant loss of body weight and decrease in locomotor activity (data not shown). From these observations, the amounts of 0.1 mg/kg and 0.5 mg/kg TAS106 were chosen for the therapeutic doses.

Though 2 Gy of X irradiation or 0.1 mg/kg TAS106 alone induced no statistically significant retardation of tumor growth, X irradiation 3 h after TAS106 treatment induced a significant retardation in the tumor growth in the Colon26 tumor model. This retardation was observed from day 12 to day 16 (P < 0.05 on day 12 and P < 0.01 on days 13-16). The inhibition rate was 50.8% on day 15 (Fig. 2A). Additionally, the combination of 2 Gy of X irradiation and 0.5 mg/kg TAS106 induced similar retardation in the tumor growth (Fig. 2B). To examine the optimal time sequencing for combining X rays and TAS106, Colon26-tumor-bearing mice were administered 0.5 mg/kg TAS106 at different times before or after X irradiation. The TAS106 administration 6 and 9 h before X irradiation had significant effects on tumor growth (6 h, P < 0.05 on days 13-14 and P < 0.01 on days 15-16;
9 h, \( P < 0.05 \) on day 14), but the inhibition rates were smaller than that for TAS106 administration 3 h before X irradiation. Furthermore, TAS106 administration 1 and 6 h after X irradiation did not enhance the tumor radioresponse (Fig. 2B). These results suggested that administration before X irradiation was a necessary condition for the enhancement of the tumor radioresponse and that TAS106 administration 3 h before X irradiation was optimal.

In the MKN45 tumor model, 0.1 mg/kg TAS106 could not enhance the radiation-induced inhibition of tumor growth (\( P > 0.05 \), Fig. 2C). However, 0.5 mg/kg TAS106 combined with X rays significantly inhibited the tumor growth (\( P < 0.05 \) on days 13-14 and \( P < 0.01 \) on days 15-20). The inhibition rate was 48.5\% on day 20 (Fig. 2D). From these observations, the amounts of 0.1 mg/kg and 0.5 mg/kg TAS106 were estimated to be minimally effective doses for Colon26 and MKN45, respectively.

Though a single treatment with X rays and TAS106 had a significant retardative effect on tumor growth, reduction of tumor growth was not observed. To investigate whether repetitive treatments with X rays and TAS106 resulted in greater antitumor efficacy, mice received fractionated treatments at days 10, 12, and 14. Though treatment with X rays alone or TAS106 alone showed a tendency to slightly retard the tumor growth, no reduction of tumor growth was observed (Figs. 3B and C). In these cases, three of the five mice in the control group and one of the five mice in the TAS106-treated group died by day 25. As severe loss of body weight (>10\%) without macroscopic and pathological lesions was observed in these mice, cachexia seemed to be responsible for the deaths. On the other hand, mice treated with X-rays/TAS106 three times showed obvious inhibition of tumor growth and, interestingly, tumor reduction was observed in three of the six unlike with a single combined treatment, and all the mice survived the experimental period (Fig. 3D).

Morphological changes of tumors exposed to X rays and/or TAS106 were examined
by TUNEL staining as well as electron microscopic observation to determine the relationship between the induction of apoptosis and the retardation of tumor growth. Figures 4A(b) and (c) show that only a few apoptotic cells appeared after either X irradiation or TAS106 treatment. In contrast, an obvious increase of apoptotic cells was detected with the combination of X irradiation and TAS106 (Fig. 4A[d]). Nuclear structures of Colon26 tumor cells treated with X rays and TAS106 were found to be smaller and accompanied by chromatin condensation, chromatin crescents and nuclear fragmentation, as shown in Fig. 4B. The percentage of TUNEL-positive cells vs. total cells demonstrated a significant increase in apoptotic cells (5.7±1.0% for Colon26, 6.6±0.3% for MKN45) compared to the control (0.8±0.2% for Colon26, 0.6±0.3% for MKN45) (Fig. 4C) 1 day after the combined treatment. These results indicated that TAS106 treatment made the X-irradiated tumor cells sensitive to apoptosis. However, the rate of apoptotic cells seemed to be too low to explain the overall suppression of tumor growth. We examined whether 2 Gy of X irradiation and/or 0.1 mg/kg TAS106 damaged normal tissues as well as tumor tissues, but few TUNEL-positive cells were observed in the epidermis and dermis of all groups (Fig. 4D).

Figure 5 shows the effects of X irradiation and/or TAS106 on the cell proliferation in tumors that were immunohistochemically examined by using the Ki-67 antigen, a nuclear antigen expressed in cells undergoing active cell division. In Colon26 tumors, the percentages of Ki–67-positive cells vs. total cells were scored as 41.3±1.4% (control), 31.4±2.7% (X rays alone), and 34.1±2.3% (TAS106 alone), indicating that a single treatment with TAS106 or X irradiation moderately suppressed Ki-67-positive cells (P < 0.05). However, when both treatments were combined, the percentage of Ki–67-positive cells significantly decreased to 11.4±3.9% compared to the control tumor (P < 0.01) or X-irradiated tumor (P < 0.01) (Fig. 5B[a]). A similar result was obtained in MKN45 tumors, and the percentages of
Ki–67-positive cells were 69.7±2.4% (control), 53.6±1.8% (X rays alone), 54.6±4.2% (TAS106 alone), and 28.0±1.5% (X-rays/TAS106) (Fig. 5B[b]). These results indicated the possibility that the decrease in the proliferative cells in tumors, especially in MKN45 tumors, was quantitatively responsible for the suppression of the tumor growth. We examined whether TAS106 enhanced the radioresponses of normal tissues due to the inhibition of cell proliferation as in tumor tissues, but TAS106 had no effect on the percentage of Ki-67-positive cells in the basal layer of the epidermis in X-irradiated mice (Fig. 5C).

To obtain further information about the molecular basis of the inhibition of cell proliferation by TAS106, we examined its effect on the expression of \textit{SURVIVIN} mRNA by real-time quantitative PCR in MKN45 xenografts (Fig. 6A). While 2 Gy of X irradiation had little effect on the expression of \textit{SURVIVIN} mRNA, 0.5 mg/kg TAS106 suppressed \textit{SURVIVIN} mRNA levels to 0.58 (TAS106 alone) and 0.51 (X-rays/TAS106) at 24 h. Suppressive effects of TAS106 on the expression of survivin at the protein level were also observed at 24h in MKN45 xenografts (Fig. 6B). X irradiation with 2 Gy alone had little effect on the survivin expression (20.3±1.8%) compared to the control (19.8±2.2%), but it was suppressed to 9.4±0.8% (TAS106 alone) and to 5.6±0.4% (X-rays/TAS106). These results were similar to those shown in Fig. 5.

Since cell survival and proliferation are closely associated with the activation of kinases like ERK1/2 and Akt, the effects of TAS106 on their activation were examined (Fig. 7). While X irradiation with 2 Gy had little effect on the phosphorylation of ERK1/2 compared to the control (19.7 ± 2.0%), TAS106 inhibited the phosphorylation of ERK1/2 to 8.2±1.7% (TAS106 alone) and 11.2±2.5% (X-rays/TAS106), but there was no significant difference between them. X irradiation with 2 Gy caused no significant inhibition of the phosphorylation of Akt compared to the control, but treatment with 0.5 mg/kg TAS106 largely
inhibited the activation of Akt (3.2±0.4% for TAS106 alone and 7.9±1.3% for X-rays/TAS106) (Fig. 7B). In this case, a significant difference between them was observed, but the reason why the suppressive effect of TAS106 treatment was greater than that of the combined treatment was not fully elucidated. These results differed from those shown in Fig. 5.
DISCUSSION

The success of radiation therapy depends on its ability to inhibit cell proliferation and induce cell death (23). However, many types of tumor cells are resistant to radiation therapy (24). These tumor cells require high doses for sufficient therapeutic efficacy. Therefore, to increase the cure rate of malignant neoplasms, it is essential to manipulate the radiosensitivity of tumors.

The role of apoptosis in radiation-induced cell killing and its significance as a predictor of tumor cell radiosensitivity have been investigated in several studies (25, 26). However, it has also been shown in several studies with cells in vitro and tissues in vivo that changing the sensitivity to apoptosis produces little or no change in the overall sensitivity of the cells or tissues to radiation or anticancer drugs (12). In the present study, TAS106 treatment increased the sensitivity of cells in tumors to apoptosis, resulting in the enhancement of the suppressive effect of X rays on tumor growth (Fig. 4). This result seemed to prove that the sensitivity to apoptosis is a critical determinant of tumor radiosensitivity, but the rate of the apoptosis induction was somewhat low to explain the suppression of tumor growth.

Immunohistochemical analysis for the cell proliferation marker Ki-67 showed that Ki-67-negative cells were largely induced in the tumors treated with X rays and TAS106 (Fig. 5). In this case, the percents of Ki-67-positive cells were about 40% and 70% in control Colon26 and MKN45 tumors, respectively, whereas about 30% of Ki-67-positive cells in Colon26 tumors and about 40% of Ki-67-positive cells in MKN45 tumors were changed to Ki-67-negative ones by the combined treatment. Therefore, the effect against the cell proliferation induced by the combined treatment was quantitatively more plausible than that of apoptotic induction alone as a cause of the suppression of tumor growth.
The mitogen-activated protein kinase signaling pathway is a key regulator of cell proliferation and survival (27). Jorgensen et al. demonstrated that the activation of ERK1/2 in malignant melanoma induced the expression of Ki-67, which was detected in the cells existing in all phases of the cell cycle except the $G_0$ phase (28). The phosphatidylinositol-3 kinase/Akt pathway was shown to be involved in the regulation of various cellular processes, including not only cell proliferation and growth but also apoptosis (29). Therefore, we examined whether TAS106 suppressed these molecules related to cell proliferation. Phosphorylation of both ERK1/2 and Akt was inhibited by TAS106 in the MKN45 xenograft as shown in Fig. 7, but the results were not in accord with that of Ki-67-immunohistochemical analysis of the MKN45 xenograft shown in Fig. 5. Therefore, the activation of ERK1/2 and Akt might not have been critical for the suppression of the tumor growth.

The radiation-induced cell killing effect is closely associated with the induction of DNA double-strand breaks (30). TAS106 might contribute to the effective suppression of X-irradiated tumors by interfering with the repair of DNA double-strand breaks. The measurement of the extent of $\gamma$-H2AX phosphorylation is used as evidence for the presence of DNA double-strand breaks (31). However, our preliminary experiments using anti-phosphorylated $\gamma$-H2AX showed that there were no differences in the number of foci per cell after X irradiation, TAS106 treatment and X irradiation combined with TAS106 treatment in cultured MKN45 cells (data not shown). Thus, the effective suppression of tumor growth by the combined treatment cannot be attributed to the inhibition of repair of DNA double-strand breaks at present.

X irradiation sometimes induces up-regulation of mRNA levels of various anti-apoptotic proteins such as survivin, a member of the IAP family, in cancer cells (32). Moreover, it has been reported that DNA damage stimulates ATM/ATR followed by the
activation of cell-cycle checkpoint kinases 1 and 2 (CHK1 and CHK2) and subsequently by the phosphorylation of CDC25C, and induction of the G2/M checkpoint (33). Slowly dying cells have a variety of cell cycle arrest profiles and, therefore, differences in the length and magnitude of radiation-induced G2/M delay may be critical determinants of cellular radiosensitivity (9, 34). In fact, an inhibitor of CHK1, 7-hydroxystaurosporine (UCN-01), was reported to enhance the antitumor activity of ionizing radiation by abrogating the G2/M checkpoint (35). Manipulation of these factors for cell survival and the G2/M checkpoint seems to be important for tumor radiotherapy.

Since TAS106 is an inhibitor of RNA synthesis (17), we hypothesized that the expression of various proteins causing the resistance of tumor cells to genotoxic agents would be suppressed by TAS106, followed by an increase in the X-ray-killing effect. Our recent experiments demonstrated that TAS106 enhanced not only the induction of apoptosis but also loss of clonogenic ability in X-irradiated tumor cells in vitro, regardless of the difference in TP53 status, at low concentrations causing no cell death (10, 11). In these studies, TAS106 suppressed the radiation-induced expression of G2/M checkpoint-related molecules CDC2, phospho-CDC2, cyclin B1, WEE1 and survivin, resulting in the shortening of G2/M delay through abrogation of the G2/M checkpoint. The present study suggested the possibility that the change of Ki-67-positive (proliferative) cells to Ki-67-negative (inactivated) cells in X-irradiated tumors treated with TAS106 was a cause of effective suppression of tumor growth. This may be explained by TAS106 suppressing the expression of survivin, resulting in reduced resistance to X irradiation, and shortening of G2/M delay via the expression of G2/M-related molecules, which led to the effective suppression of growth of X-irradiated tumors.

We showed that 0.1 mg/kg TAS106 gave no effects on normal tissues like epidermis
and dermis in X-irradiated mice (Figs 4D and 5C). To obtain more clinical information about drug toxicity, we measured the activities of marker enzymes and metabolites such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine phosphokinase (CPK), amylase (AMY), blood urea nitrogen (BUN) and creatinine (CREA) in the sera of mice. At 2 day after 10 mg/kg TAS106 administration, significant increases were observed in AST, LDH and CPK compared to control, although 0.1 mg/kg and 0.5 mg/kg TAS106 did not influence these parameters (data not shown). This result indicated that the use of TAS106 at high dose such as 10 mg/kg might cause damage of normal tissues, suggesting that there are some risks for the normal tissue in clinical situation if high dose of TAS106 is used.

In conclusion, we demonstrated that an inhibitor of RNA synthesis, TAS106, could potentiate the antitumor activity of X irradiation with 2 Gy for solid tumors. In particular, it is noteworthy that repetitive treatments with the combination of X irradiation and TAS106 induced marked inhibition of tumor growth in half of the mice (Fig. 3). Although the precise mechanisms for the suppression of the tumor growth in vivo need to be further investigated, a low dose of TAS106 in combination with radiation therapy may be a promising strategy for clinical trials.
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FIGURE LEGENDS

Fig. 1. The chemical structure of 1-(3-C-ethynyl-β-D-ribo-pentofuranosyl)cytosine (ECyd, TAS106).

Fig. 2. Growth curves of transplanted tumors ([A] and [B]: Colon26-transplanted tumor, [C] and [D]: MKN45-transplanted xenograft). Tumor volumes were monitored every day as described in Materials and Methods. Arrows indicate the day that mice were treated. Error bars represent the SE of the mean. (A) Each group of animals was treated with 2 Gy of X rays (□: n=5~6), 0.1 mg/kg TAS106 (○: n=6~7), 2 Gy of X-rays/0.1 mg/kg TAS106 (▲: n=6~7), and control (●: n=6~7). *: P < 0.05, **: P < 0.01 TAS106 → X-rays (3 h) vs. control. (B) Each group of animals (n=6 per group) was treated with X irradiation with 2 Gy (□), 0.5 mg/kg TAS106 (○), 0.5 mg/kg TAS106 administration for 3, 6 or 9 h followed by X irradiation (▲: 3 h, ■: 6 h, ♦: 9 h), 0.5 mg/kg TAS106 administration for 1 or 6 h after X irradiation (△ dashed line: 1 h, ◊ dashed line: 6h) and control (●). *: P < 0.05, **: P < 0.01 TAS106 → X-rays (3 h) vs. control. †: P < 0.05, ††: P < 0.01 TAS106 → X-rays (6 h) vs. control. ‡: P < 0.05 TAS106 → X-rays (9 h) vs. control. (C) Each group of animals (n=6 per group) treated with either 2 Gy of X irradiation (□), 0.1 mg/kg TAS106 (○), 2 Gy of X-rays/0.1 mg/kg TAS106 (▲) and control (●). (D) Each group of animals (n=6 per group) treated with either 2 Gy of X rays (□), 0.5 mg/kg TAS106 (○), 2 Gy of X-rays/0.5 mg/kg TAS106 (▲) and control (●). *: P < 0.05, **: P < 0.01 TAS106 → X-rays (3 h) vs. control. †: P < 0.05, ††: P < 0.01 TAS106-treated group vs. control.

Fig. 3. Effects of repetitive treatments with X irradiation and/or TAS106 three times on tumor growth of Colon26 tumors. Tumor volume of each animal was plotted against the day. (A)
Control group, (B) Group X-irradiated with 2 Gy three times, (C) Group treated with 0.1 mg/kg TAS106 three times, and (D) Group treated with 0.1 mg/kg TAS106 3 h before X irradiation three times. Arrows indicate days when mice were treated.

**Fig. 4.** Effects of combined treatments on the induction of apoptosis in transplanted tumors. Tumors were excised 1 day after treatment. Panel A: TUNEL assay in sections of Colon26 tumor tissues. B: Electron-microscopic structures of transplanted Colon26 tumor cells before and after treatment. (a) Normal cell in a transplanted tumor. (b) Apoptotic cell in a transplanted tumor after the combined treatment. Panel C: Percent induction of apoptosis determined by TUNEL assay in Colon26 and MKN45 tumors. The apoptotic index calculated as described in Materials and Methods is presented as mean±SE. **: P < 0.01 vs. control. Panel D: TUNEL assay in skin tissue located in the Colon26 tumor margin. (a) control, (b) tumor exposed to 2 Gy of X rays, (c) tumor exposed to 2 Gy of X-rays/0.1 mg/kg TAS106.

**Fig. 5.** Effects of combined treatments on the proliferative activity of tumors. Proliferative cells were detected by Ki-67 staining in tumor tissue sections. Tumors were excised 1 day after treatment. Panel A: Typical microscopic fields in Colon26 tumors. Panel B: Quantitative data obtained by immunohistochemical analysis in Colon26 and MKN45 tumors. Ki-67 index calculated as described in Materials and Methods is presented as mean±SE. *: P < 0.05 vs. control, **: P < 0.01 vs. control, ††: P < 0.01 X-irradiated group vs. combination group. Panel C: Representative microscopic fields in skin tissue located in the Colon26 tumor margin. (a) control, (b) tumor exposed to 2 Gy of X rays, (c) tumor exposed to 2 Gy of X-rays/0.1 mg/kg TAS106.
**Fig. 6.** Panel A: Effects of X irradiation and TAS106 on the mRNA expression of *SURVIVIN* in MKN45 xenografts. Total RNA was isolated from MKN45 tumors at 3, 12 or 24 h after X irradiation and/or TAS106 administration. The *SURVIVIN* mRNA level was detected by real-time quantitative PCR. The expression level was normalized to an endogenous reference (GAPDH) and expressed as an $n$-fold mRNA level relative to the control at each time. Data are expressed as mean±SE. Panel B: Expression of survivin molecules in MKN45 xenografts. Tumors were excised 1 day after treatment. (a) Typical microscopic fields of MKN45 xenografts. (b) Quantitative data obtained by immunohistochemical analysis. Error bars represent the SE of the mean. **: $P < 0.01$ vs. control.

**Fig. 7.** Expression of phospho-ERK1/2 and phospho-Akt in MKN45 xenografts. Tumors were excised 1 day after treatment. Panel A: Typical microscopic fields of MKN45 xenografts. Panel B: Quantitative data obtained by immunohistochemical analysis. Error bars represent the SE of the mean. *: $P < 0.05$ vs. control, **: $P < 0.01$ vs. control, †: $P < 0.05$ TAS106 administration group vs. combination group.
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A) Colon26 (0.1 mg/kg)

B) Colon26 (0.5 mg/kg)

C) MKN45 (0.1 mg/kg)

D) MKN45 (0.5 mg/kg)

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