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Acetylation of α-tubulin by a Histone Deacetylase Inhibitor, Resminostat, Leads Synergistic Antitumor Effect with Docetaxel in Non-Small Cell Lung Cancer Models

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Abstract

Background: There is a growing body of clinical evidence to demonstrate that inhibition of histone deacetylase is effective in the treatment of various types of cancer. We examined whether acetylation of a non-histone protein α-tubulin was induced by resminostat and this acetylation exerts combination effects with docetaxel since α-tubulin was a target of docetaxel.

Methods: The cytotoxicity of resminostat was evaluated in 11 human non-small cell lung cancer cell lines and the inhibitory activity of resminostat against histone deacetylase 6 was also determined. The stability of α-tubulin (the amount of polymerized and acetylated α-tubulin) was analyzed in NCI-H460 cells treated with resminostat and/or docetaxel. Caspase-3/7 activity was analyzed in NCI-H460, A549, NCI-H1975, and MOR/CPR cell lines treated with resminostat alone or in combination with docetaxel. The antitumor efficacies of resminostat and docetaxel were evaluated in xenograft mouse models.

Results: Resminostat exhibited anti-proliferative activity in all 11 cell lines tested (IC_{50}, 0.36-8.7 μmol/L) and also inhibited histone deacetylase 6 activities. The amounts of polymerized and acetylated α-tubulin and caspase-3/7 activity were increased by resminostat alone or the combination with docetaxel. This combination synergistically increased antitumor efficacy in all the tumor-bearing mice tested.

Conclusions: These results demonstrate that the combination of resminostat and docetaxel offers a promising regimen in the treatment of non-small cell lung cancer.

Keywords

Histone deacetylase inhibitor, Non-small cell lung cancer, Combination chemotherapy, Docetaxel, Acetylation of α-tubulin

Introduction

Lung cancer is the leading cause of cancer-related death worldwide [1]. A number of pharmacological agents are frequently used in the treatment of non-small cell lung cancer (NSCLC), including cisplatin (CDDP), docetaxel (DTX), and erlotinib, an epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) [2]. Among them, DTX inhibits the depolymerization of microtubules. This stabilizing effect is then followed by an increase in the polymerization of α-tubulin [3]. Monotherapy with DTX, however, has proven insufficient to produce a satisfactory response; hence clinical study is focusing on its effect in combination with other agents.

One such agent is a histone deacetylase (HDAC) inhibitor. Inhibition of HDAC activity is followed by the accumulation of acetylated histones and acetylated non-histone proteins. HDAC regulates chromatin remodeling and is crucial in the reactivation of epigenetically silenced tumor suppressor genes [4,5]. Inhibitors of HDAC have been reported to mediate transcriptional activity by modulating the acetylation status of nucleosomal histones proximal to gene promoters [6]. In addition to chromatin remodeling, HDAC activity (mainly HDAC6) has been linked to the acetylation of non-histone proteins such as tumor suppressor p53 and α-tubulin [7-9]. Excessive acetylation of α-tubulin leads to the stabilization of microtubules, resulting in cytotoxicity [10].

HDAC inhibitors and DTX stabilize microtubules in different ways [3,10]. This suggests that combination therapy with HDAC inhibitors and DTX might achieve a therapeutic effect on NSCLC from the mode of action not only of efficacy, but also of safety. We examined the efficacy of combination treatment with resminostat [11-13], an HDAC inhibitor, and DTX in four chemotherapy-resistant NSCLC cell lines: A549, NCI-H460, NCI-H1975, and MOR/CPR. The first two harbor an amino acid substitution on KRAS (G12S and Q61H, respectively), which affects activation ofthe MAPK and PI3K-AKT pathways [14]; NCI-H1975 cells harbor a T790M substitution on EGFR that renders cancer cells resistant to EGFR-TKIs [15]; and MOR/CPR cells are CDDP-resistant due to reduced accumulation of platinum complexes [16]. These cell lines represent typical chemotherapy-resistant mechanisms in NSCLC cells. One problem with combination therapies is that although they offer an increase in efficacy, they also mean a compromise in safety due to a tendency toward an increase in adverse effects. One toxic side

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effect of DTX is myelosuppression [17], whereas with resminostat, gastrointestinal disturbances are more common [12]. This suggests that combination therapy with resminostat and DTX might offer a potent therapy against NSCLC, while avoiding synergistic adverse effects. In addition, DTX-based therapy enhances the stabilization of microtubules through a different biochemical mechanism than resminostat, which acetylates α-tubulin, suggesting a potential improvement in therapeutic efficacy.

The purpose of this study was to investigate the efficacy of combination therapy with resminostat and DTX against chemotherapy-resistant NSCLC cells. Our hypothesis was that this combination might provide one of novel regimens for the clinical treatment of NSCLC.

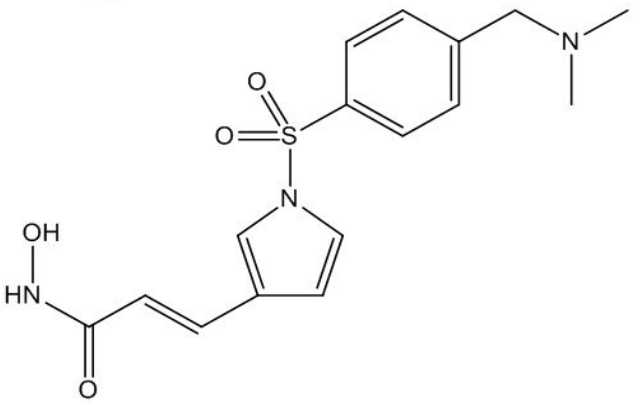

Figure 1: Chemical structure of resminostat.

Methods

Reagents and cell cultures

Resminostat (Figure 1) was obtained from Yakult Honsha Co., Ltd. (Tokyo, Japan). DTX (TAXOTERE®) was obtained from Sanofi K.K. (Tokyo, Japan). Suberoylanilide hydroxamic acid (SAHA) was obtained from Alexis Corporation (Lausen, Switzerland) and tubacin from Selleck Chemicals (Houston, TX, USA). Human NSCLC cell lines A549, NCI-H460, and NCI-H1975 were obtained from ATCC (Manassas, VA, USA). Another human NSCLC cell line, MOR/CPR, was obtained from the European Collection of Cell Cultures (Salisbury, UK). All the cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and antibiotics. The absence of mycoplasma contamination was confirmed periodically with the MycoAlert™ Mycoplasma Detection Kit (Lonza, Basel, Switzerland).

Inhibition of HDAC activity in NCI-H460 cells

The NCI-H460 cells were treated with resminostat or SAHA for 60 min to determine the level of inhibitory activity of each of them against HDAC I/II. The HDAC I/II activity in these cells were subsequently determined using the HDAC-Glo™ I/II Assay and Screening System (Promega, Madison, WI, USA). The inhibitory activity of SAHA or resminostat against HDAC6 was also determined with the HDAC6 Fluorimetric Drug Discovery Kit (Enzo Life Sciences Inc., Farmingdale, NY, USA).

Anti-proliferative activity

The cells (A549, NCI-H460, NCI-H1975, and MOR/CPR) were seeded into a 96-well culture plate (2500 cells/well; 50 μL/well) and resminostat and/or DTX added the next day. The number of viable

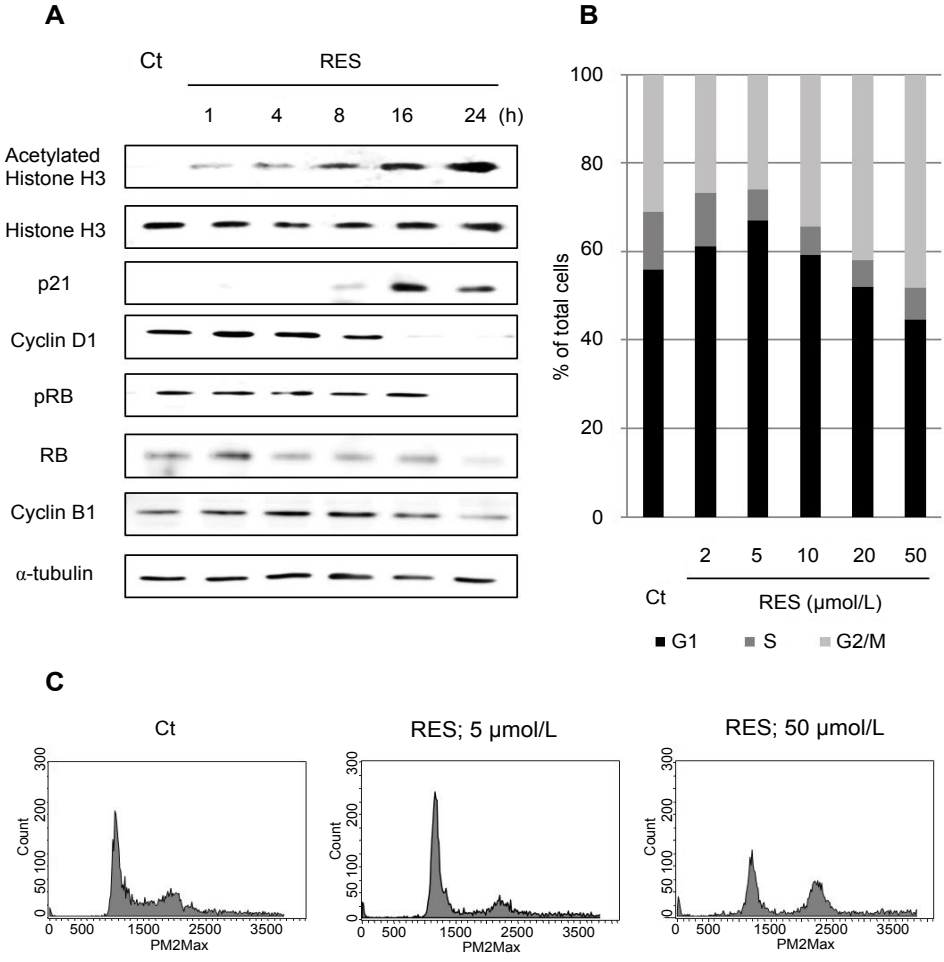

Figure 2: Change in cell cycle-related protein levels and cell cycle profiles after treatment with resminostat (RES). (A) Immunoblotting analysis of cell cycle-related proteins. NCI-H460 cells were treated with RES (20 μmol/L) for1-24 h. After treatment, cell cycle-related proteins were examined by immunoblotting; (B) Cell cycle profile; (C) typical cell cycle histograms. NCI-H460 cells were treated with RES (2-50 μmol/L) for 24 h. After treatment, cell cycle statuses of 5000 cells were determined.

cells was determined after 48 h incubation using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan).

Cell cycle in NCI-H460 cells

The NCI-H460 cells were exposed to resminostat (2-50 μmol/L) for 24 h. The cell cycle in these cells (5000 cells) was analyzed using the Cell Cycle Phase Determination Kit (Cayman Chemical Company, Ann Arbor, MI, USA) and a Guava easy Cyte™ flow cytometer (Merck Millipore, Darmstadt, Germany).

Immunoblotting of cell cycle- and apoptosis-related proteins in NCI-H460 cells

In the cell cycle-related protein analysis, the NCI-H460 cells were treated with resminostat (20 μmol/L) for 1-24 h; in the apoptosis-related protein analysis, they were treated with resminostat (10 μmol/L) and/or DTX (10, 20 nmol/L) for 24 h. After treatment, the cells were harvested and lysed in RIPA buffer (10 mmol/L Tris-HCl, pH7.4; 0.1% nonidet-P-40; 0.1% sodium deoxycholate; 0.1% SDS; 150 mmol/L NaCl; 1 mmol/L EDTA; 10 μg/mL aprotinin; and a phosphatase inhibitor cocktail) (Nacalai Tesque, Inc., Kyoto, Japan). Equal amounts of protein were subjected to SDS-PAGE on an 8-15% gel. The separated proteins were transferred to a nitrocellulose membrane. The membrane was incubated with the following primary antibodies: anti-Acetyl-Histone H3 (CST-9677; Cell Signaling Technology, Inc. CST, Beverly, MA, USA), anti-Histone H3 (CST-9715; CST), anti-p21 (sc-397; Santa Cruz Biotechnology, Dallas, TX, USA), anti-Cyclin D1 (CST-2926; CST), anti-phospho-retinoblastoma (pRB) (CST-9307; CST), anti-RB (CST-9302;CST), anti-Cyclin B1 (sc-752; Santa Cruz Biotechnology), anti-α-tubulin (T9026; Sigma-Aldrich), anti-acetylated-α-tubulin (T6793; Sigma-Aldrich), anti-X-linked inhibitor of apoptosis protein (XIAP)(CST-2045; CST), anti-B-cell lymphoma 2(BCL-2) (sc-492; Santa Cruz Biotechnology), anti-Bcl-2-like protein 11 (BIM)(CST-2933; CST), and anti-β-actin (A5316; Sigma-Aldrich). All the antibodies were used at a dilution of 1:3000. Immune complexes were detected with horseradish peroxidase-conjugated antibodies (Sigma-Aldrich) and chemiluminescence reagents (GE Healthcare, Buckinghamshire, UK). All other chemicals were of the highest purity available.

Immunoblotting of polymerized α-tubulin in NCI-H460 cells

The NCI-H460 cells were treated with resminostat (10 μmol/L), tubacin (10 μmol/L), and/or DTX (2 nmol/L) for 24 h. The cells were then suspended in lysis buffer (20 mmol/L Tris-HCl, pH7.4; 0.5% nonidet-P-40; 1 mmol/L $MgCl_2.6H_2O$; 2 mmol/L EGTA; 10 μg/mL aprotinin; and a phosphatase inhibitor cocktail) for detection of polymerized α-tubulin. Cells lysates were clarified by centrifugation at 20,000 g for 10 min at 4 °C. The precipitate was lysed in RIPA buffer. For detection of other proteins (listed in Figure 3), the cells were lysed in RIPA buffer.

Caspase-3/7 activity

The cells (A549, NCI-H460, NCI-H1975, and MOR/CPR) were seeded into a 96-well, white-walled plate (2500 cells/well; 50 μL/well) and resminostat and/or DTX added the next day. Caspase-3/7 activity was determined after 24 h incubation using Caspase-Glo™ 3/7 Assays (Promega).

Subcutaneous xenograft models

Five-week-old male BALB/c-nu/nu mice were obtained from Japan SLC, Inc. (Hamamatsu, Japan). Cultured tumor cells (2×10^6 cells/mouse) were injected subcutaneously into the flank of each mouse. Each tumor lesion was measured in two dimensions by caliper twice a week and volume calculated using the following formula: tumor volume (mm^3) = 1/2 × (major axis) × (minor axis)^2. When tumor volume reached 100-200 mm^3, the mice were randomized and treated with resminostat (60 or 90 mg/kg/dose, oral administration (p.o.), 5 times/week, formulated with 0.5% methylcellulose in water) and/or DTX (5 or 10 mg/kg/dose, intravenous administration (i.v.), once every 3 weeks, formulated under the manufacturer's instructions).

Statistical analysis

Group differences were analyzed by using a one-way analysis of variance (Dunnett).

Results

Resminostat suppresses proliferation of NSCLC cells

Resminostat and SAHA showed similar inhibitory activity against HDAC I/II (cell-based assay) and HDAC6 (enzyme assay) (Table 1). The cytotoxic activity of resminostat was determined in NSCLC cells, including those with a *KRAS* mutation and chemotherapy-resistance

Table 1: HDAC inhibitory activity of resminostat (RES).

	IC_{50} (nmol/L)	
	HDAC6	HDAC class I/II
RES	343	127
SAHA	207	76

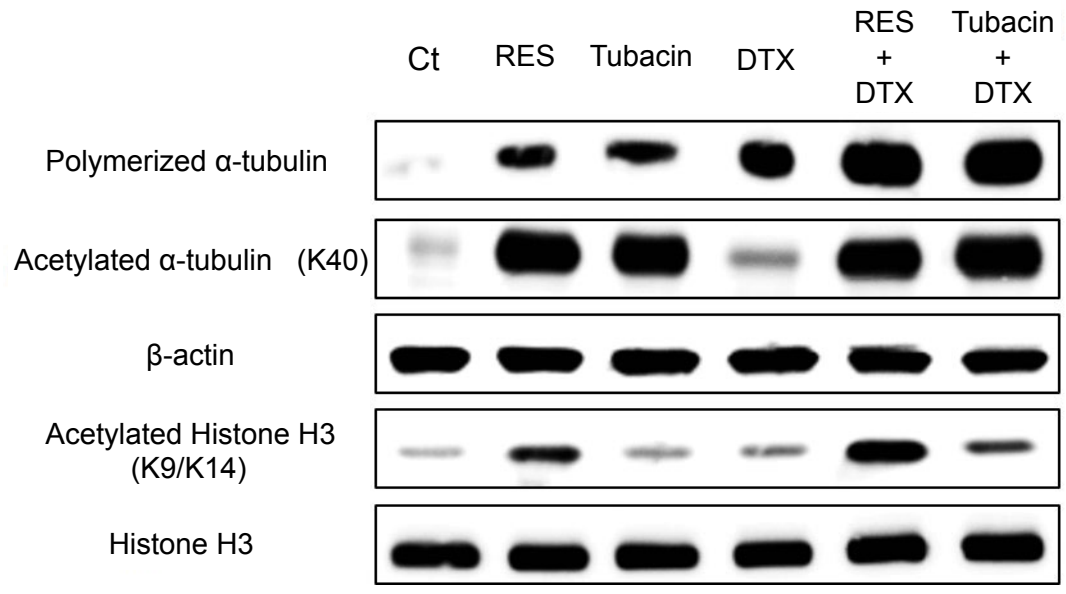

Figure 3: Levels of polymerized and acetylated α-tubulin after treatment with RES, tubacin, or docetaxel (DTX). NCI-H460 cells were treated with RES (10 μmol/L), tubacin (10 μmol/L), and/or DTX (2 nmol/L) as indicated for 24 h. Amounts of polymerized and acetylated α-tubulin were determined by immunoblotting.

Table 2: IC_{50} values for resminostat anti-proliferative activity against NSCLC cells.

Cell	IC_{50} (μmol/L)	*KRAS* status	Note
A437	5.3	wild	
Calu-3	2.0	wild	
NCI-H522	8.7	wild	
NCI-H1975	5.0	wild	EGFR-TKI-resistant
NCI-H2126	3.4	wild	
A549	3.3	G12S	
COR-L23	4.2	G12V	
MOR/CPR	0.74	G12C	CDDP-resistant
NCI-H23	4.4	G12C	
NCI-H358	0.36	G12C	
NCI-H460	4.5	Q61H	CDDP-resistant

(Table 2). The average IC_{50} value for the cytotoxicity of resminostat against the *KRAS* wild type cell line was 4.9 μmol/L (range 2.0-8.7 μmol/L), while that against the *KRAS* mutation cell line was 2.9 μmol/L (range 0.36-4.5 μmol/L) (Table 2).

Additionally, no cross-resistance to resminostat was observed in EGFR-TKI-or CDDP-resistant cells. Resminostat-resistant NSCLC cells were not detected in 11 cell lines. The influence of resminostat on the cell cycle and related proteins was also determined to investigate the relationship between re-expressed proteins and cell cycle arrest. Resminostat induced expression of p21 accompanied by acetylation of histone H3. Subsequent degradation of cyclin D1 and dephosphorylation of RB ensued (Figure 2A). The cell cycle analysis revealed that resminostat induced G1 phase arrest at lower

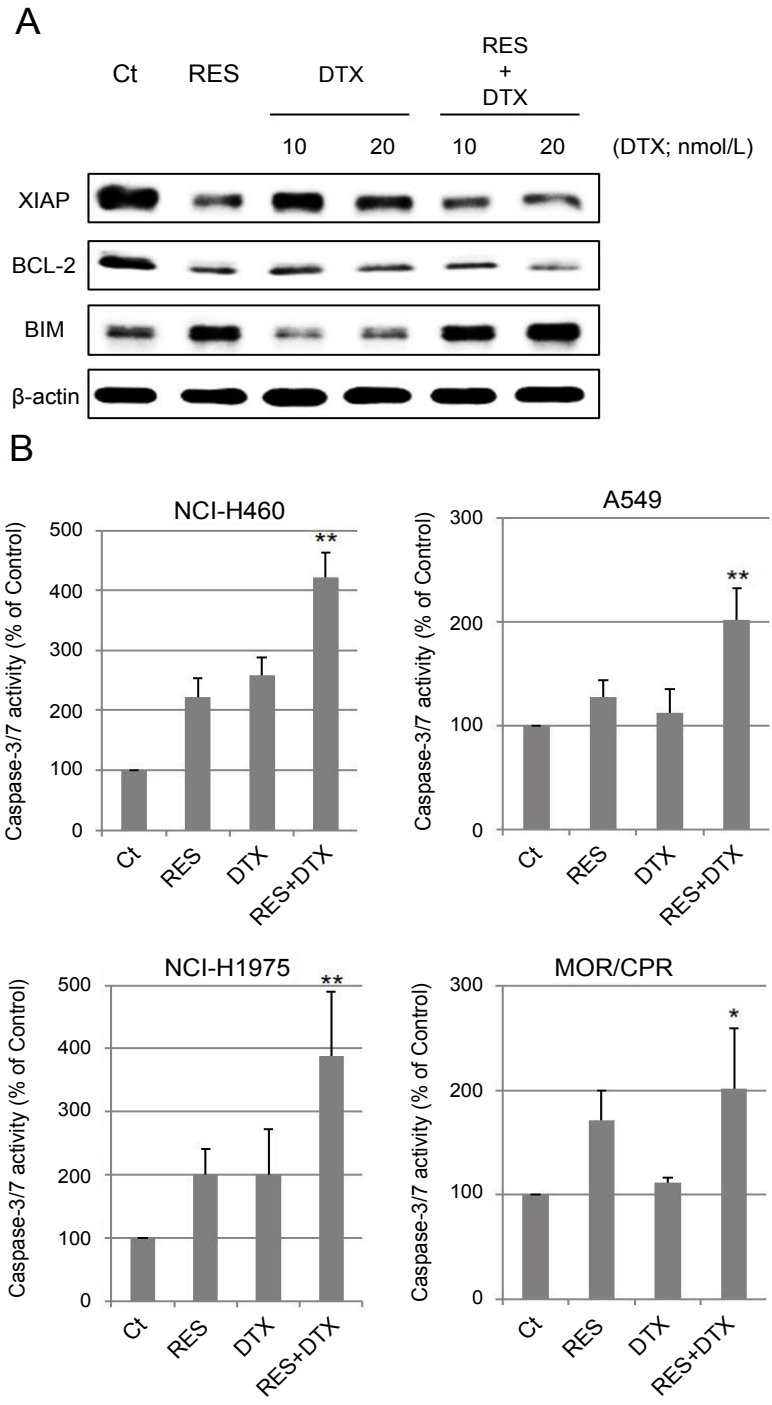

Figure 4: Change in apoptosis-related proteins and caspase-3/7 activity. (A) Immunoblotting analysis. NCI-H460 cells were treated with RES (10 μmol/L) and/or DTX (10, 20 nmol/L) for 24 h. After treatment, amounts of XIAP, BCL-2, and BIM were determined by immunoblotting; (B) Activity of Caspase-3/7. Four NSCLC cell lines were treated with RES 10 μmol/L (NCI-H460 cells), 2.5 μmol/L (A549 and NCI-H1975 cells), 0.5 μmol/L (MOR/CPR cells), and/or DTX 10 nmol/L (NCI-H460, A549, and NCI-H1975 cells) or 1 nmol/L (MOR/CPR cells) for 24 h. Apoptotic cells were detected as those exhibiting caspase-3/7 activity (calculated from 3 independent experiments).

$^{*}p < 0.05$ vs. Control, $^{**}p < 0.01$ vs. Control and single agents.

(2-5 μmol/L) and G2/M phase arrest at higher concentrations (20-50 μmol/L) (Figure 2B and Figure 2C). The status of the cell cycle and related proteins changed depending on the concentration used and duration of treatment.

Combination of resminostat and DTX synergistically promoted stabilization of microtubules and apoptosis

Excessive stabilization of microtubules leads to the suppression of cell division. The stabilization of α-tubulin in resminostat and/or DTX-treated cells was determined with respect to the polymerization and acetylation status of α-tubulin. Resminostat induced acetylation of α-tubulin and promoted its polymerization to the same extent as tubacin, a selective HDAC6 inhibitor. DTX induced polymerization of α-tubulin but not its acetylation. Combination treatment promoted polymerization to a greater extent than resminostat or DTX alone (Figure 3). Next, the effects of stabilization on apoptosis were examined. Expression of anti/pro-apoptotic proteins XIAP, BCL-2, and BIM, and caspase-3/7 activity were determined as indicators of induction of apoptosis. Resminostat decreased expression of XIAP and BCL-2, and induced expression of BIM (Figure 4A). Treatment with resminostat or DTX alone induced caspase-3/7 activity in all the tested NSCLC cells; in A549 cells, however, this effect was observed at higher concentrations (data not shown). In addition, combination treatment induced higher caspase-3/7 activity in NCI-H460, A549, and NCI-H1975 cells, than single-agent therapy, and this difference was significant (Figure 4B).

Combination of resminostat and DTX inhibits tumor growth in chemotherapy-resistant NSCLC cells in nude mice

The *in vivo* efficacy of resminostat in combination with DTX was subsequently determined. The antitumor efficacy was determined in two different schedules, intermittent administration schedule and daily administration schedule. In the former schedule, the mice were treated with resminostat on days 1-5, 8-12, 22-25, 29-33 (Figure 5A).

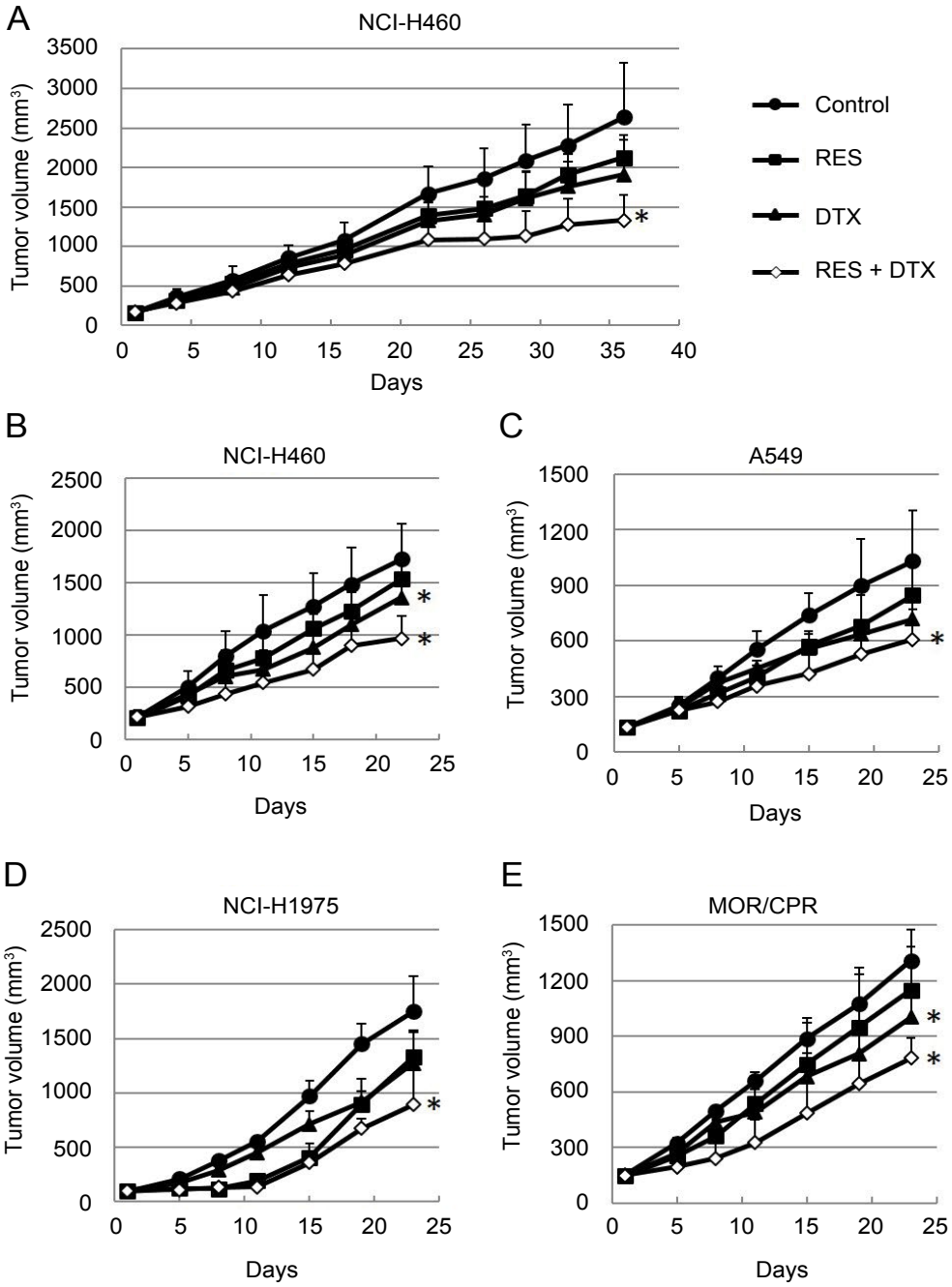

Figure 5: Antitumor efficacies of RES and/or DTX in mouse xenograft models of non-small cell lung cancers. (A) Nude mice bearing NCI-H460 tumors were treated with RES (90 mg/kg) on days 1-5, 8-12, 22-26, and 29-33 and/or DTX (5 mg/kg) on days 1 and 29; (B-E) Nude mice bearing (B) NCI-H460; (C) A549; (D) NCI-H1975; or (E) MOR/CPR tumors were treated with RES 90 mg/kg/dose (NCI-H460, NCI-H1975, and MOR/CPR tumors) on days 1-5, 8-12, and 15-19 and/or DTX 10 mg/kg/dose (NCI-H460 and A549 tumors) or 5 mg/kg/dose (NCI-H1975 and MOR/CPR tumors) on day 1. Tumor volume was measured using calipers on days indicated. Mean ± SD tumor volumes are shown for groups of 5 mice. $^{*}p < 0.05$ vs. Control.

In the latter schedule, the mice were treated with resminostat on days 1-5, 8-12, 15-19 (Figure 5B). There was no difference on antitumor efficacy. Monotherapy with resminostat or DTX yielded a moderate decrease in tumor growth, whereas combination therapy resulted in an appreciable improvement in antitumor efficacy in all the NSCLC tumors tested (Figure 5B-5E) ($p < 0.05$ vs. Control). None of the mice treated with these agents showed any severe macroscopic adverse effects.

Discussion

The present results revealed that combination therapy with resminostat and DTX was effective against chemotherapy-resistant NSCLC cells. This combination therapy exerted a synergistic effect, resulting in an increase in antitumor efficacy against several NSCLC cells both *in vitro* and *in vivo*, especially with respect to the stabilization of microtubules. Resminostat exhibitsits cytotoxicity on chemotherapy-resistant NSCLC cells through various mechanisms. In this study, no cross-resistance to resminostat was observed in EGFR-TKI-or CDDP-resistant or *KRAS* mutation cells. The difference in the cytotoxic effect of resminostat and DTX is likely due to the mode of action of each reagent. In general, tubulin-targeting agents exert strong cytotoxicity, whereas HDAC inhibitors exert moderate cytotoxicity [18,19]. HDAC inhibitors have several modes of action, including tubulin acetylation, HSP90 inhibition, and pro-apoptosis protein re-expression [20-23]. An agent with several modes of action is less likely to induced resistance due to specific mechanisms. In fact, in the present study, no cross-resistance to resminostat was detected in other chemotherapy-resistant cell lines. This suggests that the combination of resminostat and DTX could be effective in the treatment of NSCLC.

Combination therapy with resminostat and DTX appears to be a suitable option with respect to its mode of action, i.e., the stabilization of microtubules. Increased levels of acetylated α-tubulin with resminostat might be due to inhibition of HDAC6 (Table 1). Acetylated α-tubulin contributes to the stabilization of microtubules, whereas DTX inhibits α-tubulin depolymerization. Thus, used in combination, these two agents exert a synergistic effect in increasing polymerization of α-tubulin, resulting in its stabilization. In addition, these results correlated with those of the apoptosis experiments, which indicated synergistic activation of caspase-3/7.

Resminostat suppressed the proliferation of tumor cells in the G1 phase, and DTX killed other cells in the G2/M phase through stabilization of microtubules. Resminostat induced p21 expression, which was followed by a decrease in cyclin D1 expression and phosphorylation of RB. This arrest of the G1 phase may have been due to this decrease in phospho-RB and cyclin D1 [24-27]. The stabilization of α-tubulin had a significant influence on the cell cycle, resulting in arrest at the G2/M phase [28]. In the present study, acetylation of α-tubulin led to stabilization of microtubules, resulting in G2/M phase arrest in cells treated with high concentrations of resminostat. In an earlier study, resminostat induced G1 phase arrest in 3 out of 4 myeloma cell lines associated with upregulation of p21, and G2/M phase arrest in 2 out of 4 cell lines at different concentrations. The cause of this G2/M phase arrest remains to be identified [11]. However, resminostat exhibits multiple modes of action, including induction of p21 expression and acetylation of α-tubulin. Thus, both G1 and G2/M phase arrest induced by resminostat appears to be due to its exposure conditions.

The combination of resminostat and DTX exerted a synergistic antitumor effect against chemotherapy-resistant NSCLC cells in both the *in vitro* and *in vivo* studies. Resminostat alone increased expression of pro-apoptotic while decreasing expression of anti-apoptotic proteins. Induction of BIM expression might be due to enhancement of FOXO3A and E2F1 promoter activity [29,30]. It has been reported that low BIM expression can lead to resistance to TKI treatment [31]. In the present study, induction of BIM expression may have led to synergistic cytotoxicity against chemotherapy-resistant cell lines. Reduced expression of BCL-2 may be caused by decreased binding of its transcription factor to the *BCL-2* promoter due to reduced interaction between the transcription factor and HDAC2 [32]. Change in apoptosis-related proteins influences the cytotoxicity of many antitumor agents. This indicates that resminostat might offer potent synergistic efficacy when used in combination with other antitumor agents. In the present *in vivo* study, resminostat combination therapy synergistically enhanced antitumor efficacy independent of the dosing schedule and cell line used. The cell lines used here represent several chemotherapy-resistant properties in clinical practice [14-16]. The *in vivo* studies validated the effectiveness of the *in vitro* combination studies with respect to cytotoxicity and tubulin stabilization assays. No severe toxicity was observed in the mice with any of the agents used in the present study. Therefore, resminostat combination therapy is regarded as well tolerated. These results demonstrated that resminostat combination therapy offers a feasible option for NSCLC patients.

Conclusions

Our study indicates that a rational strategy in combination with resminostat and DTX is thought to a promising regimen in the treatment of chemothrapy-resistant NSCLC. Based on the present results, we are proceeding with a phase I/II study in NSCLC patients to further investigate the safety and efficacy of this regimen.

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Conflict of Interest

The authors have no conflict of interest.

Ethical Statement

All the *in vivo* experimental protocols were approved by the Animal Care Committee of the Yakult Central Institute prior to the study.

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