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Potentiation of Anticancer Activity of G_2/M Blockers by Mild Hyperthermia

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Abstract. *Background/Aim: Hyperthermia (HT), combined with chemotherapy, has been used to treat various types of cancer. This study aimed to investigate the HT-sensitivity of malignant and non-malignant cells, and then evaluate the combination effect of docetaxel (DTX) and a newly synthesized chromone derivative (compound A) with HT. Materials and Methods: The number of viable cells was determined using the MTT method. Cell cycle distribution was analyzed using a cell sorter, and DNA fragmentation pattern was detected using agarose gel electrophoresis. Results: Among 12 cultured cells, oral squamous cell carcinoma (OSCC), especially Ca9-22 cells, and myelogenous leukemia cells showed higher sensitivity to HT than lung carcinoma and glioblastoma cell lines, while normal oral cells were the most resistant. Cytotoxicity of DTX on Ca9-22 cells was maximum at 41-42˚C and 45~60 min exposure to HT. DXT, compound A, and HT induced G_2/M arrest of Ca-22 cells. Mild HT enhanced the DTX- and compound A-induced subG_1 arrest, in a synergistic fashion. Conclusion: The combination G_2/M blockers and mild-HT can potentially be used for the treatment of OSCC.*

Surgery, radiation, and chemotherapy are three standard treatments for oral cancer. Considering the postoperative impairment of physical function and appearance, minimally invasive therapies such as chemotherapy combined with hyperthermia (HT), which induces cancer cell death by heating to 42.5˚C or higher (1, 2), are promising. Treatment of malignant tumors with HT in Japan has long track record since the approval of insurance coverage in 1990. While chronic myelogenous leukemia cells have been reported to be highly sensitive to HT (3, 4), clinical reports of HT on oral cancer have been limited. For HT, cancerous tissue is placed between two electrodes, and then is exposed to Joule heat generated using the radiofrequency dielectric warming method. This method is applicable to breast cancer that can be easily clamped between the electrodes, but not to primary tumor such as oral cancer (especially tongue cancer, which moves involuntarily). Therefore, HT for head and neck cancer, including oral cancer, is generally applied to cervical lymph node metastasis rather than the primary tumor (5). We have reported favorable clinical data of superselective intra-arterial chemoradiotherapy (based of cisplatin, docetaxel (DTX) and radiation) for patients with oral squamous cell carcinoma with lymph node metastasis (6). In addition to the direct application to tumor, combination of relatively lower temperature for HT (41-42˚C) [mild hyperthermia (mild-HT)] have been reported (7, 8).

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Key Words: Oral squamous cell carcinoma, mild hyperthermia, chromone derivatives, G_2/M blocker, synergism.

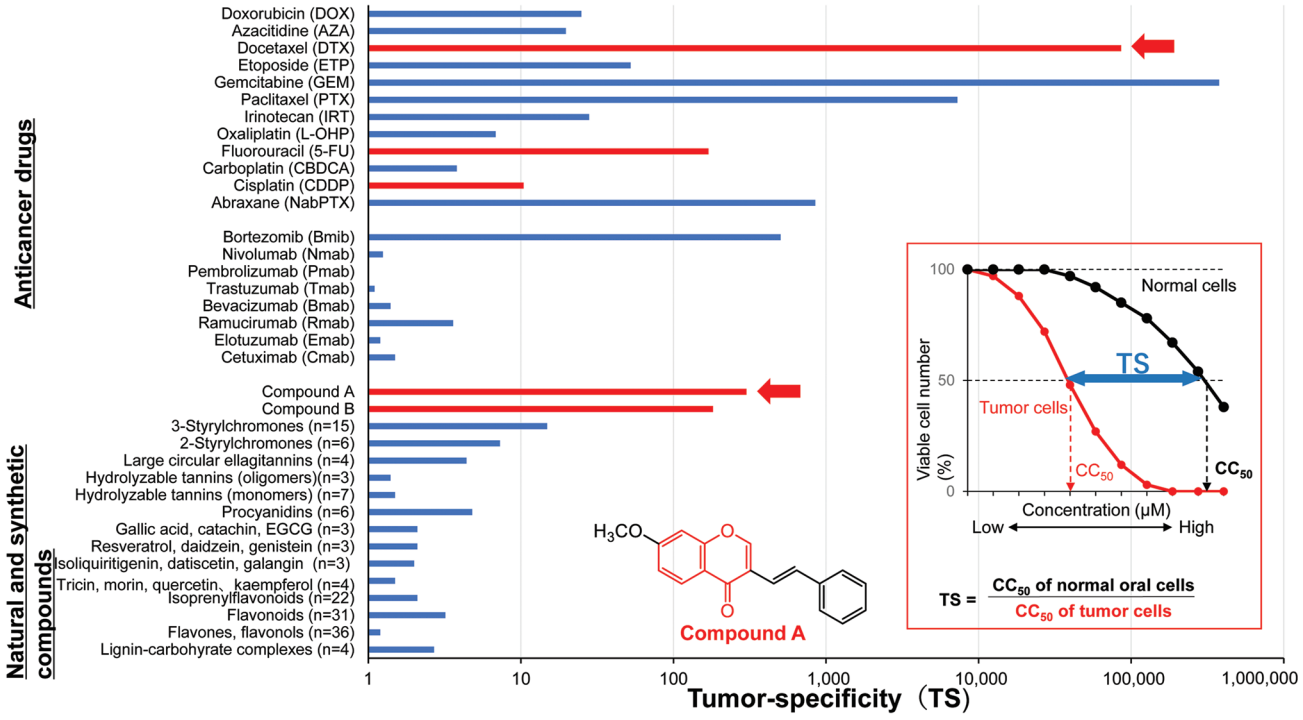

Figure 1. *Tumor-specificity of anti-cancer drugs and natural and synthetic compounds against human oral squamous cell carcinoma (OSCC) cell lines. Data have already been reported (9-13).*

In vitro experiments using human cancer cell lines and human normal cells are useful to discover novel anticancer agents. We have comprehensively measured the tumor-selectivity of natural products and synthetic organic compounds against human oral squamous cell carcinoma cells (OSCC) (9, 10). As a result, DTX (11), one of three representative chemotherapeutics for treating oral cancer, and 7-methoxy-3-[(1*E*)-2-phenylethenyl]-4*H*-1-benzopyran-4-one (compound A) (12, 13), a 3-styrylchromone derivative, showed the greatest tumor-specificity *in vitro* (Figure 1). However, few studies have investigated the combination effects of DTX or compound A with HT in cultured cells. In the present study, the relative sensitivity of various human malignant and non-malignant cells to HT was first investigated. Then, Ca9-22 cells, the most sensitive OSCC cell line, was used to establish the optimal condition of HT treatment. Finally, the combined effects of DTX or compound A and HT were investigated.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM), RPMI1640 medium and paraformaldehyde were purchased from Fujifilm Wako Pure Chemical Co. (Osaka, Japan). DTX was purchased from Toronto Research Chemicals (Toronto, Canada). Fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and ribonuclease (RNase) A were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Nonidet-40 (NP-40) was purchased from Nacalai Tesque (Kyoto, Japan). 7-Methoxy-3-[(1*E*)-2-phenylethenyl]-4*H*-1-benzopyran-4-one (compound A) was synthesized by Knoevenagel condensation of 7-methoxy-3-formylchromone with phenylmalonic acid, as described previously (13).

Cell culture dishes (100 mm) were purchased from True Line (Nippon Genetics Co., Ltd., Tokyo, Japan) whereas 96-microwell plates were purchased from TPP (Techno Plastic Products AG, Trasadingen, Switzerland).

Cell culture. Human normal oral cells (human gingival fibroblasts, HGF; human periodontal ligament fibroblast, HPLF) [established from the first premolar extracted tooth in the lower jaw and periodontal tissues of a twelve-year-old girl, according to the guidelines of Meikai University Ethic Committee (No.A0808), and used in this study at 12~20 population doubling level (PDL)] (14), human oral squamous cell carcinoma (OSCC) cell lines (Ca9-22, HSC-2, HSC-3, HSC-4), human lung cancer cell lines (A549, WA-hT, A904L), human glioblastoma (T98G) (15) (Riken Cell Bank, Tsukuba, Japan) were cultured at 37˚C in DMEM supplemented with 10% heat (56˚C, 30 min)-inactivated FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate in a humidified 5% CO_2 incubator (MCO-170 AICUVD-P, Panasonic Healthcare Co., Ltd., Gunma, Japan). Human myelogenous leukemia cell lines [promyelocytic leukemia (HL-60) (16), myeloblastic leukemia ML-1 (17)] (Riken Cell Bank) were cultured in RPMI1640 medium

supplemented with 10% heat-inactivated FBS and antibiotics as described above. Cell morphology was examined periodically under an optical microscope (EVOS FL; Thermo Fisher Scientific, Waltham, MA, USA).

Determination of viable cell number. Cells cultured in a 100-mm dish were detached with 0.25% trypsin solution (containing 1 mM EDTA), and 0.1 ml of cell suspension (2×10^4/ml) was inoculated in triplicate into 96-microwell plates. Cells were cultured at 37˚C in 5% CO_2 incubator for 48 h to achieve complete attachment to the plate. After attachment, cells were cultured for 48 h with fresh medium containing various concentrations of samples, and then viable cell numbers were measured using the MTT method (13). From the dose-response curve, 50% cytotoxic concentration (CC_{50}) was determined. All drugs were dissolved in DMSO. The toxicity of DMSO alone was subtracted.

Calculation of stimulation index of cytotoxicity (SI). Cells were incubated for 45~60 min at 41, 42 or 43˚C in fresh culture medium containing various concentrations of samples in a CO_2 incubator E-22 (AS ONE, Osaka, Japan). After replacing with fresh culture medium for 48 h at 37˚C, the CC_{50} value was calculated, and the stimulation index of cytotoxicity (SI) was calculated using the following formula. SI=CC_{50} (37˚C)/CC_{50} (41-43˚C)

Cell cycle analysis. Treated and untreated cells grown in 100-mm culture dishes (approximately 10^6 cells) were harvested, fixed for 1 h with paraformaldehyde (1%), treated for 30 min with RNase A (preheated to inactivate DNase) (0.2 mg/ml), stained with propidium iodide (0.01%) in the presence of 0.01% NP-40 in PBS (–) to prevent the cell aggregation, filtered through the cell strainers (Corning Inc., Corning, NY, USA), subjected to cell sorting (SH800 Series; SONY, Tokyo, Japan), and then analyzed with Cell Sorter Software version 2.1.5. (SONY), as described previously (13). Fragmentation of DNA in the subG_1 population was detected with ethidium bromide using agarose gel electrophoresis after DNA extraction (15, 16).

Calculation of Combination Index (CI value). CI is defined by the following equation: CI=(A+B)/C (18), where A represents the % increase in subG_1 fraction of cells treated with mild-HT alone. B the % increase in subG_1 fraction of cells treated with drug alone And C the % increase in subG_1 fraction of cells treated with both mild-HT and drug. When CI <1, CI=1, and CI>1, the combination action was judged as synergic, additive, and antagonistic, respectively.

Statistical analysis. Experimental data are presented as the mean±standard deviation (SD) of triplicate determinations. The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* test for multiple comparisons (SPSS version 27.0). Student *t*-test (paired) was performed for statistical analysis of differences between two groups (exposed to 37 or 41˚C). A value of $p<0.05$ was considered to indicate statistically significant differences.

Results

OSCC cell lines showed the highest HT sensitivity. Oral squamous cell carcinoma (OSCC) (Ca9-22, HSC-2, HSC-3, HSC-4), especially Ca9-22 cells, and myelogenous leukemia cells (HL-60, ML-1) showed higher sensitive to HT than lung carcinoma (A549, A904-L, WA-hT) and glioblastoma (T98G) cell lines, whereas normal human mesenchymal oral cells (HGF and HPLF) were the least sensitive. This trend was more apparent after 48 h rather than 24 h incubation ($p<0.05$) (Figure 2).

Optimal condition of HT treatment. Next, the optimal temperature and treatment time with HT were investigated. Treatment at 41˚C for 45 to 60 min most significantly enhanced the cytotoxicity of DTX (Figure 3A). Thermal treatment at 41˚C and 42˚C showed similar patterns. Thermal treatment at 43˚C caused strong cytotoxicity, and the additive effect with HT could not be clearly observed (Figure 3A). The cytotoxicity-enhancement effect of HT (1 h) was quantified using the SI value (Figure 3B). When the HT temperature was elevated from 37˚C to 41, 42 and 43˚C the SI value was increased by 2.9, 2.4, and 1.1-fold, respectively (Figure 3B). This demonstrated that the optimal treatment time and temperature were 45 to 60 min, and 41˚C, respectively.

Accumulation of cells in the G_2/M phase following treatment with mild HT, DTX and compound A. Morphological observation under an optical microscope demonstrated cell spreading (enlargement) 6 h after HT treatment, before the detection of cytotoxicity at 24 h (Figure 4A). The positive control, 1 mM actinomycin D, induced cell shrinkage, characteristic of apoptosis, at 41˚C and 42˚C after 24 h (Figure 4A).

Cell cycle distribution after HT treatment was monitored using a cell sorter (Figure 4B and C). Exposure to 41˚C induced significant accumulation of cells to the G_2/M and subG_1 phases only after 24 h. The positive control, 1 mM actinomycin D, also showed an increase in the subG_1 populations (composed of DNA fragments produced by activated DNases) (Figure 4B and C). Exposure to 42˚C induced significant accumulation of cells to the G_2/M phase already after 6 h, followed by higher number of cells in the subG_1 phase after 24 h. The transition from G_2/M to the subG_1 phase was more pronounced at 42˚C than at 41˚C at 24 h (Figure 4C).

DTX (A) and compound A (B) also induced the accumulation of cells to G_2/M and subG_1 phases (Figure 5). DTX induced the accumulation of nearly 30% of total DNA in the subG_1 (Figure 5A), while the remaining 70% of the DNA was distributed into G_1, S, and G_2/M phases of the cell cycle. Since DTX significantly ($p<0.05$%) reduced the number of cells in the G_1 and S phases, the number of cells in the G_2/M phase should be larger than that in the G_1 and S phases, confirming the increase in the relative distribution into G_2/M phase cell by DTX.

Synergistic enhancement of the subG_1 population by DTX or compound A with HT. Finally, whether mild-HT further enhances the accumulation of cells to subG_1 phase by DTX

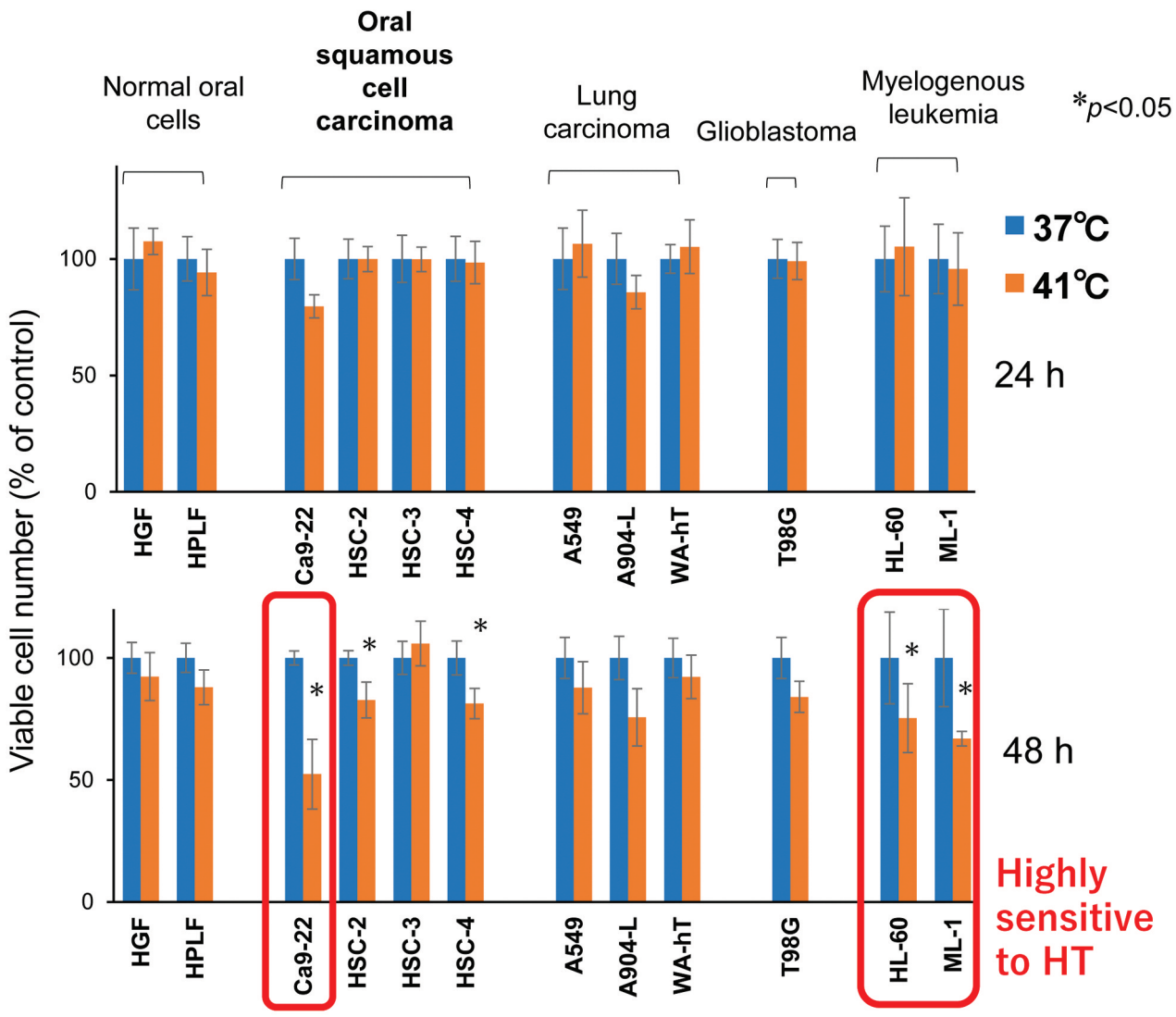

Figure 2. *Heat-sensitivity of malignant and non-malignant cell lines. Near confluent cells were incubated for 24 or 48 h at 37 or 41˚C, and then viable cell numbers were determined using the MTT method. Each value represents mean±S.D. of triplicate determinations, and is expressed as % of that determined at 37˚C. Significant difference (*p<0.05) between 37˚C and 41˚C was detected using student t-test.*

or compound A was examined using the combination index (CI). Ca9-22 cells were incubated at 37 or 41˚C for 1 h without or with 200 nM DTX (A) or 20 μM compound A (B). Cells were then incubated for 23 h in drug-free medium at 37˚C and subjected to cell cycle analysis. Elevation of the temperature from 37 to 41˚C synergistically increased the accumulation of cells in the subG$_1$ phase (CI=0.61 and 0.63, respectively) (Figure 6).

To achieve the elevation of intracellular temperature more rapidly, a heat block was used instead of an HT-incubator. Ca9-22 cells detached using trypsin were treated with drugs at 37˚C or 41˚C for 60 min. After replacement with fresh medium, cells were cultured at 37˚C for 72 h to ensure the cell death and subjected to cell sorter. Treatment with HT (41˚C) together with DTX or compound A produced synergistic accumulation of cells in the subG$_1$ phase (CI=0.55, 0.63) (Figure 7). These two experiments (Figure 6 and Figure 7) demonstrated that mild HT and DTX or compound A, all G$_2$/M blockers, synergistically increased the accumulation of cells in the subG$_1$ phase (Figure 7).

Discussion

The present study revealed that OSCC (Ca9-22, HSC-2, HSC-4) and human myelogenous leukemia (HL-60, ML-1) cells were more sensitive to mild HT (41˚C) than lung cancer

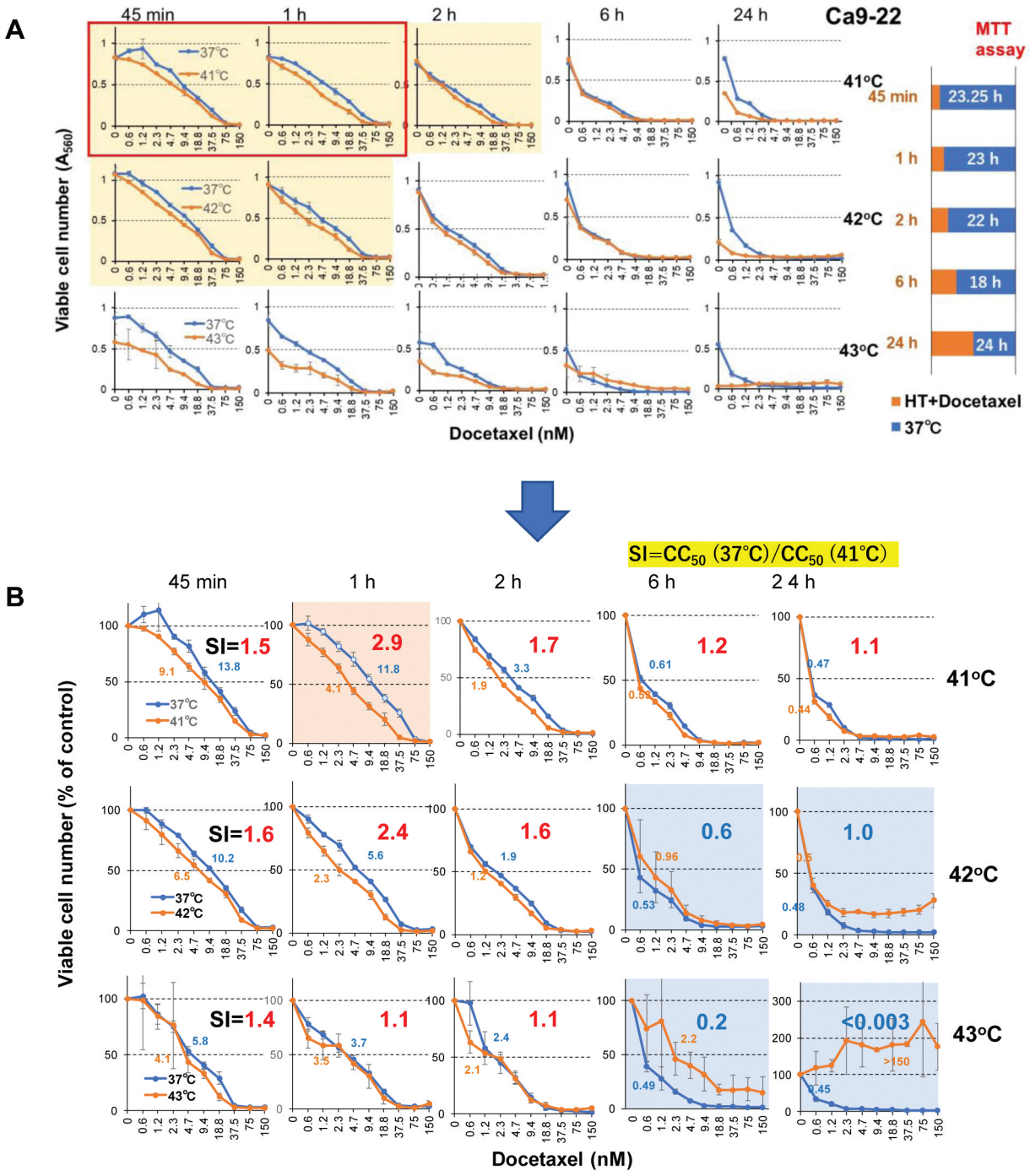

Figure 3. *Determination of optimal temperature and exposure time of HT. Ca9-22 cells were treated for 45 min, 1, 2, 6, or 24 h at 37 or 41˚C in the presence of the indicated concentrations of docetaxel, and then incubated for 23.3, 23, 22, 18 or 24 h in fresh DMEM medium. The viable cell numbers were determined using the MTT method and expressed as absorbance at 560 nm (A) or % of control (37˚C) (B). Each value represents mean±S.D. of triplicate determinations, and is expressed as % of that determined at 37˚C. Significant difference (*p<0.05) between 37˚C and 41˚C.*

(A549, A904L, WA-hT) and glioblastoma (T98G) cells, while normal mesenchymal oral cells (HGF and HPLF) were the least sensitive (Figure 2). Recently, we have reported that OSCC (Ca9-22, HSC-2, HSC-4) cells were also the most sensitive to UVC, followed by glioblastoma (T98G) and lung carcinoma (A549, WA-hT, A904L, LC-1/sq), whereas normal

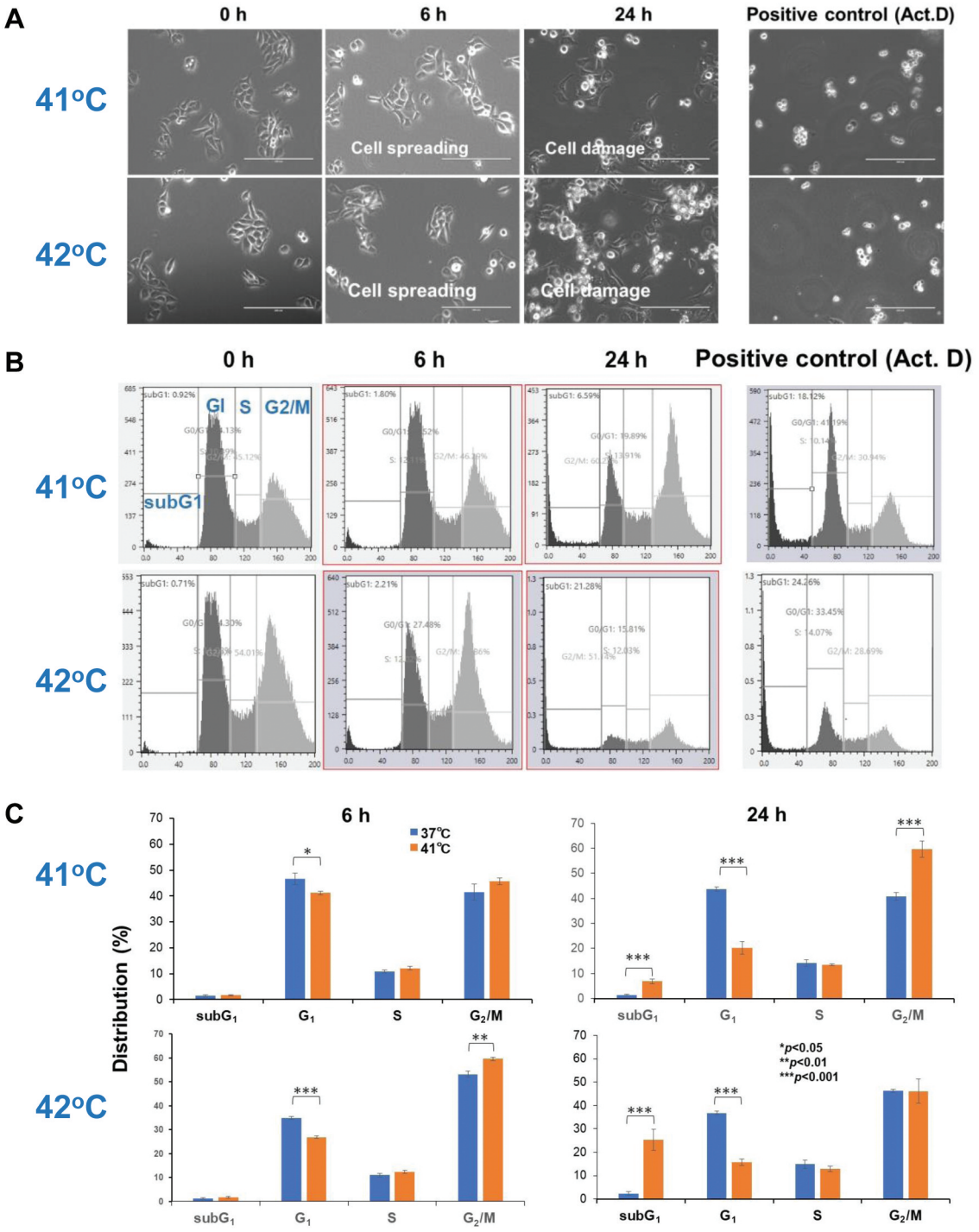

Figure 4. *Induction of G_2/M cell accumulation by mild HT. Ca9-22 cells were incubated for 0, 6, 24 h at 41 or 42˚C, and then investigated for morphological change (A) and cell cycle distribution (B, C). Each value represents mean±S.D. of triplicate determinations. *p<0.05, **p<0.01, ***p<0.001 (Student t-test, paired).*

oral cells again were the most resistant to UVC (19). These results suggest that OSCC may be vulnerable to external stresses such as HT and UVC.

Human normal epithelial cells are ideal for comparison of their sensitivity to mild HT with that of OSCC. However, most of anticancer drugs (DTX, CPT, SN-38, DOX, DNR,

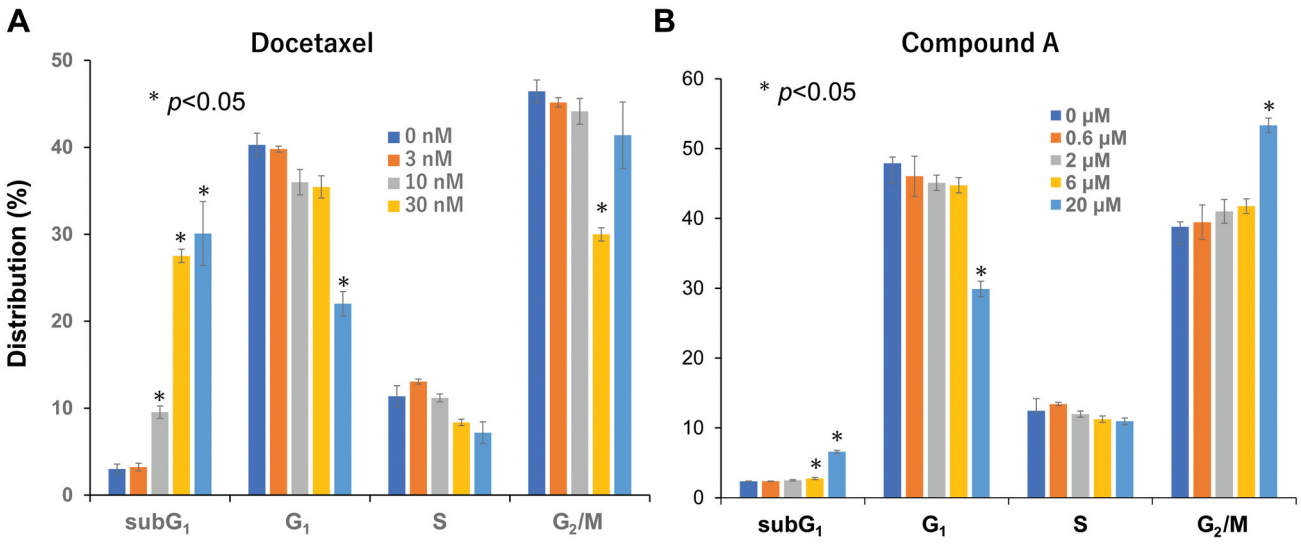

Figure 5. *Induction of accumulation of cells to G_2/M and subG$_1$ by docetaxel (A) and compound A (B). Cells were incubated for 24 h with the indicated concentrations of docetaxel or compound A. Each value represents mean±S.D. of triplicate determinations. *p<0.05 vs. control (Bonferroni's post-hoc test).*

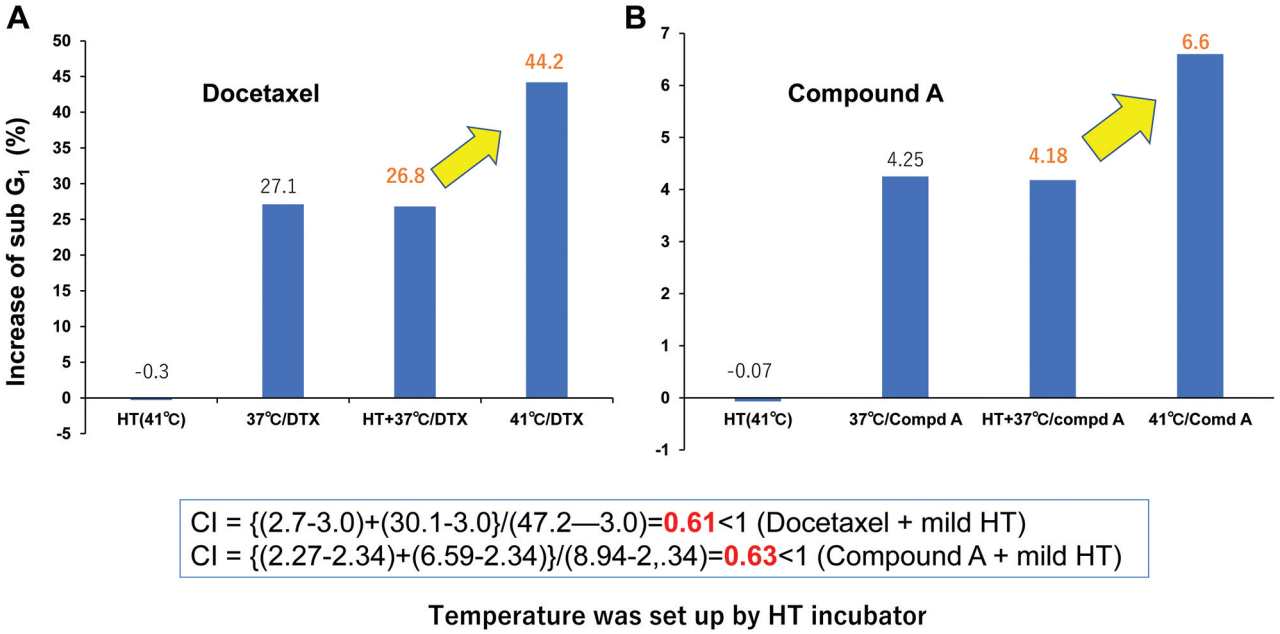

Figure 6. *Synergistic action of mild HT on DTX- and compound A- induced cytotoxicity against Ca9-22 cells (Temperature was set up using an HT incubator). Cells were incubated at 37 or 41˚C for 1 h without or with 200 nM docetaxel or 20 μM compound A, and then incubated for 47 h in drug-free medium at 37˚C. Non-adherent cells and adherent cells (harvested using with trypsinization) were combined, and then subjected to cell cycle analysis. CI index was then determined.*

ETP, MMC, 5-FU, DOC, Melphalan, Gefitinib) killed both OSCC and normal epithelial cells [oral keratinocyte (HOK), human gingival epithelium progenitor (HGEP)] to comparable extent (20). Therefore, normal human mesenchymal cells rather than normal epithelial cells were used in the present study for the comparison of sensitivity against HT.

The present study demonstrated that treatment at 41˚C for 40 to 60 min most significantly enhanced the cytotoxic activity of DTX against Ca9-22 cells. This treatment

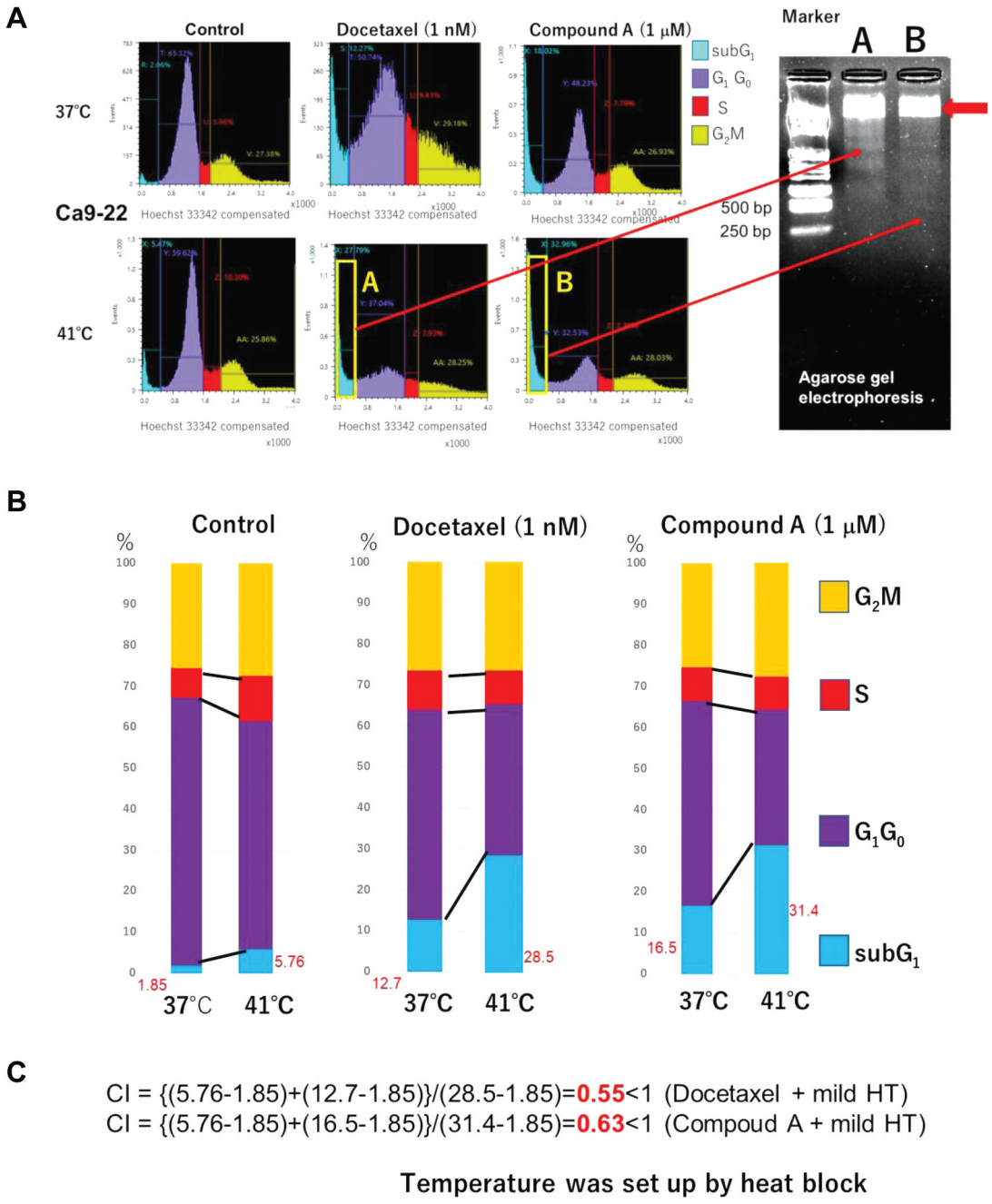

Figure 7. *Synergistic action of mild HT on DTX- and compound A-induced cytotoxicity against Ca9-22 cells (Temperature was set up using heat block). Cells (harvested by trypsinization) were incubated at 37 or 41˚C for 1 h without or with 1 nM docetaxel or 1 μM compound A, and incubated for 71 h. Cells were then harvested as described in Figure 6 and then subjected to cell cycle analysis in triplicate. The CI was then determined.*

condition is similar to that of mild HT used in clinical cases. The temperature range from 40.5˚C to 42.5˚C can increase the tissue blood flow and lead to a substantial thermal enhancement of the cytotoxic chemotherapeutic agents (21). This mild HT exposure synergistically enhanced Ca9-22 cell death induced by DTX or compound A (as determined by the percentage of cells in the $subG_1$ phase (Figure 7). DTX, compound A, and mild HT induced the accumulation of Ca9-22 cells in the G_2/M and $subG_1$ phases of the cell cycle. DTX, known as a G_2/M blocker (22), induced the mitotic cell arrest through the excessive polymerization of microtubules (23) and the appearance of $subG_1$ population,

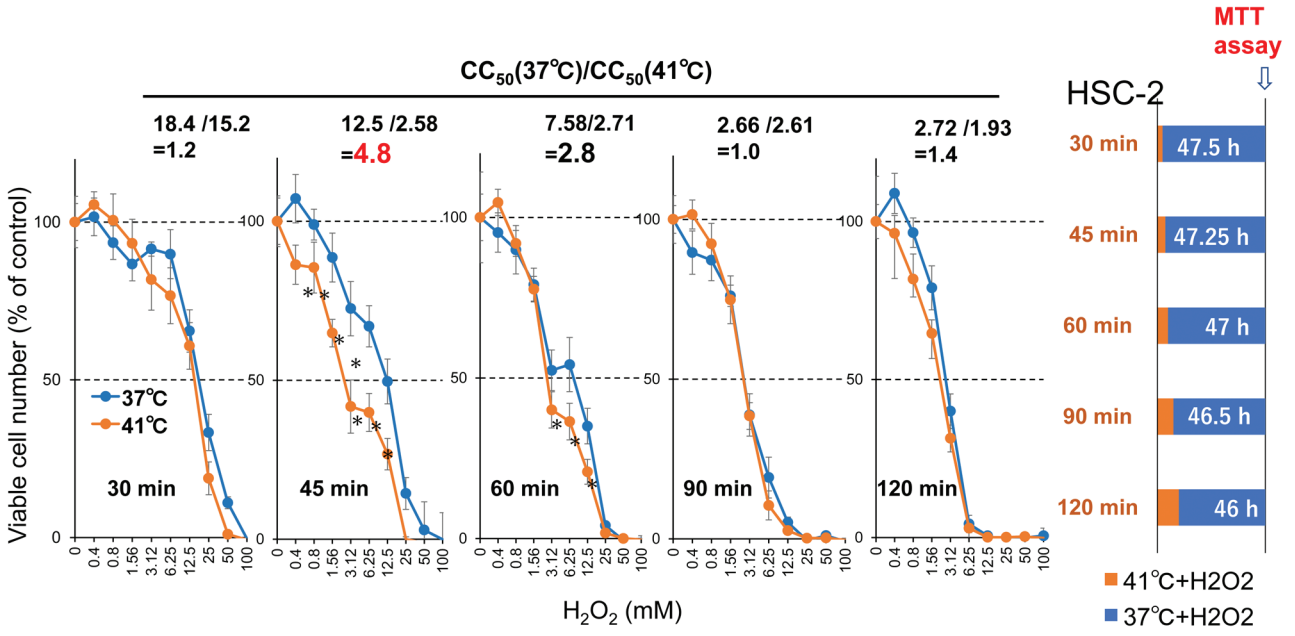

Figure 8. *Enhancement of H_2O_2-induced oral cancer cell death with Mild HT. HSC-2 cells were exposed to the indicated concentrations of H_2O_2 for 30, 45, 60, 90 or 120 min in sextuplicate at 37 or 41˚C, and then cultured for 47.5, 47.25, 47, 46.5 or 46 h in fresh culture medium to determine the viable cell number using the MTT method. CC_{50} ±S.D. (n=6) was determined from the dose-response curve. The SI value [=CC_{50} (37˚C)/CC_{50} (41˚C)] was determined.*

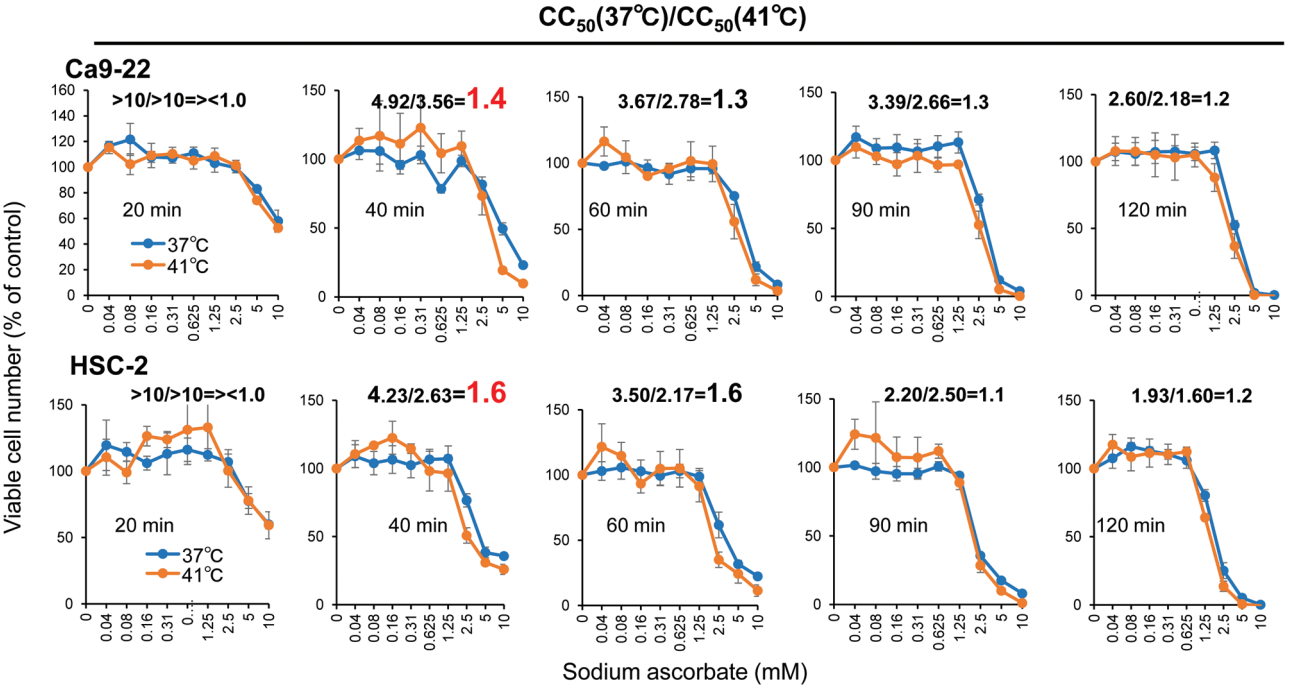

Figure 9. *Enhancement of sodium ascorbate-induced oral cancer cell death with Mild HT. Ca9-22 (upper column) and HSC-2 cells (lower column) were exposed to the indicated concentrations of sodium ascorbate for 20, 40, 60, 90 or 120 min in triplicate at 37 or 41˚C, and then cultured for 47.7, 47.3, 47, 46.5 or 46 h in fresh culture medium to determine the viable cell number using the MTT method. CC_{50} ±S.D. (n=3) was determined from the dose-response curve. The SI value [=CC_{50} (37˚C)/CC_{50} (41˚C)] was determined.*

leading to growth inhibition and cell death. The subG$_1$ population contains either internucleosomal DNA fragments, characteristic of apoptosis (24), random DNA fragments characteristic of necrosis (25), or large DNA fragments detected by pulse-field gel electrophoresis (26), depending on the type of cell and drug (27-29). In Ca9-22 cells, used in this study, the combined use of DTX or compound A and mild HT induced much less internucleosomal DNA fragmentation rather than fragmentation of DNA into large fragments (Figure 7, red arrows). To confirm whether apoptosis was actually induced, apoptosis markers such as caspase activation should be investigated.

We have reported that pyoktanin blue (30), 1,5-diaryl-3-oxo-1,4-pentadienes (31), 4,6,8-trimethyl azulene amide derivatives (32), and pyrazole-based hybrids carrying hydrazone and zinc-binding benzenesulfonamide pharmacophores (33) showed high tumor selectivity for OSCC, however induced only slight activation of caspase-3 in Ca9-22 cells, suggesting non-apoptotic cell death.

Our preliminary study demonstrated that the anti-tumor activity of hydrogen peroxide (H_2O_2) (Figure 8) and sodium ascorbate (that produces H_2O_2 in the presence of oxygen) (Figure 9) was stimulated by mild-HT. Whether H_2O_2 is involved in the synergistic interaction of DTX or compound A with mild-HT remains to be investigated.

In conclusion, the present study demonstrated that combination of two G$_2$/M blockers, DTX (22, 23) or compound A (13), that showed very high tumor-selectivity against OSCC, with mild HT (34) (also this study) that also induced G$_2$/M accumulation of cells, synergistically enhanced the cytotoxic effect against OSCC. Such combination is expected to reduce the cost of treatment by reducing the required doses of anticancer drugs, and alleviates their side effects such as keratinocyte toxicity (20) and neurotoxicity (35). It is necessary to investigate the cytotoxicity of mild HT alone against normal epithelial cells and nerve cells, whether it can be applied to tongue cancer, and its synergistic effects with anticancer agents other than G$_2$/M blockers.

Conflicts of Interest

The Authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Authors' Contributions

YT, HS, SA, SU, and KB performed the experiments. KT and YS synthesized chromone derivatives. YT and HS wrote the article. ST, MT, YU, NY, HidS, RN, TK, KM, and IT reviewed the article. HS provided interpretation of experimental results and edited the article. All Authors read and approved the final version of the article.

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