# Antitumor efficacy of sequential treatment with docetaxel and 5-fluorouracil against human oral cancer cells

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**Abstract.** Docetaxel (DOC) and 5-fluorouracil (5-FU) are important anticancer agents widely used in the treatment of a variety of cancers including oral squamous cell carcinoma (OSCC). The purpose of this study was to determine the antitumor efficacy of the sequential administration of DOC and 5-FU against OSCC cells (B88 and CAL27 cells) *in vitro* and *in vivo*. In *in vitro* growth inhibition assays, sequential treatment with DOC followed by 5-FU was more effective in inhibiting cancer cell growth than 5-FU followed by DOC, single treatment with DOC or 5-FU, or combined treatment with DOC and 5-FU. Furthermore, DOC followed by 5-FU significantly inhibited tumor growth *in vivo* compared to 5-FU followed by DOC. To understand the mechanisms underlying the enhanced growth inhibitory effect of the administration sequence, DOC followed by 5-FU, we examined the expression of 5-FU metabolic enzymes such as thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD) and orotate phosphoribosyl transferase (OPRT), which were known to regulate the antitumor effect of 5-FU, by real-time RT-PCR and western blot analysis. Downregulation of TS and DPD expression and upregulation of OPRT expression were induced by DOC treatment, suggesting that DOC enhanced the efficacy of 5-FU by altering the expression of its metabolic enzymes. These results indicate that sequential treatment with DOC followed by 5-FU could be a promising therapeutic strategy for oral cancer.

## Introduction

A variety of treatments have been used for oral squamous cell carcinoma (OSCC), including surgery, radiotherapy, and chemotherapy administered alone or in combination. For patients with locally advanced OSCC that were unresponsive to induction chemotherapy (1-3), new chemotherapeutic treatment strategies are needed for improving the treatment outcome and cure rates (4-6).

5-Fluorouracil (5-FU) is widely used as an anticancer agent and considered a key drug in chemotherapeutic treatments for OSCC, colorectal, gastric, and oesophageal cancer (5-8). Thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD), and ortate phosphoribosyl transferase (OPRT) are key enzymes in the regulation of 5-FU metabolism (9). Two main action mechanisms have been proposed for 5-FU through its active metabolites, 5-fluorodeoxyuridine monophosphate (FdUMP) and 5-fluorouridine triphosphate (FUTP) (Fig. 1), with the main mode of action being through FdUMP (10). FdUMP suppresses TS by forming covalent ternary complexes with 5,10-methylenetetrahydrofolate (CH_2THF), which then inhibits DNA synthesis. RNA function is inhibited when 5-FU is modified by OPRT to form 5-fluorouridine monophosphate (FUMP), which is then converted to FUTP. FUTP is incorporated into cellular RNA, resulting in RNA dysfunction. Thymidine phosphorylase (TP) anabolizes 5-FU to FdUMP. DPD is the initial enzyme in the catabolism of 5-FU to 2-fluoro-$\beta$-alanine, primarily in the liver. DPD is also the rate-limiting enzyme of 5-FU catabolism, degrading 85% of the administered dose of 5-FU into inactive metabolites (10). Therefore, downregulation of TS and DPD expression and upregulation of OPRT expression enhance the anti-tumor effect of 5-FU (9-12). Hence, the pharmacogenetic variability of these enzymes might be a major determinant of the variations in outcome among cancer patients treated with 5-FU (9). The relative expression levels of the TS, DPD, and OPRT genes were reported as a predictive factor for the prognosis and survival of oral cancer patients treated with 5-FU (13,14).

DOC is also an effective agent against OSCC (15). We selected DOC as the combination agent in this study because of its overlapping antitumor spectrum including breast, oesophageal, gastric, and oral cancers (15,16). Additionally, DOC has a different mechanism of action from 5-FU and acts as a potent anti-mitotic agent by promoting abnormal microtubule stabilization, which results in inhibition of mitosis between metaphase and anaphase, and in the initiation of apoptosis (17).

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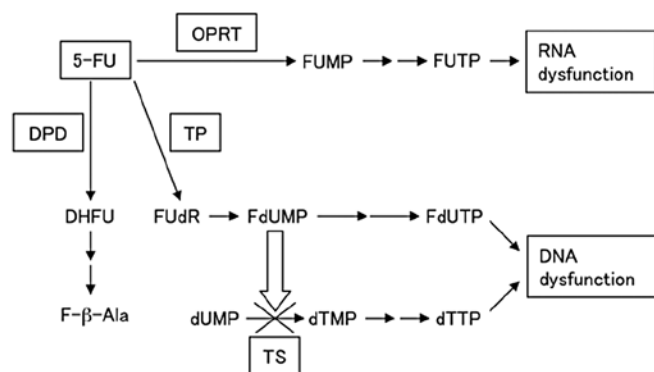E-mail: ttama@dent.tokushima-u.ac.jp

Figure 1. 5-FU, metabolic pathway of 5-fluorouracil; OPRT, ortate phosphoribosyl transferase; FUMP, 5-fluorouridine monophosphate; FUTP, 5-fluorouridine triphosphate; TP, thymidine phosphorylase; FUdR, 5-fluorodeoxyuridine; FdUMP, 5-fluorodeoxyuridine monophosphate; FdUTP, 5-fluorodeoxyuridine triphosphate; TS, thymidylate synthase; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; dTTP, deoxythymidine triphosphate; DPD, dihydropyrimidine dehydrogenase; DHFU, 5-fluoro-dihydrouracil; F-b-Ala, a-fluoro-b-alanine.

The combined treatment of 5-FU and DOC has been reported to improve response rates (14,15), however, little is known about sequential treatment with DOC and 5-FU.

The aim of this study was to evaluate the anti-tumor effects of sequential treatment with DOC and 5-FU against OSCC. Furthermore, to elucidate the mechanisms underlying the enhanced growth inhibitory effect of DOC followed by 5-FU, we examined the expression of the 5-FU metabolic enzymes TS, DPD and OPRT.

**Materials and methods**

*Cell lines and cell culture*. B88 cells were previously established from an OSCC patient in our laboratory (18). CAL27 cells were obtained from American Type Culture Collection (Rockville, MD, USA). Both cell lines produce tumors when subcutaneously inoculated into nude mice. The cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and 5% antibiotic-antimycotic solution in a humidified atmosphere containing 5% $CO_2$ at 37˚C.

*In vitro cell growth assay*. Cells ($3\times10^3$ cells per well) were seeded in 96-well plates. Twenty-four hours later, cells were treated with various concentrations of 5-FU or DOC for 24 h. Then, they were treated either with sequential treatment, 100 pg/ml DOC for 24 h followed by 4 µg/ml 5-FU for 24 h, 4 µg/ml 5-FU for 24 h followed by 100 pg/ml DOC for 24 h or with combined treatment, 4 µg/ml 5-FU and 100 pg/ml DOC at the same time for 48 h. A 10 µl aliquot of 5 mg/ml 3-(4,5-dimethylthiol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich) was added to each well and the cells were incubated for 4 h. The blue dye taken up by cells was dissolved in dimethyl sulfoxide, and the absorbance was measured with a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at 540 nm. All assays were run in triplicate.

*Flow cytometry*. Cells ($1\times10^6$) were cultured in 100-mm Petri dishes and treated with 4 µg/ml 5-FU or 100 pg/ml DOC alone, in combination or in sequence. The cells were collected and fixed with 70% ethyl alcohol and kept at -20˚C until analyzed. Then, they were treated with propidium iodide (40 µg/ml) and RNase A (1 µg/ml) at 37˚C for 30 min. Samples were kept on ice and the analysis of the sub G1 population was completed by measuring propidium iodide-stained DNA content with a Coulter® Epics® XL-MCL cytometer (Beckman Coulter, Brea, CA, USA).

*In vivo tumor growth assay*. The tumorigenic potential of cancer cells was assessed by inoculation of cells into 5- to 6-week-old female athymic BALB/c nude mice (Japan Clea Inc., Osaka, Japan). Cells ($5\times10^6$) were inoculated subcutaneously into the backs of mice, 5 mice per group. When tumors reached 50-100 mm$^3$ in volume, they were treated with sterile saline, 15 mg/kg 5-FU, and 10 mg/kg DOC by intraperitoneal (i.p.) injection. The treatment protocol of the six experimental groups of mice is shown in Fig. 2. Tumor volume and body weight of mice were measured 3 times a week. The tumor volumes were calculated by the formula: 0.5 x largest diameter x (smallest diameter)$^2$. The mice were maintained under pathogen-free conditions and handled in accordance with the Guidelines for Animal Experimentation of Tokushima University.

*RNA isolation and quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR)*. When cells reached subconfluence in culture, they were treated with 4 µg/ml 5-FU or 100 pg/ml DOC for 3, 6, 12, 24 or 48 h. Total-RNA was extracted by using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized from total-RNA using the Advantage cDNA PCR kit® (Invitrogen). For quantitative real-time PCR, equal aliquots of cDNA were amplified with TaqMan universal (50 µl) PCR master mix using the ABI prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The primer set and TaqMan probe used for the experiments were purchased from TaqMan gene expression assay systems (TS; Hs00426591_m1, DPD; Hs00559278_m1, and OPRT; Hs00165978_m1). Data were normalized using RT-PCR glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (Applied Biosystems).

*Western blot analysis*. After cells were treated with 4 µg/ml 5-FU or 100 pg/ml DOC alone, in combination or in sequence, they were collected and lysed. Mice treated with 15 mg/kg 5-FU or 10 mg/kg DOC alone or in sequence were sacrificed on the 21st day, then tumors were collected from the mice and proteins were isolated from the tumors. Whole cell lysate was subjected to electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated with rabbit polyclonal antibodies against TS, DPD and OPRT (Taiho Pharma, Tokyo, Japan). After rinsing membranes, the antibodies were detected using a chemiluminescent western blotting detection system (Amersham, Tokyo, Japan) according to the manufacturer's instructions.

*Statistical analysis*. Statistical analysis was performed by Mann-Whitney U test; values of $p < 0.05$ were considered statistically significant.
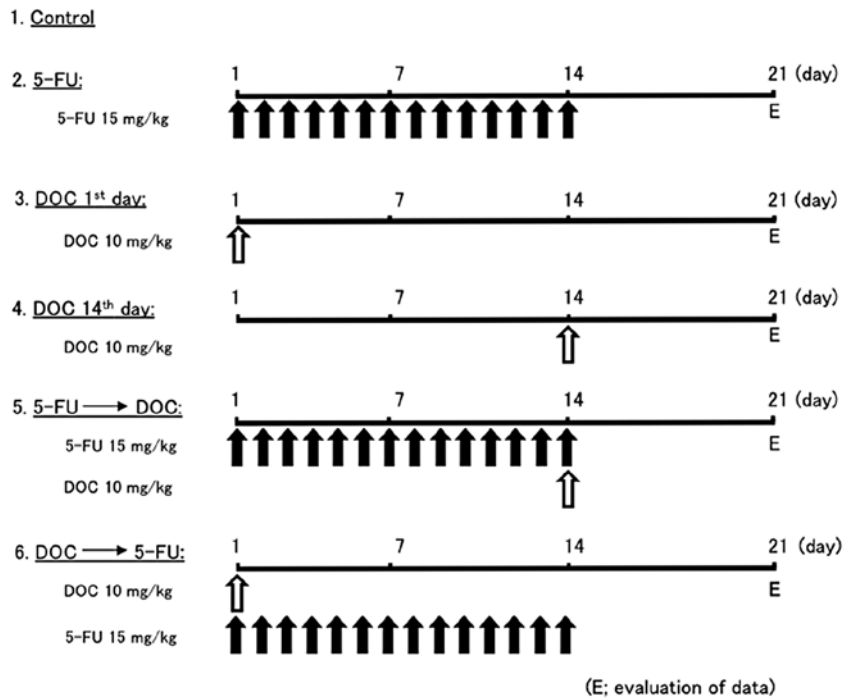
Figure 2. The experimental protocol for the *in vivo* tumor growth assay. The groups were as follows: 1) control group, treated with sterile saline for 2 weeks; 2) 5-FU group, treated with 5-FU by i.p. for 14 days; 3) DOC 1st day group, DOC injected on day 1 of the treatment cycle; 4) DOC 14th day group, DOC injected on day 14; 5) 5-FU-DOC group, 5-FU injected for 14 days and DOC injected on day 14; and 6) DOC-5-FU group; DOC injected on day 1 and 5-FU injected for 14 days.
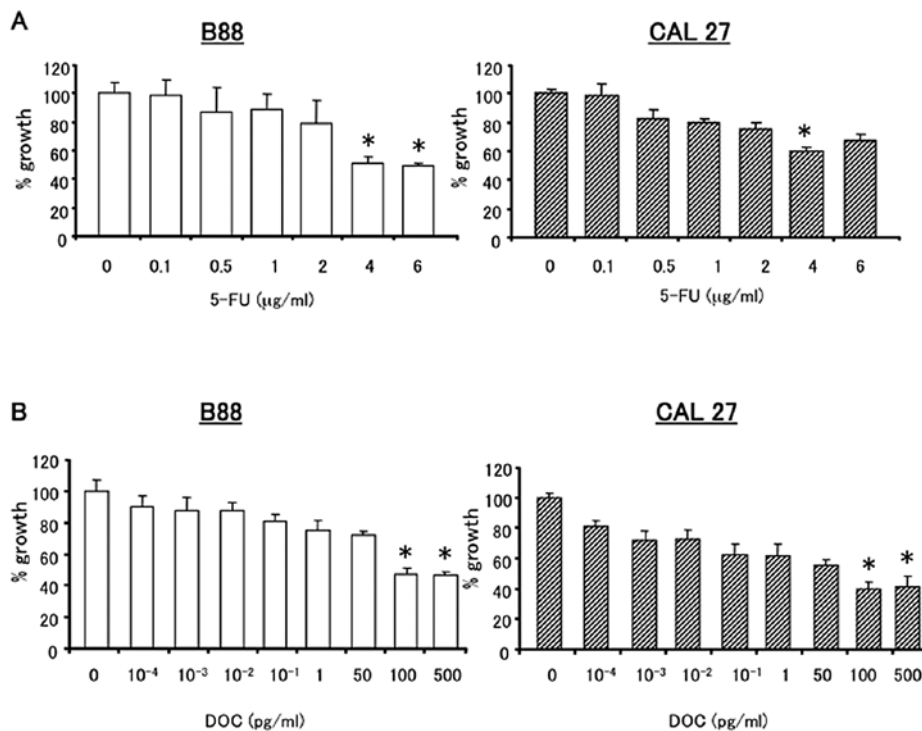


Figure 3. Growth inhibitory effects of 5-FU and DOC *in vitro*. Cells ($3 \times 10^3$ cells per well) were seeded on 96-well plates. Twenty-four hours later, the cells were treated with (A) 5-FU or (B) DOC for 24 h. *In vitro* cell growth was evaluated by the MTT assay and reported as percentage growth. *p<0.05.

## Results

*Growth inhibitory effects of sequential and combined treatment with 5-FU and DOC in oral cancer cells in vitro.* The growth inhibitory effects of 5-FU and DOC on B88 and CAL27 cells were analyzed by the MTT assay. Cells were treated with various concentrations of 5-FU or DOC alone for 24 h (Fig. 3) and 48 h (data not shown). 5-FU and DOC inhibited the growth of B88 and CAL27 cells in a dose-dependent manner. For sequential treatment, a concentration of 4 $\mu$g/ml
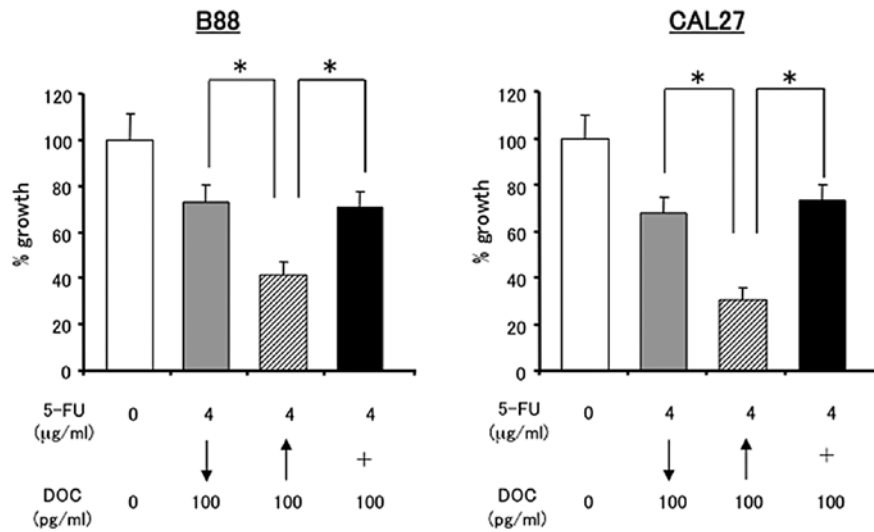
Figure 4. Growth suppression by sequential or combined treatment with 5-FU and DOC in B88 and CAL27 cells. The cells were untreated (for 48 h), or treated with 5-FU for 24 h followed by DOC for 24 h, with DOC for 24 h followed by 5-FU for 24 h, or with 5-FU and DOC for 48 h. $^*p<0.05$.
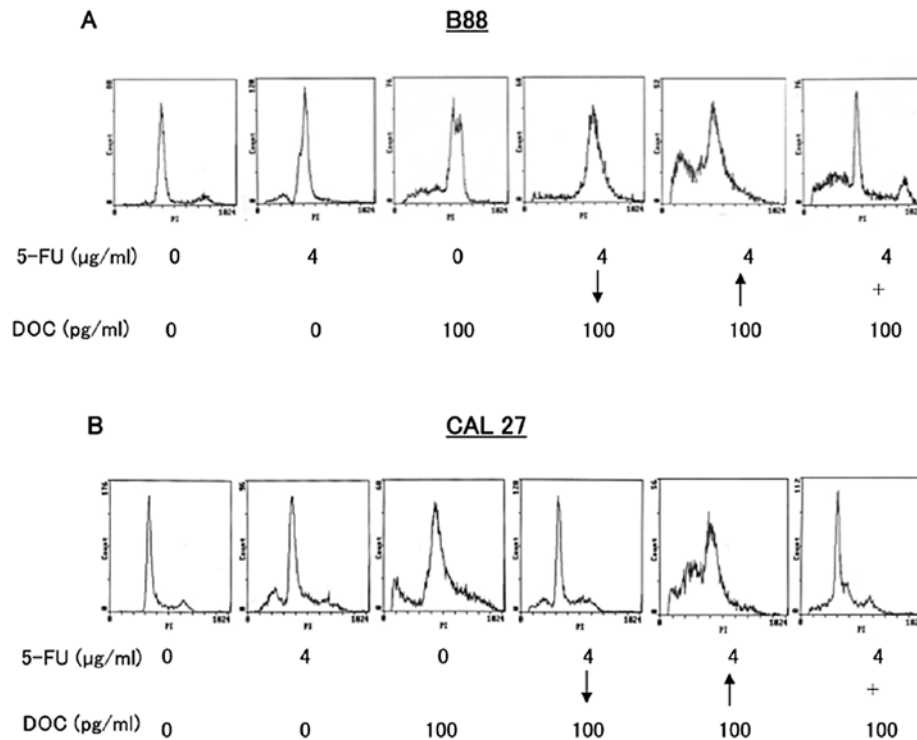


Figure 5. Flow cytometric analysis of treatment with 5-FU, DOC, in combination, or in sequence in (A) B88 and (B) CAL27 cells. The sub-G1 population of cell cycle shows apoptosis.

5-FU and 100 pg/ml DOC were selected. These concentrations showed a growth inhibitory rate of approximately 40-60% in both cancer cell lines (Fig. 3A and B). Thereafter, the effects of the sequential treatment with 5-FU and DOC were evaluated using following sequence. Cells were treated either with 5-FU (24 h) followed by DOC (24 h), with DOC followed by 5-FU, or with 5-FU and DOC at the same time (48 h). DOC followed by 5-FU sequential treatment was more effective in inhibiting cancer cell growth than 5-FU followed by DOC treatment or combined treatment (Fig. 4). To investigate whether this enhanced cytotoxicity of sequential treatment was due to

apoptosis, the sub G1 population of cancer cells was examined by flow cytometry (Fig. 5). B88 and CAL27 cells were treated with either 5-FU, DOC, or both of the drugs simultaneously or sequentially. The population of cells in the sub G1 phase was significantly increased in DOC followed by 5-FU sequential treatment than 5-FU followed by DOC or combined treatment in both cancer cells (Fig. 5A and B).

*Anti-tumor effects of sequential treatment with DOC and 5-FU on the human tumor xenografts in nude mice.* To investigate the efficacy of the DOC followed by 5-FU sequence
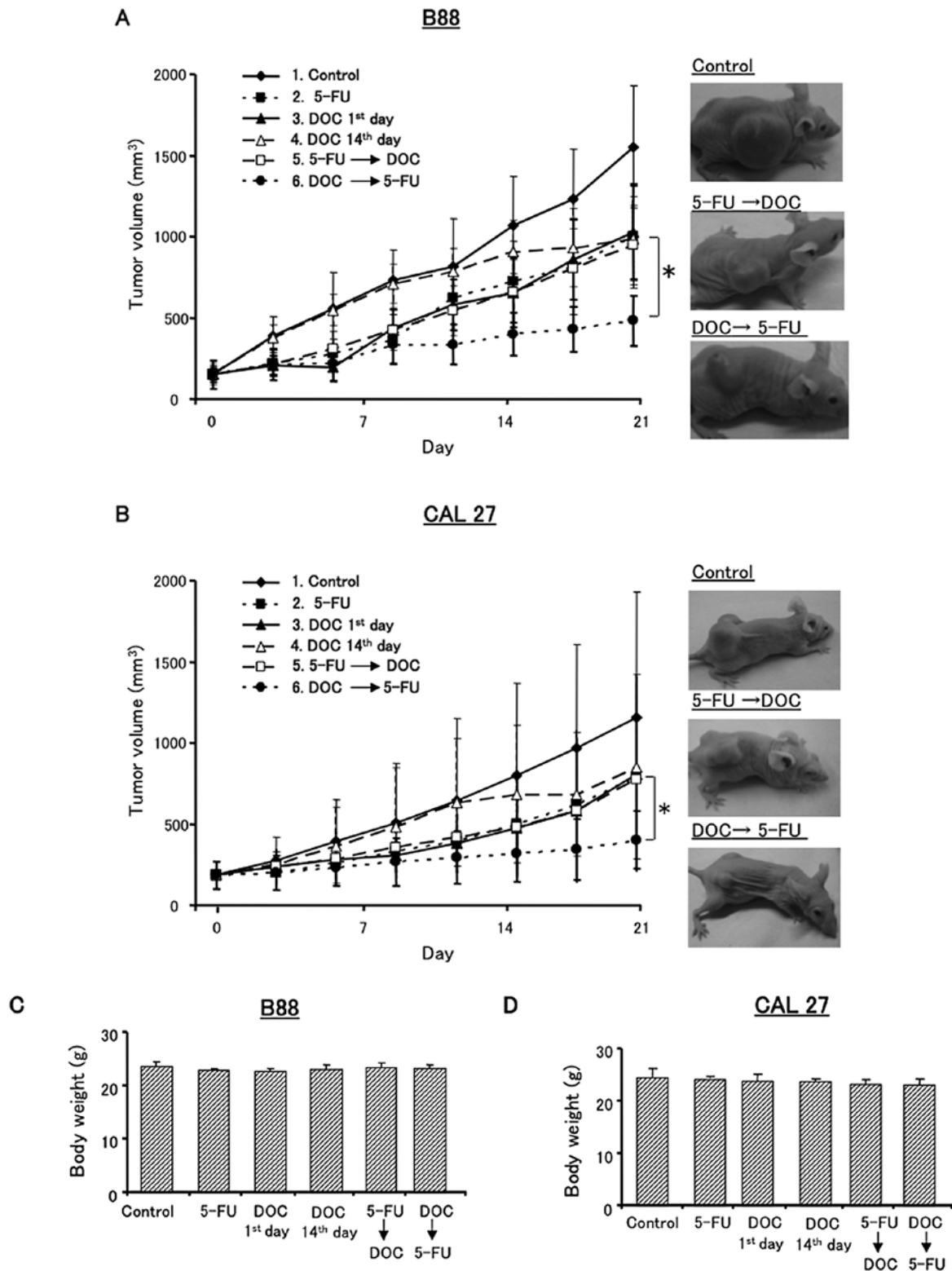
Figure 6. Anti-tumor effects of sequential treatment with 5-FU and DOC on tumor xenografts. B88 or CAL27 cells ($5 \times 10^6$) were inoculated subcutaneously into the back of mice. When tumors reached 50-100 mm³ in volume, they were treated according to the protocol shown in Fig. 2. Tumor volume and the body weight of mice were measured 3 times a week. The tumor volumes were calculated by the following formula: 0.5 x largest diameter x (smallest diameter)² in (A) B88 and (B) CAL27 xenografted mice. Right side shows the pictures of tumors in the untreated, 5-FU followed by DOC, and DOC followed by 5-FU groups. The body weight of (C) B88 and (D) CAL27 xenografted mice was measured on the 21st day. *p<0.05.

*in vivo*, experiments with B88 and CAL27 tumor xenografts were performed. The treatment plan is shown in Fig. 2. Control mice were injected with saline (group 1). The mice in groups 2-6 were injected with 15 mg/kg 5-FU alone, 10 mg/kg DOC alone, or sequential treatment with DOC and 5-FU. Fig. 6 shows the antitumor effects of the various treatment with DOC and/or 5-FU. In B88 tumor xenografts, DOC followed by 5-FU sequential treatment significantly reduced tumor growth
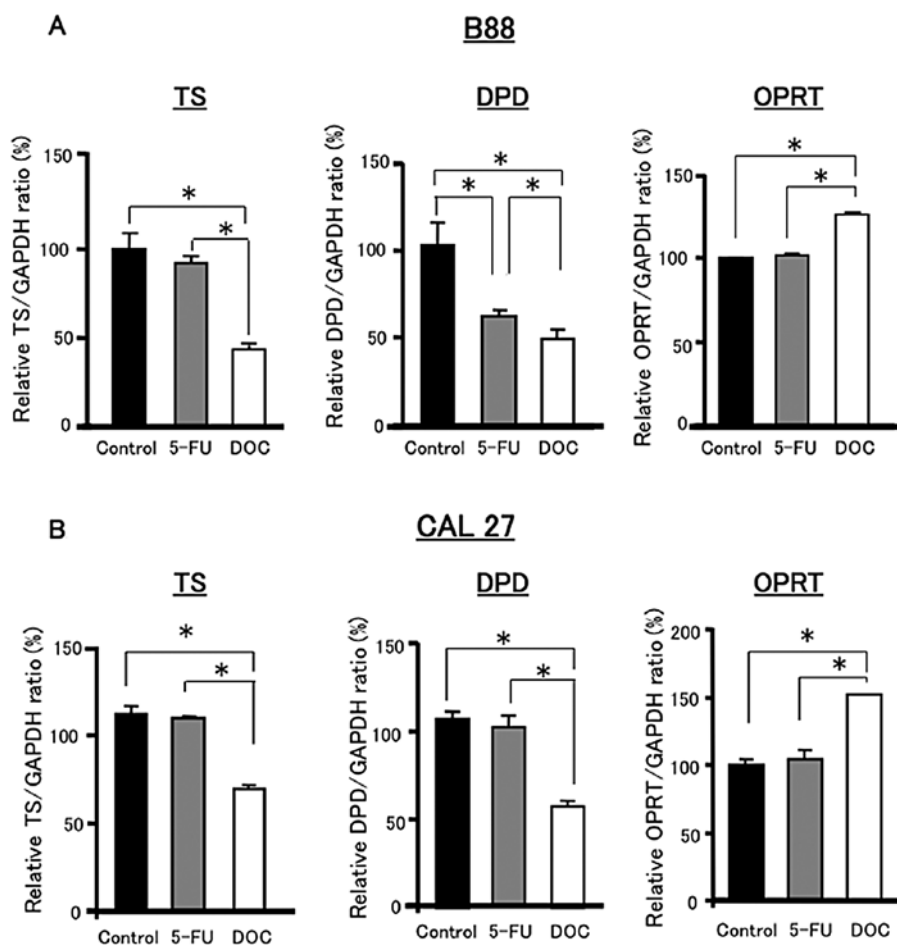
Figure 7. Effects of treatment with 5-FU or DOC on the mRNA expression of TS, DPD, and OPRT in (A) B88 and (B) CAL27 cells. The data are shown as the ratio of the GAPDH-normalized fold change in the PCR product in control cells. The results are representative of the mean of three independent data. *p<0.05.

compared to the control, 5-FU followed by DOC or other treatment groups (Fig. 6A). However, there was no significant difference between DOC 1st day (group 3) and DOC 14th day (group 4). The results for the CAL27 tumor xenografts were similar to those for the B88 tumors (Fig. 6B). In addition, drug toxicity did not cause carcass weight loss in any of the treated mice in these experiments (Fig. 6C and D). Altogether, these results showed that DOC followed by 5-FU was the most effective treatment sequence *in vivo*.

*Effects of 5-FU or DOC treatment on the expression of TS, DPD and OPRT.* To further identify the mechanisms underlying the enhanced growth inhibition by the sequential treatment, DOC followed by 5-FU, the expression levels of 5-FU metabolic enzymes, TS, DPD and OPRT were examined in cancer cells. These expression profiles were determined by real-time RT-PCR and western blot analysis, following treatment of cancer cells with 5-FU or DOC alone, in combination or in sequence.

Fig. 7 shows the mRNA expression levels of TS, DPD and OPRT in B88 and CAL27 cells after 12 h of treatment with 4 $\mu$g/ml 5-FU or 100 pg/ml DOC alone. There were no significant differences in TS expression between the control and 5-FU treatment. However, DOC treatment significantly decreased the expression of TS compared to the control and 5-FU treatment in B88 and CAL27 cells. The expression of DPD was also

reduced by DOC treatment compared to the control and 5-FU treatment in both cell lines. In contrast, DOC significantly increased the expression of OPRT compared to the control and 5-FU treatment in both cell lines.

To examine the expression of TS, DPD and OPRT at the protein level, western blot analysis was performed. As shown in Fig. 8A, the expression of TS, DPD and OPRT after 5-FU or DOC treatment in B88 and CAL27 cells was examined. The expression of TS and DPD was reduced by the treatment with DOC compared to the control and 5-FU treatment, whereas, the expression of OPRT was slightly increased by DOC treatment (Fig. 8A). Fig. 8B shows the expression of TS, DPD and OPRT in B88 and CAL27 cells after 24 h of 5-FU and DOC combined and sequential treatment. The expression of TS and DPD was downregulated by DOC followed by 5-FU compared to 5-FU followed by DOC or combined treatment. OPRT expression was also upregulated by DOC followed by 5-FU in both cell lines (Fig. 8B).

To investigate the expression of TS, DPD and OPRT *in vivo*, western blot analysis was performed with tumors extirpated from mice used in the xenograft experiment shown in Figs. 2 and 6. As shown in Fig. 9, DOC followed by 5-FU down-regulated the expression of TS and DPD and upregulated the expression of OPRT compared to 5-FU followed by DOC or 5-FU alone. These results show that downregulation of TS and
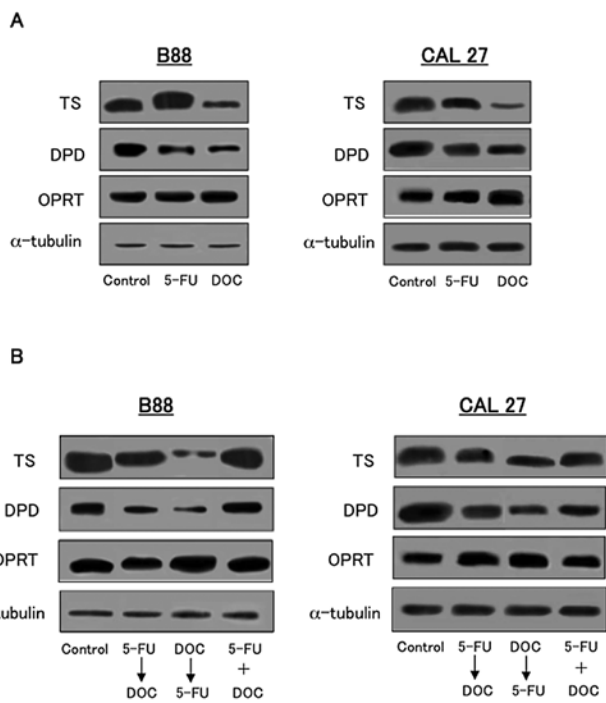
Figure 8. Effects of treatment with 5-FU and/or DOC on the protein expression of TS, DPD, and OPRT in B88 and CAL27 cells by western blot analysis. The cancer cells were treated with 5-FU or DOC alone, (A) in sequence or (B) in combination. Whole cell fractions were subjected to western blot analysis.
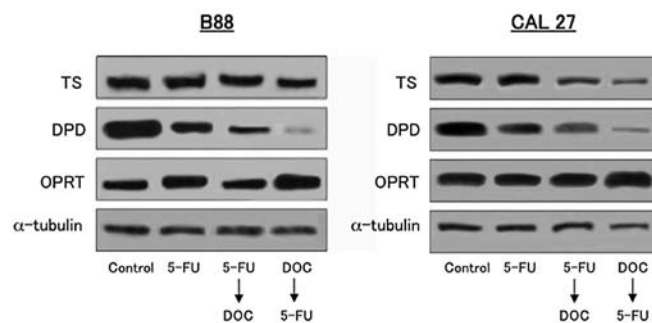


Figure 9. Effects of treatment with 5-FU and/or DOC on the protein expression of TS, DPD, and OPRT in mouse tumors. Tumors were collected from B88 and CAL27 xenografted mice on the 21st day. Proteins isolated from the tumors were subjected to western blot analysis.

DPD expression and upregulation of OPRT expression were induced by DOC treatment *in vitro* and *in vivo*.

## Discussion

In this study, the anti-tumor effects of sequential treatment with DOC and 5-FU against OSCC were investigated. It was clearly demonstrated that DOC followed by 5-FU treatment more effectively inhibited tumor growth *in vitro* and *in vivo* compared to 5-FU followed by DOC treatment. Furthermore, to elucidate the mechanisms underlying the enhanced growth inhibitory effect of DOC followed by 5-FU, the expression of the 5-FU metabolic enzymes TS, DPD, and OPRT was examined. Thus, DOC downregulated the expression of TS and DPD and upregulated OPRT expression in cancer cells, and these alterations of 5-FU

metabolic enzyme expression could enhance anti-tumor effects of 5-FU in DOC followed by 5-FU treatment.

5-FU metabolic enzymes regulate the anti-tumor efficacy of 5-FU (9-11). High expression of TS and DPD in tumors has been associated with its resistance to 5-FU (19-21). TS, of these enzymes, is the most important regulator of the sensitivity of cancer cells to 5-FU. TS plays important roles in cellular proliferation and growth, catalyzing the methylation of FdUMP to deoxythymidine monophosphate (dTMP), an essential precursor for DNA synthesis (22). Therefore, TS inhibiting drugs could augment the efficacy of 5-FU. The present study demonstrated that the expression of TS protein and mRNA was decreased by DOC, however, the expression of TS protein was enhanced by 5-FU in B88 cells. The precise mechanisms responsible for the induction of TS expression by 5-FU and downregulation of TS expression by DOC are not fully understood (19). It was reported that the transcriptional activator E2F1, a cell cycle regulatory protein forming complexes with Rb, encodes a representative transcriptional enzyme that transcribes the messages of TS (23). In addition, several studies using cDNA microarray demonstrated that the expression of E2F1 and Rb was decreased by DOC in head and neck squamous cell carcinoma (HNSCC) (24,25). Therefore, DOC could lead to suppression of TS expression via inhibition of E2F1/Rb expression. On the other hand, DPD expression was also downregulated by DOC. However, the mechanisms behind this downregulation of DPD have not been fully analyzed. Recently, Ukon *et al* (26) reported that activation of protein (AP)-1 accelerated DPD gene transcription in gastric cancer cells. In addition, Yoo *et al* (24,25) reported that DOC downregulated the expression of c-Jun N-terminal kinase (JNK) and phosphorylated JNK in HNSCC cells. Therefore, DPD expression could be downregulated by DOC via inhibition of the JNK-AP-1 pathway.

It was reported that combined treatment with DOC and 5-FU had synergistic inhibitory effects on the growth of breast and gastric cancer cells (16), however, sequential treatment with DOC and 5-FU was not examined. In the present study, the effects of administration sequence on drug efficacy with DOC and 5-FU were evaluated. In *in vivo* study, it is clearly demonstrated that DOC followed by 5-FU treatment more effectively inhibited tumor growth compared to 5-FU followed by DOC. But, the possibility was considered that this result was affected by the different timing of the DOC injection into the mice in DOC followed by 5-FU and 5-FU followed by DOC treatment. Thus, DOC was injected on day 1 in DOC followed by 5-FU treatment, whereas on day 14 in 5-FU followed by DOC treatment. It means that the enhanced anti-tumor effect of DOC followed by 5-FU may be caused by the difference in duration of DOC action. However, there was no significant difference in tumor growth rates between DOC 1st and DOC 14th groups on the evaluated day. Therefore, this result suggested that timing of the DOC injection did not appear to affect the anti-tumor effect of those two sequential treatments. Thus, the enhanced efficacy of DOC followed by 5-FU could be caused by the effect of DOC, which directly regulated 5-FU metabolic enzymes.

The mechanisms underlying the enhanced growth inhibitory effect of DOC followed by 5-FU, compared to 5-FU followed by DOC could be explained by considering two possibilities. One is that DOC affects the expression of 5-FU metabolic enzymes

or 5-FU regulated genes. The other is that 5-FU provides the effects on DOC regulated genes. Yoo *et al* (25) reported that DOC induced the expression of the cell cycle regulator proteins p19 and cyclin-dependent kinase 2, but reduced the expression of cyclin A, B, C, D2 and D3, E2F1 and bcl-2. Among these genes, the overexpression of bcl-2 is correlated with upregulation of TS expression and resistance of colorectal cancer cells to DOC (27). On the other hand, resistance to DOC also appears to be caused by the high expression of P-glycoproteins, thioredoxin, and ribophorin 2 (RPN2) (19) and by the low expression of p27 (19,28). However, effects of 5-FU on the expression of DOC resistance related genes, RPN2, P-glycoprotein, bcl-2, and DOC induced genes were not examined in this study. Studies on effects of 5-FU on genes related to DOC resistance will be important to understand the mechanisms of DOC and 5-FU sequential treatment.

A more effective chemotherapy based on 5-FU and DOC may be developed by using various modulators for metabolic enzymes of 5-FU and resistance related genes of DOC. Several pathways could be considered, including the phosphatidylinositol 3-kinases (PI3K)-Akt mammalian target of rapamycin (mTOR) pathway, which is related with various types of malignancies (28). It has been reported that activation of the PI3K-Akt-mTOR pathway induces TS expression and could be responsible for the incomplete response of cancer for DOC and 5-FU (28,29). Moreover, Shigematsu *et al* (28) reported that an mTOR inhibitor, rapamycin, downregulated the expression of TS and showed enhanced anti-tumor effects in combination with DOC and 5-FU in gastric cancer. The TS inhibitors TOM and Thymitaq, and the DPD inhibitor eniluracil, have been used in combination with 5-FU for their enhanced anti-tumor effects against various types of cancers (29-31). Insufficient inhibition of TS and DPD could be the cause of poor outcomes of 5-FU and DOC based treatments, therefore, novel combinations of TS or DPD inhibitors with 5-FU and DOC could provide important new opportunities for improving the clinical outcome for oral cancer patients. Moreover, the overexpression of bcl-2 and constitutive activation of NF-κB have been reported to cause the resistance to 5-FU and DOC in cancer cells (32-34). Therefore, understanding the effects of these modulators on the efficacy of 5-FU and DOC treatment, and inhibition of the signaling pathways related to bcl-2 or NF-κB would facilitate the development of new therapeutic strategies based on 5-FU and DOC.

In conclusion, this study clearly showed that sequential treatment with DOC followed by 5-FU more effectively inhibited the tumor growth of oral cancer cells. The mechanisms underlying the growth inhibitory effect of DOC followed by 5-FU sequential treatment could be downregulation of TS and DPD expression, and upregulation of OPRT expression induced by DOC treatment. Thereby, anti-tumor effect of 5-FU could be enhanced in DOC followed by 5-FU treatment. These findings demonstrated that sequential treatment with DOC followed by 5-FU can be more effective for the patients with OSCC than that with 5-FU followed by DOC.

## Acknowledgements

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