Effects of D-allose in combination with docetaxel in human head and neck cancer cells

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Abstract. In this study we investigated the combined effects of docetaxel and D-allose in HSC3 human oral carcinoma cells. The dose enhancement ratios at the 25% survival level were 1.3 and 1.71 for combined treatment with 10 or 25 mM D-allose, respectively. Apoptosis was significantly increased by addition of D-allose. Additionally, a synchronous increase in the G_2/M-phase population was observed after docetaxel plus D-allose treatment. *In vivo* experiments revealed that docetaxel plus D-allose was more effective than either agent alone. Thus, D-allose enhanced the anticancer effects of docetaxel, and combined treatment may be useful to achieve clinical efficacy with reduced toxicity.

Introduction

Docetaxel, the most potent taxane, is derived from extracts of European yew needles. Docetaxel has been shown to have significant antitumor activity. The main mechanism of action is through stabilization of tubulin, arresting cells in the G_2/M phase of the cell cycle (1). In head and neck cancer patients, docetaxel is now widely applied as first- and second-line induction chemotherapy or used in combination with other anticancer drugs or radiation (2-7). However, some patients develop resistance to docetaxel. Although the causes and mechanisms of docetaxel resistance are still unknown, activation of the redox system is thought to be involved (8). Thioredoxin (TRX), a small redox-active multifunctional protein, acts as a potent antioxidant and redox regulator in signal transduction (9). TRX has been reported to be overexpressed in various types of cancers (10-12) and its overexpression is associated with a poor prognosis in patients (13,14). Indeed, Kim *et al* (15) reported that breast tumors with high TRX expression show a significantly lower response rate to docetaxel treatment than those with low TRX expression.

D-allose is a rare sugar that is found only in very small quantities in nature. In recent studies, we reported that D-allose inhibited the growth of head and neck cancer cells by inducing of cell cycle arrest, apoptosis and competition with glucose uptake (16). In addition, D-allose stimulates the overexpression of TRX-interacting protein (TXNIP) and enhances the effects of radiation (17). TXNIP is known to interact with TRX and is involved in the regulation of the cellular redox state (18). Moreover, the *TXNIP* gene is a tumor suppressor (19) and metastasis suppressor (20) and its expression is lower in various cancer cells when compared to normal cells (21-23). Therefore, we speculated that the induction of TXNIP expression by D-allose treatment may enhance the anticancer effects of docetaxel.

In the present study, we evaluated the combined effects and mechanisms of docetaxel and D-allose in head and neck cancer *in vitro* and *in vivo*.

Materials and methods

Cell culture. The human head and neck carcinoma cell line HSC3 (tongue carcinoma) was obtained from the Health Science Research Resources Bank, Osaka, Japan. HSC3 cells were cultured at 37˚C in an atmosphere containing 5% CO_2 in Eagle's minimal essential medium (EMEM), 10% heat-inactivated fetal bovine calf serum and 1% penicillin-streptomycin.

Determination of cell survival. D-allose was kindly supplied by Dr K. Izumori at the Department of Biochemistry and Food Science, Faculty of Agriculture, Kagawa University, Kagawa, Japan. Docetaxel was obtained from Sanofi (Paris, France) and stored in frozen aliquots. Before use, docetaxel was thawed and diluted to the desired concentrations in the cell culture medium. The growth inhibitory effects of docetaxel plus D-allose were compared with those of docetaxel or D-allose alone. The cells were seeded in 96-well plates at a density of $2.5x10^3$ cells/100 μl (n=5 wells/treatment) and were cultured for 24 h. Medium was then removed, and fresh medium containing 0.1 ng/ml docetaxel, 10 mM D-allose or 0.1 ng/ml

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docetaxel plus 10 mM D-allose were added. Cells were incubated for an additional 24-96 h. The net number of viable cells was then determined using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The absorbance was measured by a microplate reader at 450 nm after 2 h of incubation.

To investigate the enhancement of docetaxel-dependent anticancer effects by D-allose, $5x10^3$ cells/500 μl were plated into 6-well plates and cultured for 24 h (n=3 wells/treatment). The cells were treated with 0, 10 or 25 mM D-allose and various concentrations of docetaxel and were incubated at 37˚C for 96 h. Colonies were fixed with 3:1 methanol/acetic acid and stained with 0.5% crystal violet in methanol. Colonies were counted under a microscope, with a cut-off of 50 viable cells. The survival fraction was calculated as (mean colonies)/(cells inoculated) x (plating efficiency). The docetaxel dose enhancement ratio (DER) was calculated as the dose (ng/ml) for docetaxel alone divided by the dose (ng/ml) for docetaxel plus D-allose for a survival fraction of 0.25.

For three-dimensional (3D) culture experiments, the 3D culture BME cell proliferation assay (Trevigen, Gaithersburg, MD, USA) was used. Each well of a 96-well plate was coated with 35 μl of 3D Culture BME, and plates were then incubated at 37˚C for 1 h to promote gel formation. Cells were then seeded in the coated 96-well plates at a density of $5x10^3$ cells per 100 μl and cultured for 48 h. After the establishment of 3D structures, 100 μl of fresh medium containing 0.1 ng/ml docetaxel, 25 mM D-allose or 0.1 ng/ml docetaxel plus 25 mM D-allose was added. Cells were then incubated at 37˚C for an additional 5 days. Colonies growing >5 fold were scored as survivors. Error bars indicate the standard deviation calculated after pooling the results of 3 independent experiments.

Measurement of apoptosis. TUNEL assays were performed using the Apoptosis Detection System Fluorescein kit (Promega, Madison, WI, USA). Briefly, treated HSC-3 cells were spread on a poly-l-lysine slide (Sigma, St. Louis, MO, USA), fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Cells were incubated in 50 μl TdT incubation buffer [nucleotide mix (fluorescein-12-dUTP) and TdT enzyme prepared according to the manufacturer's protocol] for 60 min at 37˚C in a humidified chamber. The reaction was terminated by washing the cells in 2X SSC followed by 2 washes in PBS. Cells were counterstained with 1 μg/ml propidium iodide and then washed in distilled water. Staining was observed under a fluorescence microscope. Green fluorescence indicated DNA fragmentation due to fluorescein-12-dUTP labeling. For each experimental time point, 10 fields, each containing 100 cells, were scored for the appearance of apoptosis; two chambers were scored in this manner for each time point.

Cell cycle analysis. Flow cytometry was performed using a FACSEpics XL flow cytometer (Beckman Coulter, Fullerton, CA, USA). Cells were washed twice with PBS, fixed in 1 ml of 70% ethanol for 2 h at 4˚C, treated with 200 g RNase A, and stained with 50 μg propidium iodide. Cell cycle distribution was analyzed using System II software (Beckman Coulter).

Analysis of mRNA expression. To investigate the effects of docetaxel, D-allose and docetaxel plus D-allose on the expression of *TXNIP* and *TRX* transcripts, cells were cultured in 6-cm dishes with 0.1 ng/ml docetaxel, 25 mM D-allose or 0.1 ng/ml docetaxel plus 25 mM D-allose and incubated for an additional 24 h. Real-time polymerase chain reaction (PCR) was carried out using TaqMan gene expression assay primers and an ABI7700 real-time PCR system. Each reaction was performed in duplicate. The *GAPDH* gene was used to normalize expression across assays and runs, and a threshold value (Ct) for each sample was used to determine the expression level of the gene.

Western blot analysis. After treatment with 0.1 ng/ml docetaxel, 25 mM D-allose or 0.1 ng/ml docetaxel plus 25 mM D-allose for 24 h, cells were scraped into lysis buffer (1% NP40, 150 mM NaCl, 50 mM NaF, 20 mM Tris-HCl, pH 7.5, 1 mM Na_3VO_4, 10 μM Na_2MnO_4, 1 mM PMSF, 10 μg/ml leupeptin, 1% aporotinin) with protease inhibitors and sonicated. Samples were centrifuged for 10 min at 14,000 rpm and supernatants were collected. For western blot analyses, proteins were separated on 10% SDS-PAGE gels, transferred to nitrocellulose membranes, blocked with 5% (w/v) non-fat dried milk in PBS and incubated with anti-TXNIP (MBL, Nagoya, Japan), anti-TRX (MBL) or anti-β-actin antibodies (Sigma). Membranes were probed with a horseradish peroxidase-conjugated anti-mouse IgG (Amersham, Tokyo, Japan), and signals were detected with an enhanced chemiluminescence system (Amersham).

Detection of reactive oxygen species (ROS) detection. Intracellular ROS generation was measured using the Total ROS Detection kit (Enzo Life Sciences, Plymouth Meeting, PA, USA) according to the manufacturer's instructions. Cells were incubated with dye from the kit at 37˚C for 1 h. Immediately after staining, the cells were analyzed using a fluorescence microscope (Olympus BX-51, Tokyo, Japan) equipped with a standard green filter (490/525 nm).

In vivo xenograft experiment. HSC3 cells were used in a xenograft model with female athymic nude mice (BALB/c nu/nu, 5-6 weeks old). A suspension of $1x10^6$ cells in 0.1 ml volume was injected subcutaneously into the posterior flanks of mice using a 1-cc syringe with a 27-G needle. Tumors were grown for 14 days until attaining an average size of 100-150 mm^3 (Day 0). Treatment groups were as follows: a) control; b) treatment with 500 mM D-allose; c) 12 mg/kg docetaxel; and d) 12 mg/kg docetaxel plus 500 mM D-allose. Each treatment group contained 6 mice. D-allose was prepared by dissolving compound in normal saline to reach a final concentration of 500 mM and aliquots (0.2 ml) were injected around tumors 5 times/week for 3 weeks. Docetaxel was diluted in normal saline to reach a final concentration of 1 mg/ml, and aliquots (0.2 ml) were administered by intraperitoneal injection on Days 0 and 7. Mice from the control group were injected with 0.2 ml normal saline at the same time points. This research was approved by the Animal Care and Use Committee of Kagawa University.

Statistical analysis. Comparisons between groups were carried out using the Student's t-test. Differences with P-values of <0.05 were considered statistically significant.

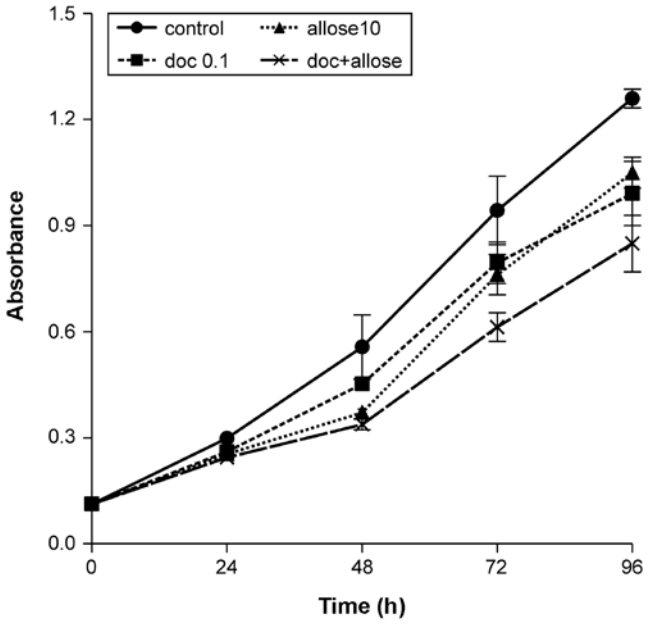

Figure 1. CCK-8 assay to analyze the effects of docetaxel, D-allose and docetaxel plus D-allose on HSC3 cells. Cells were seeded in 96-well plates. After 24 h, 0.1 ng/ml docetaxel, 10 mM D-allose or 0.1 ng/ml docetaxel + 10 mM D-allose were added. Cells were incubated for an additional 24-96 h.

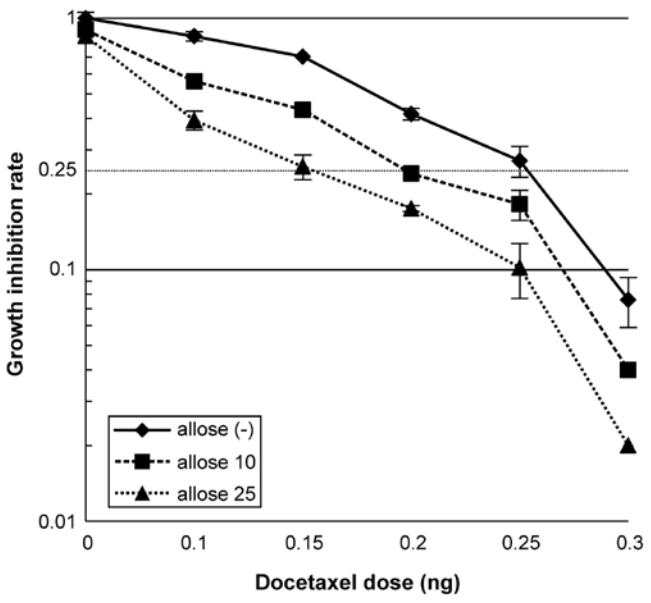

Figure 2. The combined effects of docetaxel and D-allose on the survival of HSC3 cells, based on the clonogenic assay. Cells were incubated with 0, 10 or 25 mM D-allose and various concentrations of docetaxel. Colonies were counted, and growth inhibition curves were constructed. The dose enhancement ratio (DER) was calculated as docetaxel alone divided by docetaxel plus D-allose for a survival fraction of 0.25.

Results

Effects of D-allose in combination with docetaxel. Ninety-six hours after treatment with docetaxel, D-allose or docetaxel plus D-allose, growth of HSC3 cells was decreased to 78.7, 83.3 and 67.4% that of the control, respectively. The combination of docetaxel plus D-allose significantly inhibited cell proliferation when compared to treatment with docetaxel or D-allose alone (P<0.001 and P<0.001, respectively; Fig. 1). As shown in Fig. 2, treatment of cells with 10 mM D-allose resulted in a docetaxel dose enhancement ratio (DER) of 1.3, while treatment with 25 mM D-allose resulted in a DER of 1.71. Analysis of the morphology and growth of cells in 3D cultures, as shown in Fig. 3, revealed that treatment with docetaxel alone, D-allose alone or docetaxel plus D-allose reduced cell survival to 78, 49 and 28% that of the control group, respectively. Moreover, the combination of docetaxel and D-allose also induced the highest percentage of apoptosis in comparison to either docetaxel alone or D-allose alone (P<0.0001; Table I).

Modification of the cell cycle. Cell cycle modification by docetaxel and D-allose treatment was analyzed by flow cytometry. Accumulation of cells in the G_1 phase of the cell cycle was significantly decreased after treatment with docetaxel alone, D-allose alone or docetaxel plus D-allose as compared to that of control group. Although the G_2/M-phase cell populations tended to increase after treatment, no significant differences were found (Fig. 4).

Regulation of mRNA and protein expression. The mRNA expression of *TXNIP* was markedly increased in HSC3 cells following treatment with D-allose. Additionally, the expression of *TXNIP* mRNA was enhanced after treatment with the combination of docetaxel plus D-allose treatment, while no significant increase was observed following treatment with docetaxel alone. Although the mRNA expression of *TRX* was increased by docetaxel treatment, combined treatment with D-allose and docetaxel significantly suppressed the expression of *TRX* mRNA (Fig. 5).

Table I. Docetaxel dose enhancement ratios and percent apoptosis induced by each treatment.

Treatment	Ratio to docetaxel	% Apoptosis
No treatment	0.78	0.55±0.1
Docetaxel	1.00	1.71±0.22
Allose	1.58	1.22±0.27
Docetaxel + allose	2.81	4.25±0.54

TXNIP and TRX protein levels were also evaluated by western blot analysis (Fig. 6). The expression of TNNIP was significantly increased by D-allose treatment, while docetaxel had no effect on TXNIP expression. Although no apparent changes were observed by docetaxel plus D-allose treatment, the protein expression levels of TXNIP and TRX were comparable to the expression levels of their corresponding genes.

Effects of docetaxel and D-allose on ROS production. The intracellular ROS levels following treatment with D-allose were the same as those of the positive control. No excitation emission was observed by exposure to docetaxel in the HSC3 cells. Compared with docetaxel treatment alone, the addition of D-allose induced ROS generation (Fig. 7).

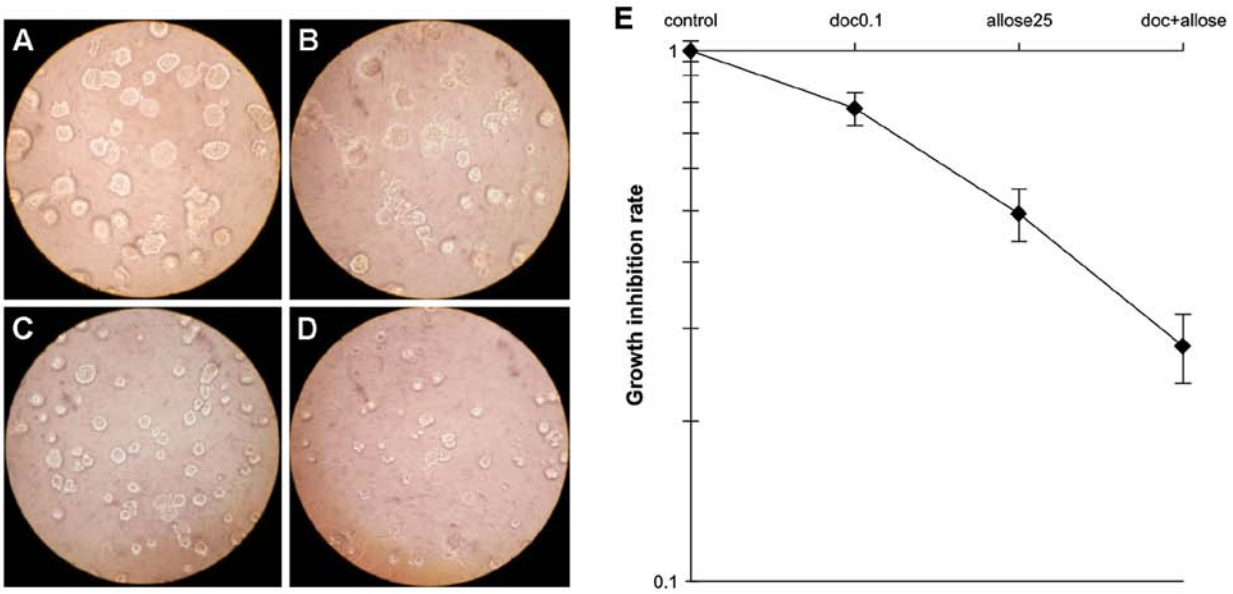

Figure 3. The cell morphology of 3D cultures. (A) Control (no treatment) cells 7 days after the establishment of colonies. (B) Docetaxel (0.1 ng/ml)-treated cells. (C) D-allose (25 mM)-treated cells. (D) Docetaxel plus D-allose-treated cells on 3D cell culture. (E) The growth inhibition of cells exposed to docetaxel alone, D-allose alone or docetaxel plus D-allose were 78, 49 and 28%, respectively.

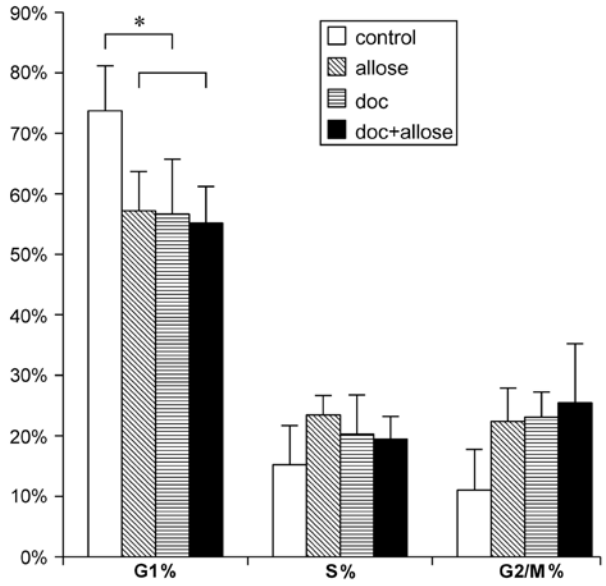

Figure 4. Cell cycle modification of HSC3 cells after treatment. All treatments tended to increase the G_2/M-phase cell population and significantly decreased the G_1-phase cell population. $^{*}P<0.05$.

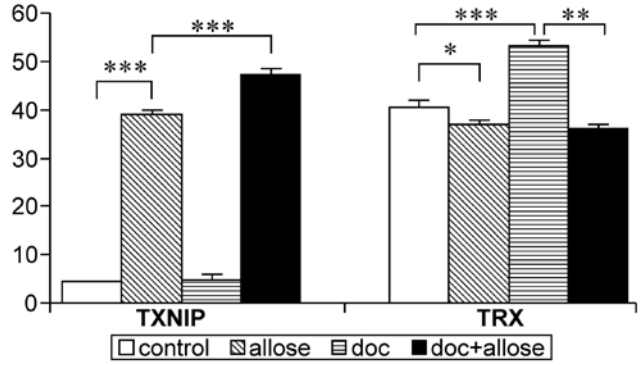

Figure 5. Changes in mRNA expression after treatment with docetaxel, D-allose or docetaxel plus D-allose. Combined treatment with D-allose significantly increased *TXNIP* mRNA expression and suppressed *TRX* mRNA expression. $^{*}P<0.05$, $^{**}P<0.001$, $^{***}P<0.0001$.

Effects of D-allose on cell proliferation in vivo. Results of tumor growth assays *in vivo* are presented in Fig. 8. Administration of 500 mM D-allose for 3 weeks resulted in a significant inhibition of tumor growth compared with that of the control group at Day 20 ($p<0.005$). Moreover, docetaxel treatment also strongly inhibited tumor growth, and combined treatment with D-allose and docetaxel markedly reduced tumor volumes compared to tumor volumes at the beginning of the treatment. No significant tissue damage (such as skin erythema or inflammation) was observed in the treatment groups.

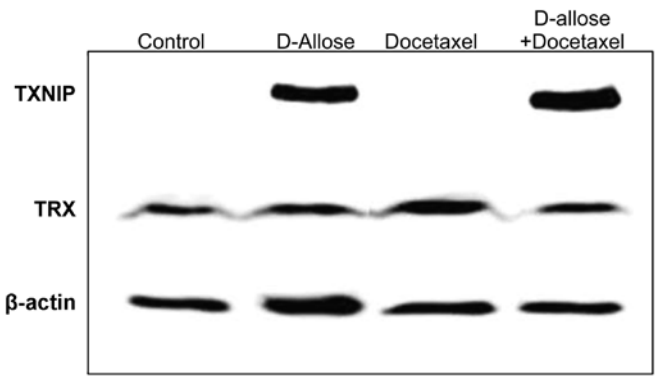

Figure 6. The protein levels of TXNIP and TRX were evaluated 24 h after the treatment. TXNIP expressions were increased by D-allose alone and docetaxel plus D-allose treatment. Combined use of D-allose seemed to decrease the expression of TRX in comparison to docetaxel alone.

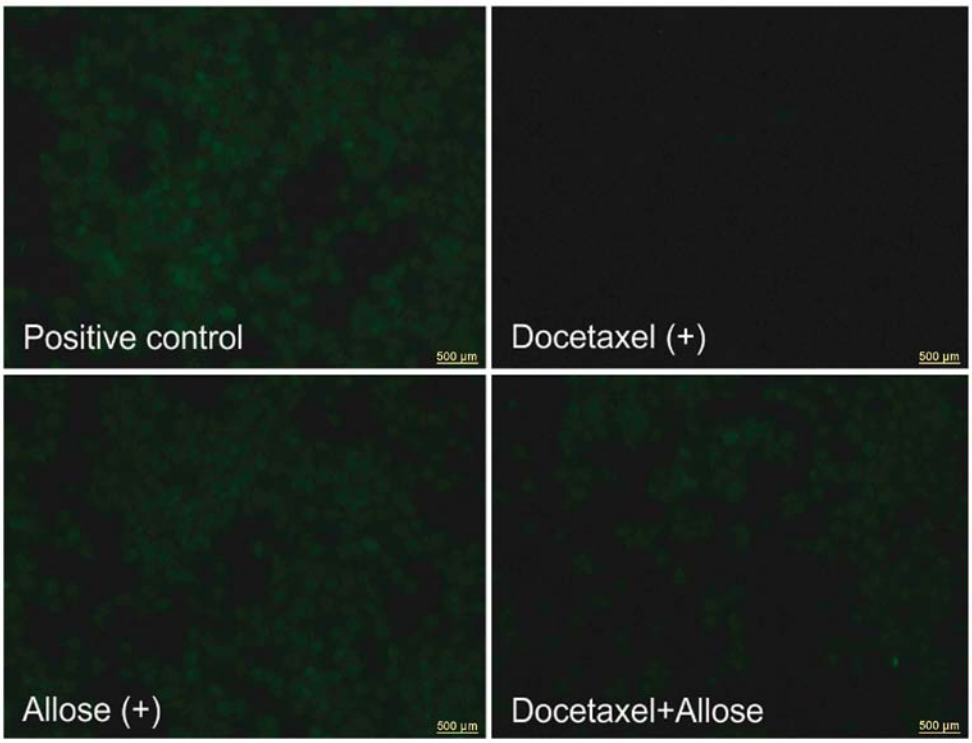

Figure 7. ROS induction was observed by fluorescence microscopy. No staining was observed with docetaxel treatment. The staining under docetaxel was reversed with D-allose treatment.

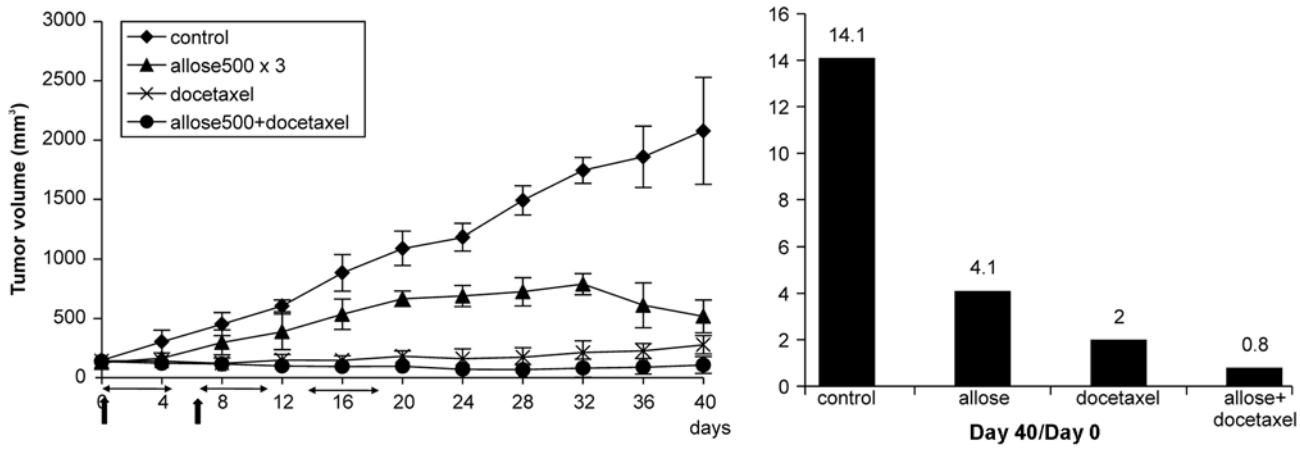

Figure 8. The effects of docetaxel and D-allose on the growth of HSC3 cancer xenografts in nude mice. D-allose was injected around tumors 5 times/week (double-sided arrows). Docetaxel was injected intraperitoneally at Days 0 and 7 (bold arrows).

Discussion

Docetaxel is a common anticancer drug used in a variety of cancers. In this study, we investigated whether D-allose, a rare sugar that possesses diverse biological effects in cells, could enhance the anticancer effects of docetaxel in head and neck cancer cells. Indeed, our results supported that combined treatment with D-allose plus docetaxel inhibited cell growth and survival to a greater extent than treatment with either compound alone.

The results of the present study showed that docetaxel treatment induced G_2/M-phase cell cycle arrest and enhanced activation of the apoptotic pathway in head and neck cancer cells. Stabilization of microtubules by taxanes results in phosphorylation and inactivation of Bcl-2, leading to increases in Bax levels and a consequent increase in apoptosis (24). Naha *et al* (25) reported that D-allose induces apoptosis by altering the expression of Bcl-2/Bax. In a previous study and in the present study, we clarified that D-allose modulates cell cycle regulatory proteins, G_2/M cell cycle arrest and apoptosis. These results suggested that the induction of G_2/M cell arrest and enhancement of the apoptotic pathway by combined treatment of docetaxel plus D-allose promoted the inhibition of cell growth.

TRX expression has been shown to be increased after docetaxel therapy and is thought to protect cells against docetaxel (14). Therefore, tumors showing increased TRX expression in response to docetaxel are expected to be more resistant to docetaxel than those showing no increase in TRX. Consistent with this, we observed an increase in *TRX* mRNA expression following docetaxel treatment, without an apparent increase in the generation of ROS. Thus, cancer cells may prevent ROS generation by upregulation of TRX expression. On the other hand, combined use of D-allose and docetaxel resulted in upregulation of TXNIP expression and downregulation of TRX expression compared to treatment with docetaxel alone. These results suggested that induction of TXNIP and suppression in TRX following D-allose treatment may prevent HSC3 cells from becoming resistant to docetaxel.

The radiosensitizing potential of docetaxel has been explored *in vitro*, *in vivo* and in clinical trials (5,6,26,27). However, several reports have shown that docetaxel resistance depends on redox regulation, as mediated by increased expression of TRX and a reduction in ROS generation (28). Khan and Ludueña (29) have shown that TRX can reduce a disulfide bridge within the tubulin dimer and inhibit microtubule assembly *in vitro*. These antioxidant molecules are also thought to contribute to radiation resistance (30-33). Therefore, regulation of the redox state is one of the key mechanisms maintaining radiosensitivity. Recently, we demonstrated that the induction of TXNIP by D-allose can enhance the effects of radiation by increasing both the intracellular ROS level and radiation-induced apoptosis (17). In addition, if D-allose inhibits the attenuation of docetaxel toxicity in cancer cells, we can expect to observe highly enhanced effects by a 3-drug combined therapy.

Docetaxel should be administered 24 h before irradiation to achieve optimal enhancement of the effects of radiation (27). This is because accumulation in the radiosensitive phase of the cell cycle, i.e., the G_2/M phase, is most likely observed after 24 h with docetaxel administration. However, one report demonstrated that preradiation in head and neck cancer cells significantly enhanced docetaxel cytotoxicity by arresting cells in the S phase (34). They concluded that irradiation followed by docetaxel may be the most effective sequence for head and neck cancer therapy. On the other hand, overexpression of TXNIP occurs at 6 h after D-allose treatment and persists for 24-48 h after treatment (35). Further studies are needed to clarify the most effective sequence of combined treatment with docetaxel, D-allose and irradiation.

In the present study, our *in vivo* experiment revealed that 500 mM D-allose injection for 3 weeks prolonged the tumor-suppressive effect after D-allose treatment was completed. In our previous study, tumor regrowth was observed after the completion of D-allose administration for 2 weeks (36). These results suggested that the dosing period may be more important than the application of high doses of D-allose. Tumor volumes in the docetaxel treatment group doubled at 40 days after the initiation of treatment, while those in the control group grew up to 14 times their original size. Moreover, tumor volumes in the combined treatment group were markedly smaller than those at the initiation of treatment. These results suggested that D-allose treatment enhanced the anticancer effects of docetaxel and may reduce the side effects of the chemotherapeutic drug by reducing the total dose of docetaxel required. Major toxicities of docetaxel are neutropenia, mucositis, peripheral neuropathy and pulmonary disorders (37). Concurrent radiation therapy may increase docetaxel toxicity. In particular, radiation-induced mucositis can result in interruption of radiation therapy. However, some reports have shown that D-allose protects the retina and neurons against ischemia-induced damage by attenuating oxidative stress (38,39). It is unknown whether such contradictory responses occur in normal tissue and malignant tumors. However, combined treatment with D-allose may be helpful to prevent radiation-induced mucositis if D-allose suppresses oxidative stress in normal mucosa surrounding the tumor. The mechanism of the redox regulation in normal mucosa by D-allose remains to be elucidated. Further studies are needed to evaluate whether D-allose acts as an antioxidant to protect against radiation and anticancer drugs in normal mucosa.

In conclusion, D-allose enhanced the anticancer effects of docetaxel by inducing changes in the cell cycle and stimulation of apoptotic pathways. Control of the redox state by D-allose may strengthen the radiosensitivity of docetaxel.

Acknowledgements

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