1-(3-C-Ethynyl-β-D-ribo-pentofuranosyl)cytosine (ECyd, TAS-106), a novel potent inhibitor of RNA polymerase, potentiates the cytotoxicity of CDDP in human cancer cells both in vitro and in vivo

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Abstract. 1-(3-C-Ethynyl-β-D-ribo-pentofuranosyl)cytosine (ECyd, TAS-106) is a novel antitumor ribonucleoside that inhibits RNA polymerase. In the present study, we investigated the cellular and molecular interactions between TAS-106 and cisplatin (CDDP) in vitro using A549 human lung cancer cells and the in vivo antitumor effect of combined treatment using OCC-1 and LX-1 human tumor xenografts. The treatment effects were determined by evaluating cytotoxicity, the cell cycle distribution, apoptosis induction and the expression of checkpoint-associated proteins. In vitro, the combination of TAS-106 and CDDP synergistically inhibited the growth of A549 cells, as determined using isobologram analysis. TAS-106 potently inhibited the expression of Chk1 protein and the phosphorylation of Chk1 and Chk2. Moreover, based on the inhibition of checkpoint-associated protein, TAS-106 abrogated the CDDP-induced S- and G2M-checkpoints and induced apoptosis in A549 cells. In vivo, TAS-106 alone showed antitumor activity; however, its combination with CDDP significantly enhanced the growth inhibition of OCC-1 and LX-1 tumors. Moreover, combination therapy with TAS-106 and CDDP in the OCC-1 xenograft model resulted in significant life-prolongation. These findings provide a rationale for combination chemotherapy using TAS-106 and CDDP in clinical settings.

Introduction

1-(3-C-Ethynyl-β-D-ribo-pentofuranosyl)cytosine (3’-ethynylcytidine, ECyd, TAS-106) is a new antitumor cytidine analogue possessing potent cytotoxic and antitumor activities in preclinical therapeutic models (1-4) and phase I/II clinical trials examining the use of TAS-106 as monotherapy and in combination with other cytotoxic drugs have recently begun. Previous studies have revealed that the cytotoxic effects of TAS-106 are mainly related to the inhibition of RNA biosynthesis (5,6). TAS-106 is converted into 3’-ethynylcytidine-5’-monophosphate (ECMP) by uridine-cytidine kinase (UCK, EC 2.7.1.48) (7) and this product is subsequently phosphorylated to 3’-ethynylcytidine-5’-diphosphate (ECDP) and finally to 3’-ethynylcytidine-5’-triphosphate (ECTP). Moreover, RNA polymerase was inhibited competitively by ECTP in the presence of isolated nuclei from FM3A mouse tumor cells. The Ki value of ECTP was 20 nM, while the apparent Km value of RNA polymerase for CTP was 8 μM (8). ECTP was found to be a major intracellular metabolite that accumulates abundantly in cells exposed to TAS-106 because of its slow elimination from these cells.

Cis-diaminedichloroplatinum (II) (CDDP) is one of the most effective antitumor agents available for the treatment of testicular, ovarian, bladder, head and neck, small cell lung and cervical cancer. The main mechanism of CDDP cytotoxicity is likely exerted through covalent binding to DNA. The binding of CDDP induces both interstrand and intrastrand cross-links in DNA structure, inhibiting replication and transcription processes. However, the presence of a DNA repair system means that these structural lesions are only transient. In many instances, a high level of DNA repair may significantly abolish the antitumor activity of CDDP (9). When CDDP damages
DNA, cell cycle progression is temporary blocked at G1 phase to facilitate DNA repair. If the DNA lesions are not repaired, however, the cells may enter an apoptotic pathway (10).

In general, combination chemotherapy with other agents that have different mechanisms of activity is performed in the hope of attaining a high antitumor efficacy. For this reason, accumulating rationale for combination chemotherapy is a useful endeavor for designing effective combination regimens with other anticancer agent(s).

The purpose of this study was to clarify whether the combination of TAS-106 and CDDP would produce a more potent antitumor effect. Indeed, the combination of both compounds was very effective, not only in an in vitro system but also in an experimental xenograft model in vivo.

Materials and methods

Chemicals. TAS-106 was synthesized at Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan). Cis-diamminedichloroplatinum(II) (CDDP, Briplatin™ injection) was obtained from Bristol-Myers Squibb Co., Ltd. All other chemicals were of analytical grade and were purchased from commercial sources.

Cell lines and cell culture. Human lung cancer A549 cells were obtained from the American Type Culture Collection (Rockville, MD). A549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; ICN Biomedicals, Inc., Aurora, OH) at 37°C in 5% CO₂.

Tumor xenografts. LX-1 (human lung cancer) and OCC-1 (human oral cancer) xenografts were obtained from the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research and the Central Institute for Experimental Animals, respectively.

Analysis of the effects of combination chemotherapy in vitro. For simultaneous exposure to TAS-106 and CDDP, A549 cells were treated with both drugs for 24 h. After treatment, the cells were washed three times with culture medium and subsequently incubated for 2 days. For sequential exposure to TAS-106 and CDDP, A549 cells were treated with TAS-106 (or CDDP) for 24 h and then washed three times with culture medium. Subsequently, the cells were treated with CDDP (or TAS-106) for 24 h. After treatment, the cells were washed three times with culture medium and were subsequently incubated for 1 day.

Viable cell growth was evaluated using a tetrazolium salt-based (MTT, C₄₃H₂₇N₃SBr) semi-automated colorimetric assay (11). The measurement of MTT-formazan product, absorbed at 540 nm, was performed using a Model 3550 microplate reader (Bio-Rad Laboratories, Hercules, CA).

Dose response interactions between TAS-106 and CDDP at the point of IC₅₀ were evaluated using the isobologram method of Steel and Peckham (12,13).

Cell cycle analysis. A549 cells were treated with TAS-106, CDDP or both drugs using various schedules. The cells were then collected and stained with propidium iodide (PI) using the Cycle Test Plus DNA Reagent kit, according to the protocol provided by the manufacturer (Becton-Dickinson Immunocytochemistry Systems, San Jose, CA). Flow cytometric analysis was performed using a FACScalibur flow cytometer (Becton-Dickinson, Franklin Lake, NJ) and the data were subsequently analyzed using CellQuest™ software (Becton-Dickinson).

DNA fragmentation (TUNEL assay). DNA fragmentation was analyzed using the APO-BRDU™ assay (14), according to the protocol provided by the manufacturer (PharMingen, San Diego, CA). Briefly, A549 cells were treated with TAS-106, CDDP or both drugs for 72 h and then collected. Subsequently, the cells were stained with FITC-labeled deoxyuridine triphosphate (dUTP) using terminal deoxynucleotidyl transferase (TdT). The percentage of apoptotic cells in the tested samples was estimated using a FACScalibur flow cytometer and subsequently analyzed using CellQuest software.

SDS-PAGE and Western blotting. A549 cells were treated with TAS-106, CDDP or both drugs for 24 h and then collected in SDS-PAGE sample buffer containing protease and phosphatase inhibitors after washing the cells in PBS. Cell lysates at the same protein concentration were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel using electrophoresis and were transferred to a polyvinylidene difluoride membrane. The membranes were probed with anti-Chk1 (G-4) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:500, anti-Chk2 (H-300) antibody (Santa Cruz Biotechnology, Inc., Beverly, MA) diluted 1:1000, anti-phospho-Chk1 (Ser345) antibody (Cell Signaling Technology, Inc., Santa Cruz, CA) diluted 1:500, anti-phospho-Chk2 (Ser161) antibody (Cell Signaling Technology, Inc.) diluted 1:1000, and anti-ß-actin (clone AC-74) antibody (SIGMA) diluted 1:5000 overnight at 4°C. The specific protein signals were detected using enhanced chemiluminescence by the appropriate secondary horseradish peroxidase-conjugated antibodies and the SuperSignal™ West Dura Extended Duration Substrate (Pierce).

Antitumor effect and life-prolonging effect of combination therapy. LX-1 and OCC-1 tumor fragments ~250 mm³ in size were transplanted s.c. into male F344/N Jcl-rnu nude rats (CLEA Japan, Inc., Tokyo). After reaching a tumor volume of ~250 mm³, the rats were randomly assigned to control group and drug treated groups, each consisting of six animals (day 0) in the case of LX-1 human tumor xenografts. In the case of the study on OCC-1 tumor xenografts, 11 rats were assigned to each experimental group and then tumor fragments were transplanted into rats (day 0). Drugs were administered intravenously at a volume of 1 ml/100 g body weight starting the next day (day 1). TAS-106 was intravenously administered once a week for 2 weeks on LX-1, and intermittently (three times a week) for 2 weeks on OCC-1, respectively. CDDP was intravenously administered on Day 1. Tumor volume was measured using microcallipers two times weekly. The tumor growth inhibition rate (IR, %) was calculated as the ratio of the mean tumor volume of the tumors treated to that of the control tumors (T/C) according to the formula: (1-T/C) x 100. A life prolonging effect, indicated as an increase in life-span
(ILS, %), was determined using the following formula: ILS (% ) = \( \frac{\text{mean survival time of treatment group}}{\text{mean survival time of control group}} - 1 \) x 100. All animal experiments were carried out in accordance with the Guidelines for the Welfare of Animals in Experimental Neoplasma.

**Statistical analysis.** The statistical analysis was performed using the Student's t-test and the Wilcoxon test for the animal experiments. A linear regression model using JMP ver.7 software (SAS Institute Inc.) was used to evaluate the results of the DNA fragmentation assay. In all of the statistical evaluations, P<0.05 was considered to denote a significant difference.

**Results**

**Combination effect of TAS-106 and CDDP in vitro.** A549 cells were exposed to TAS-106 and CDDP for 24 h according to various schedules, e.g., simultaneous or sequential exposure. Isobolograms at the IC\(_{50}\) level were generated using dose response curves for the combinations. For simultaneous exposure (Fig. 1a) and sequential exposure to TAS-106 followed by CDDP (Fig. 1b), the data points for the combination fell within the envelope of additivity, suggesting the presence of additive effects. However, prior exposure of the cells to CDDP and then to TAS-106 resulted in a strong interaction, suggesting an almost supra-additive effect (Fig. 1c).

**Variation in cell cycle according to exposure schedules.** We compared the cell cycle profiles of cells subjected to various exposure schedules using the IC\(_{50}\) value of each drug after 72 h of exposure. For the monotherapy investigations, the cells were treated with TAS-106 or CDDP for 24 h and subsequently washed three times with culture medium and cultured for 24 h with drug-free medium. TAS-106 treatment of the A549 cells caused arrest at the G1-S boundary in addition to a significant reduction of cells in S-phase. The cells treated with CDDP maintained a significant increase in S-phase or G\(_{2}\)M-phase. On the other hand, when we investigated concomitant treatment, the cell cycle analyses were performed after simultaneous or sequential exposures using A549 cells collected at 24 and 48 h, respectively. Significantly different perturbations in the cell cycle were also observed after these treatments. These results are shown in Fig. 2.

**Augmentation of apoptosis by the combination of TAS-106 and CDDP.** The extent of apoptosis was determined based on DNA fragmentation (TUNEL assay). To show the apoptotic events and the associated molecular changes more clearly, we used the drugs at higher concentrations. These results are shown in Fig. 3. The rate of apoptotic events (%) after 72 h of exposure to 0.1 μM TAS-106, 0.25 μM TAS-106 and 12.5 μM CDDP alone were 0.67, 5.46 and 0.56, respectively. The combination of these agents at the same concentrations resulted in a dramatic increase in the number of cells undergoing apoptosis. The TUNEL positive rates for combined treatment with either 0.1 μM or 0.25 μM TAS-106 and 12.5 μM CDDP were 12.4 and 55.6%, respectively. These results suggest that the combination of TAS-106 and CDDP exert a synergistic augmentation of the cytotoxic effect (p<0.05). Such strong induction of apoptosis is expected to contribute significantly to the antitumor activity.

**TAS-106 abrogates CDDP-induced cell cycle checkpoints.** The changes observed in the cell cycle after combined treatment prompted us to study whether those effects resulted from an eventual imbalance in molecular events controlling cell cycle progression. The changes in the expression of the checkpoint-associated proteins were evaluated after 24-h exposure under the same conditions which were used in the experiments on the induction of the apoptotic events. Such approach might allow us to confirm an association between early molecular events and a process of cell death. The effects
on checkpoint-associated protein are presented in Fig. 4. The treatment of cells with TAS-106 decreased the expression of checkpoint kinase 1 (Chk1), while the expression of checkpoint kinase 2 (Chk2) apparently was unaffected. Upon incubation of the A549 cells with CDDP, a DNA damaging agent, the kinases Chk1 and Chk2 became phosphorylated at Ser-345 and Ser-19, respectively. The phosphorylation of checkpoint-associated proteins causes a delay in cell cycle progression until the completion of DNA repairs. When cells were treated with a combination of TAS-106 and CDDP, the phosphorylated forms of the checkpoint-associated proteins were lower than after CDDP treatment alone. In this case, the down-regulation of the expression of the phosphorylated forms of the checkpoint-associated proteins meant that cell cycle progression occurred without sufficient repair of the DNA lesions. Therefore, these results indicated that the potentiation of the tumor growth inhibitory effect of CDDP by TAS-106 was associated with checkpoint abrogation and the subsequent increase in apoptosis induction in the tumor.

TAS-106 as cell cycle abrogator and trigger of apoptosis. We confirmed the cell cycle profiles under the conditions used in the TUNEL assay (Fig. 5). Cells treated with TAS-106 were arrested in G1 phase, while CDDP treatment resulted in S- and G2/M-phase arrest. On the other hand, cells treated with a combination of TAS-106 and CDDP increased the sub-G1 cell

![Table and diagrams showing cell cycle progression](image-url)
population in addition to a significant reduction in cells in S- and G2M-phase, compared with the effects of CDDP monotherapy. The population in S- and G2M-phase decreased to the level observed in control cells. In other words, cells were deprived of the protective mechanism whereby G2 blockade allows the cells to repair DNA damage and instead the cells progressed through the cell cycle.

**Antitumor effect of combination therapy against subcutaneously implanted LX-1 tumors.** In this experiment, we used non-toxic doses of TAS-106 and CDDP and evaluated the tumor growth inhibition on Day 15. Treatment with 0.3 mg/kg/day TAS-106 (Days 1, 8) and 3 mg/kg/day CDDP (Day 1), administered as single agents, resulted in IR (%) of 69.0 and 80.0%, respectively. These effects were statistically significant when compared with the corresponding tumor volume in the control group (p<0.05, Student’s t-test). On the other hand, the tumor volume after combined treatment with 0.3 mg/kg/day TAS-106 and 3 mg/kg/day CDDP was dramatically reduced, with a tumor growth inhibition rate of 89.3%; this value was
significantly better than those of the single drugs (p<0.01, Student's t-test). Moreover, the combined treatment was able to induce tumor regression, a phenomenon that is strongly desired in antitumor drug evaluations (Fig. 6). The strong antitumor effects were not associated with an increase in toxicity. The time course of body weight changes in the treated animals, an indicator of drug toxicity, did not exceed a drop of 10%. The hematological effects (WBC, % Cont.) in all the groups remained at 60% or more, compared with the control values. Together, these findings suggest that the effective dose levels of both monotherapies and the combined therapy were not harmful.

Antitumor effect and life-prolonging effect of combination therapy against subcutaneously implanted OCC-1 tumors. In this experiment, we used maximum tolerated doses (MTD) of TAS-106 and CDDP and measured the tumor volume from Day 8 to Day 22. The results are shown in Fig. 7a. Tumor volume measurement was completed on Day 22, because many of the rats in the control group died before Day 22. The antitumor efficacy of the combination therapy with TAS-106 and CDDP against OCC-1 tumor xenografts was significantly enhanced, compared with that of either monotherapy (p<0.01, Student's t-test). Moreover, because OCC-1 tumors are suitable for evaluating antitumor efficacy using the survival time of the animals, we evaluated the life-prolonging effects. The median survival time in the control group was 21 days. The increased life-spans (ILS) values for the animal groups treated with 0.5 mg/kg/day TAS-106, 5.0 mg/kg/day CDDP, or a combination of both drugs were 57.1, 38.1 and 123.8%, respectively (Fig. 7b). Thus, combination therapy with
TAS-106 and CDDP significantly prolonged the lives of the rats (p<0.05, Wilcoxon test).

Discussion

TAS-106 has been found to exert very strong antitumor effects, prompting us to initiate its clinical evaluation. However, effective cancer treatment using single agent chemotherapy is very seldom observed; in most instances, treatments consist of a combination of several drugs. Therefore, to further explore the potential antitumor activity of a drug, positive interactions with other clinically available anticancer drugs are usually sought. As a result of such a search, synergistic cytotoxic effects were found between TAS-106 and CDDP after 72-h simultaneous exposure in A549 human lung cancer cell line (data not shown). Although the combination of TAS-106 and CDDP was found to be sufficiently effective, it remained important to establish an optimal schedule and sequence for drug exposure. Since TAS-106 cytotoxic effects are cell cycle-independent (15), it seemed reasonable to use this compound in combination with a drug(s) causing DNA damage and leading to an accumulation of cells in S- or G_{2}M-phase. On the other hand, the exposure of cancer cells to TAS-106 caused a decrease in the cell population in S-phase; therefore, combination with agents specifically affecting cells in S-phase is not advised. An example is the case of CPT-11, an S-phase-specific agent (16), whose cytotoxic effects against TAS-106 pretreated cells were reduced because of the decreased cell population in S-phase. Thus, we investigated an optimal sequence for various schedules by using 24-h treatment of each drug.

In the present study, the expression of Chk1 and Chk2 were evaluated at higher concentration than those in the investigation of the optimal exposure schedule using isobologram; the reason was to demonstrate the molecular change clearly. Such reasons may not be free of doubt that the interaction between the two drugs might have different characteristics in different concentration range. However, sequential exposure; first to CDDP and then to TAS-106 appeared to be extremely effective, and this result agrees with the present finding that TAS-106 abrogates the cell cycle checkpoints despite the presence of CDDP-induced DNA damage (Fig. 4). Thus, we concluded that TAS-106 should be applied with DNA damaging agents, either simultaneously or after pre-exposure to achieve an optimal combination effect.

The arrest of the cell cycle in S- or G_{2}M-phase may play a protective role by allowing cells to repair DNA damage (17). The process of DNA repair is carefully controlled by a system of enzymes including Chk1, a serine/threonine kinase that is reported to be involved in S- and G_{2}M-phase arrest (18-20). In response to a variety of genotoxic stressors, such as a replicative block induced by UV light, hydroxyurea and DNA strand breaks, Chk1 is activated by phosphorylation at Ser^{317} and Ser^{45} by an upstream kinase, ATR (21). Phosphorylated Chk1 regulates cell cycle progression through checkpoints such as the cdc25A at S-phase and both the cdc25A and cdc25C phosphatases at G_{2}M-phase, respectively. The cdc25A and cdc25C play critical roles in mitotic entry (22,23). On the other hand, another kinase, Chk2, is activated by phosphorylation at Thr^{345} and Ser^{45} by the upstream kinase ATM, in the case of ionizing radiation-induced DNA damage (24).

In the present study, we confirmed that TAS-106 decreased the expression level of Chk1. As reported by Shannon and Greg (25), Chk1 interference RNA combined with the partial inhibition of DNA replication was sufficient to evoke a response to DNA damage. Both cyclin-dependent kinases (Cdk) and Chk1 inhibitors enhanced the cytotoxicity of etoposide, a DNA-damaging agent. Similar effects were reported by Bartz et al (26), where the silencing of checkpoint genes, ATR and Chk1, also enhanced sensitivity to cisplatin. These studies indicate that the down-regulation of the Chk1 expression level is one of the important factors for antitumor efficacy. Although the main mechanism of TAS-106 involves the inhibition of RNA synthesis, its additional ability to abrogate a checkpoint of the cell cycle seems to be an important feature, particularly for combined treatment. Many G_{2} checkpoint abrogators have already been reported in the case of anticancer drugs (27). In fact, Chk1, Chk2, ATM, ATR and PP2A belong to this type of target, and Chk1, in particular, is targeted by many anticancer drugs: staurosporin (28), UCN-01 (28,29), Go6976 (30) and SB-218078 (28), to name a few. Moreover, the dysregulation of cell cycle checkpoints is now recognized as a salient feature of the malignant transformation process. Some studies have suggested the involvement of Chk1 ablation in oncogenesis and/or advancing tumor grade (31,32).

The careful analysis of the cell cycle profiles, performed under the conditions used in the TUNEL assay, indicated the presence of S- and G_{2}M-phase arrests and G1 block induced by CDDP and TAS-106, respectively (Fig. 5). The treatment of the cells simultaneously with CDDP and TAS-106, on the other hand, resulted in the significant reduction of the cell population in S- and G_{2}M-phase induced by CDDP alone, in consequence leading to an increase of sub-G1 phase population. Probably, the cells damaged by CDDP were able to progress into next phase due to the lack of cell cycle checkpoints, however most of them entered the apoptotic stage because of the DNA damaging effect of CDDP. Moreover, when we observed morphology, the acridine orange/ethidium bromide staining of the cells revealed no remarkable change in TAS-106-treated cells, however, cell swelling was observed in CDDP-treated cells without obvious apoptotic events (data not shown). The combined treatment resulted, however, in very dramatic induction of apoptosis. At this stage, it is difficult to clarify the exact mechanism of this important phenomenon. These promising in vitro results prompted us to initiate in vivo evaluation of these interesting properties.

The OCC-1 tumors grew by ~20-fold in one week; in other words, the proliferation of this tumor was very rapid. So, in the study using OCC-1 tumors, TAS-106 was administered three times a week using MTD for this schedule. The OCC-1 cancer responded poorly to CDDP treatment, a golden standard for that type of tumor. However, combined treatment with CDDP and TAS-106 significantly increased the antitumor effect, both in terms of tumor size reduction (Fig. 7a) and the prolongation of the life-spans of the treated animals (Fig. 7b). Moreover, 5-FU, a key drug for head and neck cancer, was not effective against OCC-1 tumors (data not shown), while TAS-106, even administered alone, exerted a strong effect.
Thus, TAS-106 may be an effective therapeutic against head and neck cancers that are refractory to CDDP and 5-FU. A similar strong antitumor activity was demonstrated in the case of LX-1 cancer, where significant antitumor activity was even associated with tumor regression (Fig. 6). The dosage of TAS-106 used for the study on LX-1 was 1/20 of MTD, so that the efficacy of the drug at low dose exposures could be confirmed. Despite this low dose of TAS-106, which produced no significant toxic manifestations, TAS-106 enhanced the antitumor activity of CDDP, resulting in tumor regression.

In conclusion, we have shown that TAS-106 was able to potentiate the effects of CDDP, both in vitro and in vivo. Furthermore, the rationale for this drug combination is based not only on a difference in the main mechanisms of action of TAS-106 and CDDP, but also on the additional ability of TAS-106 to abrogate the cell cycle checkpoints, thus preventing the repair of damaged DNA. These results show that TAS-106 is a potential candidate drug for combination therapy with CDDP.

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