

4-hexylresorcinol inhibits NF- κ B phosphorylation and has a synergistic effect with cisplatin in KB cells

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Abstract. Cisplatin is a representative anti-cancer drug and 4-hexylresorcinol (4-HR) is known as an antiparasitic and antiseptic agent. The aims of this study were to evaluate the effect of 4-HR on the activation of nuclear factor- κ B (NF- κ B) in cell cultures, to evaluate the antitumor effect of 4-HR plus cisplatin combination therapy in a xenograft model, and to evaluate transglutaminase-2 (TG-2) and phosphorylated NF- κ B (pNF- κ B) expression in the xenograft model. To determine the effect of 4-HR on NF- κ B phosphorylation, co-immunoprecipitation and Western blot analysis were done in KB cells. To examine the *in vivo* effect of the cisplatin plus 4-HR combination therapy, KB cells were grafted into nude mice. Drugs were injected into the peritoneal cavity daily. Tumor size, body weight, and duration of survival were checked daily. Specimens from main mass were used in immunohistochemical staining for the analysis of TG-2 and pNF- κ B expression. In the *in vitro* test, as the 4-HR concentrations increased, the fraction of the bound complex NF- κ B-inhibitory- κ B (I κ B) increased. Consequently, the level of free I κ B decreased. In the xenograft model, the cisplatin plus 4-HR group exhibited a significantly decreased tumor growth rate than in the saline group ($P=0.039$). The mean survival time of the cisplatin plus 4-HR group was 51.20 ± 3.96 days and was significantly prolonged compared with the other groups ($P<0.05$). The body weight of the cisplatin plus 4-HR group had significantly less weight loss than the cisplatin only group ($P=0.045$). In the immunohistochemical analysis, the cisplatin plus 4-HR group had a significantly lower expression of TG-2 and pNF- κ B compared to the saline group ($P<0.05$). In conclusion, cisplatin plus 4-HR combination therapy had

clear advantages over the cisplatin only treatment such as similar tumor growth inhibition compared to the cisplatin only treatment despite the reduced dosage of cisplatin, less body weight loss, and prolonged survival time.

Introduction

Chemotherapy has been the main treatment modality for squamous cell carcinoma (SCC) (1). It has been used for the initial treatment of advanced cancer for neoadjuvant purposes (2) or for the adjuvant treatment of other treatments for palliative purposes (3,4). However, chemotherapy leads to many complications in the patient because of its toxic side effects (5,6). Chemotherapy agents affect not only the growth of tumor cells but also the proliferation of normal cells such as bone marrow (7) and oral mucosal cells (8). When severe complications are observed, dose reduction or alternative agents should be considered (5).

Combination chemotherapy has been used for enhancing antitumor effects and reducing toxic side effects (9). Combination therapy may avoid tumor cell resistance to chemotherapy. The failure of chemotherapy is mainly due to resistance to chemotherapy. Tumor cells may show chemotherapeutic resistance through various pathways (10-12). Recently, transglutaminase-2 (TG-2) mediated nuclear factor- κ B (NF- κ B) activation has been introduced as one of the pathways for chemotherapeutic resistance (13). TG-2 can polymerize inhibitory- κ B (I κ B) (14). Inactivated forms of NF- κ B are bound to I κ B (15). However, TG-2 mediated I κ B polymerization can increase the levels of free NF- κ B (14). Phosphorylated NF- κ B (pNF- κ B) will translocate into the nucleus and transcription will begin (16). The downstream signaling molecules of NF- κ B will help tumor cells to resist chemotherapy (17,18).

In this study, two agents, cisplatin and 4-hexylresorcinol (4-HR), were used. 4-HR has been used as a food additive for the prevention of melanosis in fresh fruit products (19) and seafood (20). Medically, it is known as an antiparasitic and antiseptic agent (21). In the past, 4-HR had been mainly used for antiparasitic purposes (22). Since much more effective antiparasitic agents have been developed, its use as an antiparasitic agent has decreased (23,24). Now, 4-HR is marketed as an ingredient of topical antiseptics, mouthwashes, and throat lozenges (21). Some studies suggested that 4-HR

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could be considered as a potential antitumor agent (21,25). In an *in vivo* study using rats and mice, the 4-HR administered group had less tumorigenesis than the control group (21). In a recent publication, 4-HR was shown to inhibit TG-2 and it exhibited synergy with cisplatin in KB cells (26). If TG-2 was inhibited by 4-HR, nuclear translocation of pNF- κ B should have decreased (13). However, this is unclear from previous publications. In addition, the effect of the 4-HR plus cisplatin combination therapy has not been studied in a xenograft model. The aims of this study were to confirm the decreased level of pNF- κ B after treatment with 4-HR, to evaluate the antitumor effect of 4-HR plus cisplatin combination therapy in a tumor xenograft model, and to evaluate TG-2 and pNF- κ B expression in the xenograft model.

Materials and methods

Cell culture. Cell line KB cells (human nasopharyngeal carcinoma; ATCC; Manassas, VA, USA) were maintained as monolayer cultures in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum containing L-glutamine, vitamins (Life Technologies, Inc., Grand Island, NY, USA), and penicillin-streptomycin (Flow Laboratories, Rockville, MD, USA). The cells were incubated in a mixture of 5% CO₂ and 95% air at 37°C. The cultures were maintained for no longer than 12 weeks after recovery from frozen stocks.

Co-immunoprecipitation (Co-IP). Cells were harvested, treated with 100 μ g/ml of dithiobis succinimidyl propionate (DSP) (Thermo Scientific, Rockford, IL, USA), a reducible chemical cross-linker, for 20 min at 37°C, and then washed twice in PBS containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Cells were lysed in cold immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA pH 8.0, 0.2 mM sodium orthovanadate, protease inhibitor cocktail, 0.5% IGEPAL CA-630). Total protein concentrations were determined using the Bradford Protein Assay kit (Bio-Rad, Richmond, CA, USA). For immunoprecipitations, 500 μ g of cellular lysates were incubated with antibodies at 4°C for 1 h on a rotator. Protein A-Sepharose beads were added, incubated for 1 h at 4°C, and washed 4 times with immunoprecipitation buffer, then resuspended in Laemmli buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.01% bromophenol blue), and boiled.

Western blot analysis. Whole cells were lysed in ProteoJET™ Mammalian Cell Lysis Reagent (Thermo Scientific) containing protease inhibitor cocktail (Sigma-Aldrich). Proteins were separated on 10% SDS-polyacrylamide gels and transferred to PVDF membranes. Blots were blocked with 5% skim milk powder in Tris-buffered saline (20 mM Tris-HCl, 137 mM NaCl, pH 7.6) containing 0.1% Tween-20 (TBS-T buffer) for 1 h at RT. Western blot analyses were done with anti-phospho-NF- κ B p65 (Cell Signaling Technology, Danvers, MA, USA), anti-NF- κ B p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-I κ B- α (Santa Cruz Biotechnology), and anti- β -actin (Sigma-Aldrich) antibodies. Primary antibodies were added to the TBS-T buffer at a 1:1,000 dilution and incubated for 90 min at RT prior to incubation with HRP-conjugated secondary anti-

bodies (1:5,000 dilution; Santa Cruz Biotechnology) for 1 h at room temperature. Proteins were detected using an enhanced chemiluminescence detection system (GE Healthcare UK, Ltd., Buckinghamshire, UK).

Xenograft study. Seven-week-old male athymic nude mice were purchased from Orient Bio (Seoul, Korea). The mice were used in accordance with the Animal Care and Use Guidelines of the College of Dentistry at Gangneung-Wonju National University (GWNU 2010-5). To produce tumors, KB cells were harvested from subconfluent cultures. A viable suspension of cells was used for the injections. To induce anesthetic conditions, the mice were intra-muscularly injected with 1.5 mg/kg of zolazepam (Zoletil®; Virbac Laboratories, Carros, France) and 3.5 mg/kg of xylazine hydrochloride (Rumpun®; Bayer Korea, Seoul, Korea) various numbers of 5x10⁵ cells were resuspended in 50 μ l of Ca²⁺ and Mg²⁺ free Hanks' balanced salt solution (HBSS) and injected into the submandibular gland in the flank using a 27-gauge hypodermic needle with a 1 ml syringe as shown in a previous study (27). After the injections, nude mice were randomly assigned to 3 groups (cisplatin, cisplatin plus 4-HR, and saline). After a tumor mass was definitively identified, the treatment was started. Tumor mass size was calculated using the following formula; mass volume = a (long distance) x b (short distance)²/2 (28).

Cisplatin was obtained from Choongwae (Seoul, Korea) and 4-hexylresorcinol from Sigma-Aldrich (Cat#, 20946-5). The drug was administered daily in the peritoneal cavity. The dosage were as follows; i) 2.5 mg/kg of cisplatin, ii) 1.25 mg/kg of cisplatin plus 2.5 mg/kg of 4-HR, and iii) 5 ml/kg of saline. Moreover, the change in mass size and body weight was observed daily. For the comparison of mass size and body weight, the mass size and body weight at the beginning of the treatment was set to 1. The relative mass size and body weight at each observation point were calculated. The difference in mass size or body weight between groups was compared by independent sample t-test. The death of a mouse was recorded and the data were used for the survival analysis. The comparison of survival was done by the Kaplan-Meier method and the difference between the groups was evaluated by log-rank test. The significance level was set at P<0.05.

Immunohistochemical staining. Primary antibodies for immunohistochemical analysis were purchased as follows: mouse monoclonal antibody to TG-2 (ab2386) (Abcam®, Cambridge, UK) and rabbit polyclonal antibody to pNF- κ B p65 (phospho T254) (ab28810) (Abcam®). The dilution rates were 1:50 for TG-2 and 1:100 for pNF- κ B. Tumor main mass from each mouse was used for immunohistochemical staining. Sections of 4 μ m were prepared for immunohistochemistry. Immunohistochemical staining was performed with Universal LSAB + kits (Dako, Glostrup, Denmark) and the subsequent procedures according to the manufacturer's protocols. Immunostaining without primary antibodies was used as a negative control. The sections were counterstained with Mayer hematoxylin. Two pathologists blinded to the original group classification reviewed all the slides. The slides were evaluated for intensity and area of staining. The intensity scales were (-) for invisible or trace staining in a focal area, (+) for visible staining in a moderate area, and (++) for dense,

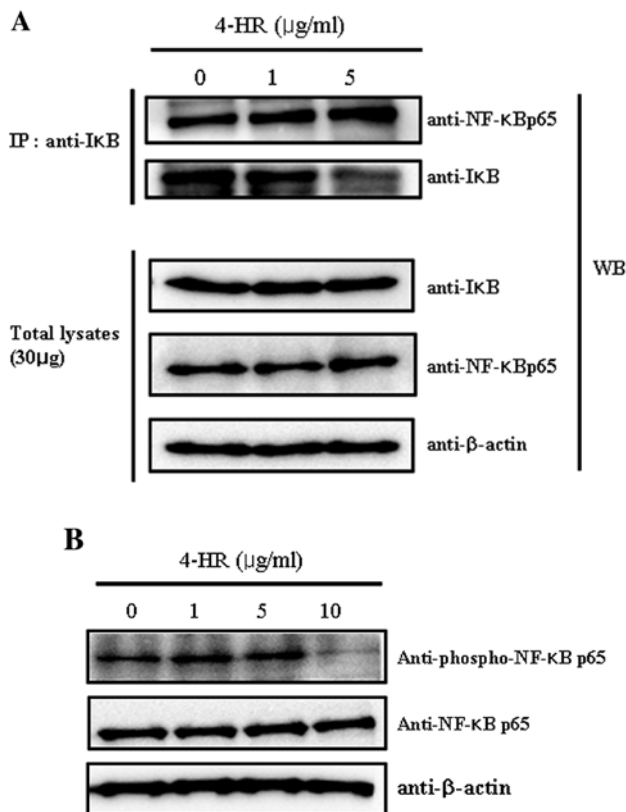


Figure 1. Co-immunoprecipitation and Western blot analysis. (A) As the 4-HR concentration increased from 1 to 5 $\mu\text{g/ml}$, the fraction of the bound complex NF- κB -I κB increased (IP, immunoprecipitation; WB, Western blot analysis). (B) Western blot analysis for whole cell lysates showed that pNF- κB significantly decreased with 4-HR treatment at a concentration of 10 $\mu\text{g/ml}$.

strong staining in an extensive area. Three different sections were used per specimen for the analysis. The average value of a sample was used as the immunohistochemical activity of the sample. The difference between the groups was analyzed by independent samples t-test and significance was set at $P < 0.05$.

Results

4-HR treatment decreases the levels of free I κB and pNF- κB . The Co-IP experiments showed that 4-HR increased interaction between NF- κB and I κB (Fig. 1A). As 4-HR concentrations increased from 1 to 5 $\mu\text{g/ml}$, the fraction of the bound complex NF- κB -I κB increased (Fig. 1A). Consequently, the level of free I κB decreased. However, total lysates showed a similar amount of NF- κB and I κB among the groups. Western blot analysis for the whole cell lysates showed that pNF- κB was significantly decreased when 4-HR was at a concentration of 10 $\mu\text{g/ml}$ (Fig. 1B). However, the level of NF- κB itself did not decrease from the treatment of 4-HR.

Cisplatin plus 4-HR combination therapy reduces body weight loss and increases survival time. In the comparison of the relative tumor mass size at 24 days after the initial treatment, the saline group, cisplatin only group, and cisplatin plus 4-HR group had a relative tumor mass size of 12.31 ± 6.63 , 4.24 ± 1.64 , and 3.40 ± 0.89 , respectively (Fig. 2A). There was a significant difference between the saline group and

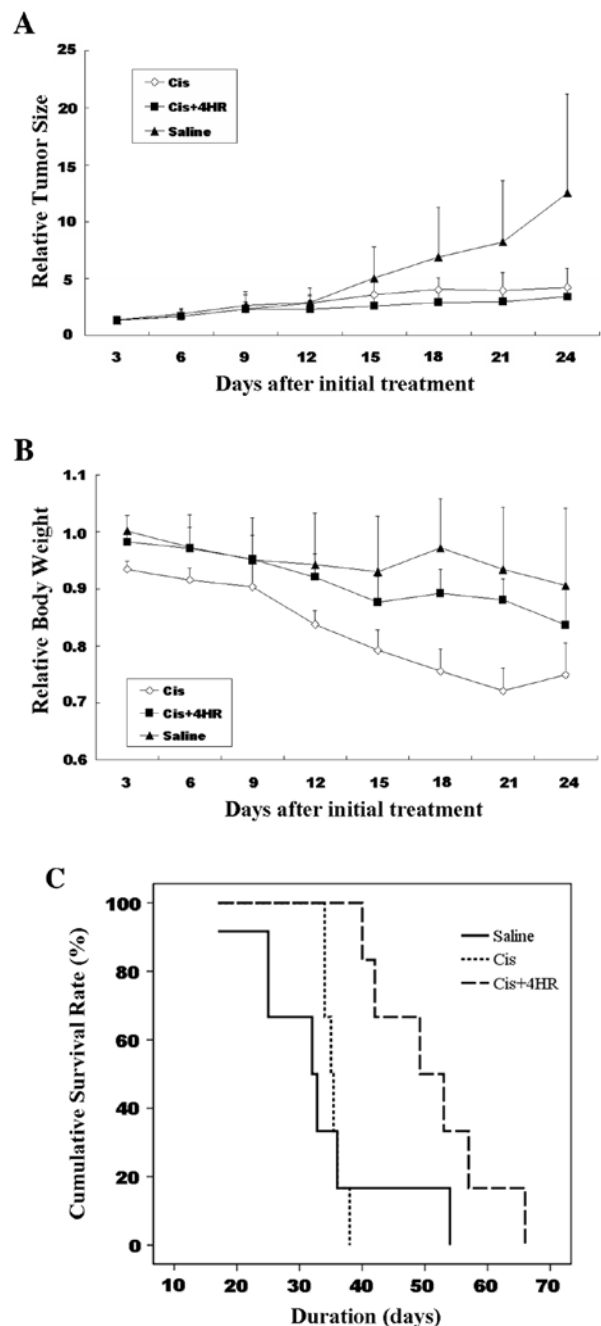


Figure 2. Tumor xenograft in nude mice. (A) Relative tumor size. (B) Relative body weight. (C) Cumulative survival curve. (Cis, cisplatin only; Cis+4HR, cisplatin plus 4-hexylresorcinol).

cisplatin plus 4-HR group ($P = 0.039$). However, there was no significant differences between the other groups ($P > 0.05$). In the comparison of the relative body weight at 24 days after the initial treatment, the saline group, cisplatin only group, and cisplatin plus 4-HR group had a relative body weight of 0.91 ± 0.14 , 0.75 ± 0.06 and 0.84 ± 0.07 , respectively (Fig. 2B). There was a significant difference between the cisplatin only group and saline group ($P = 0.029$). In addition, there was a significant difference between the cisplatin only group and cisplatin plus 4-HR group ($P = 0.045$). However, there was no significant difference between saline group and cisplatin plus 4-HR group ($P > 0.05$). In the survival analysis, mean survival time of the saline group was 32.80 ± 5.07 days (Fig. 2C). The

Table I. Immunoreactivity of TG-2 and pNF-κB.

	Saline group			Cisplatin only group			Cisplatin plus 4-HR group		
	0	+	++	0	+	++	0	+	++
TG-2	0	0	5	0	1	4	1	3	1
pNF-κB	0	1	4	2	1	2	2	3	0

TG-2, transglutaminase-2; pNF-κB, phosphorylated nuclear factor-κB; 4-HR, 4-hexylresorcinol.

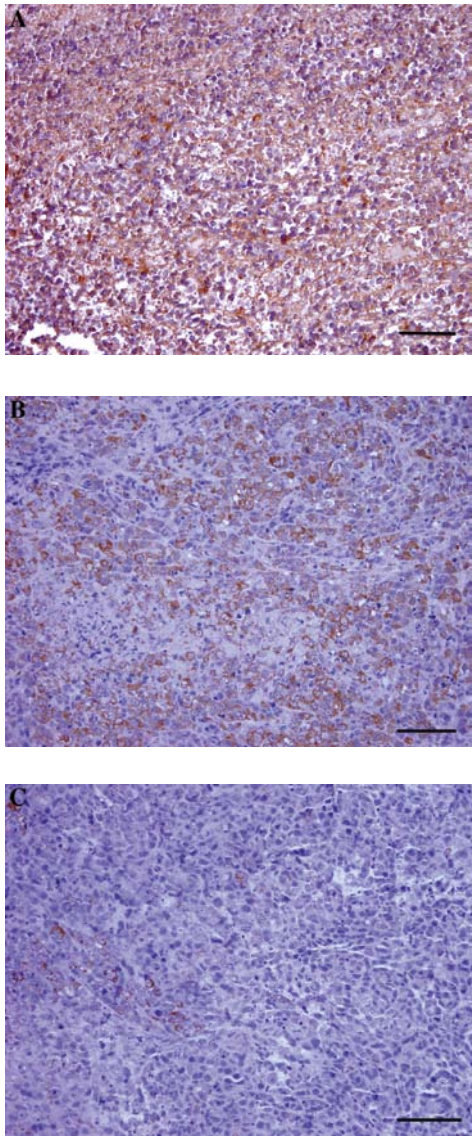


Figure 3. Immunohistochemical staining for TG-2. (A) Saline group (bar, 20 μm). (B) Cisplatin only group (bar, 20 μm). (C) Cisplatin plus 4-HR group (bar, 20 μm).

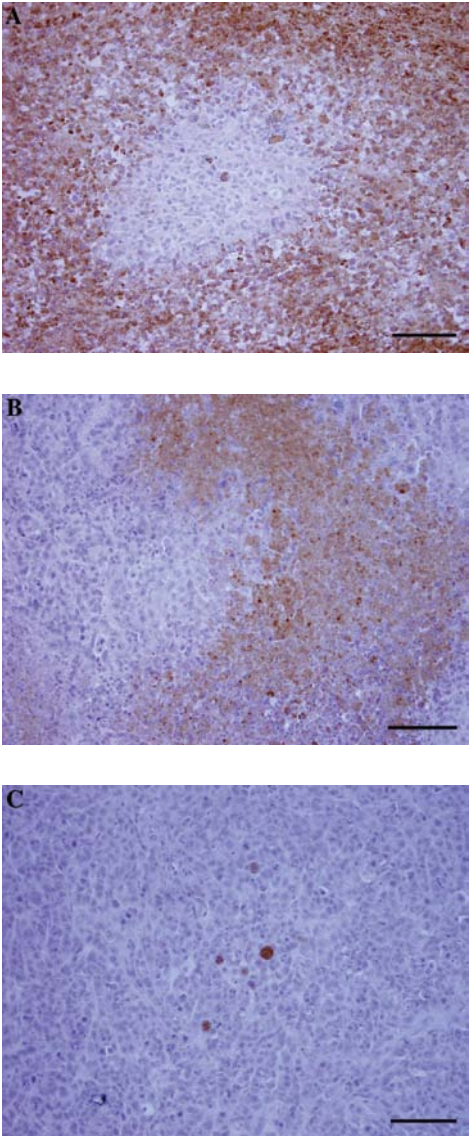


Figure 4. Immunohistochemical staining for pNF-κB. (A) Saline group (bar, 20 μm). (B) Cisplatin only group (bar, 20 μm). (C) Cisplatin plus 4-HR group (bar, 20 μm).

mean survival time of the cisplatin only group and cisplatin plus 4-HR group was 35.40±0.61 days and 51.20±3.96 days, respectively. In the comparison of the survival time, there was no significant difference between the saline group and cisplatin only group (P>0.05). However, there were significant differences between the saline group and cisplatin plus 4-HR

group (P=0.035) and between the cisplatin only group and cisplatin plus 4-HR group (P=0.001).

Cisplatin plus 4-HR combination therapy decreases TG-2 and pNF-κB in a tumor mass of xenograft model. The TG-2 and pNF-κB expression patterns are presented in Table I. The saline

group and cisplatin only group had a relatively high expression of TG-2 (Fig. 3A and B). The saline group showed a strong expression of TG-2 in the sections from all mice (5/5 mice). The cisplatin only group also showed that the expression of TG-2 was visible in the section from one mouse and had strong staining in the sections from the 4 other mice. For most of the cisplatin plus 4-HR group, however, the expression of TG-2 was negligible (1/5 mice) with a (-) degree of staining or was visible (3/5 mice) with a (+) degree staining (Fig. 3C). When the expression of TG-2 in the cisplatin plus 4-HR group was compared to that of the saline group, the difference was statistically significant ($P=0.013$). However, there were no statistically significant differences between the other groups ($P>0.05$).

The saline group showed strong expression of pNF- κ B in the section from 4 mice (80.0%). Intranuclear localization of pNF- κ B was observed to a high degree in the saline group (Fig. 4A). The cisplatin only group showed a binominal pattern of expression for pNF- κ B. The sections from 2 mice of the cisplatin only group showed negligible expression of pNF- κ B and the sections from 2 mice showed strong expression of pNF- κ B (Fig. 4B). In most of the cisplatin plus 4-HR group, however, the expression of pNF- κ B was negligible (2/5 mice) with a (-) degree of staining or was visible (3/5 mice) with a (+) degree of staining (Fig. 4C). When the expression of pNF- κ B in the cisplatin plus 4-HR group was compared with that of the saline group, the differences were statistically significant ($P=0.005$). However, there were no statistically significant differences between the other groups ($P>0.05$).

Discussion

4-HR induces apoptosis in cancer cells. However, the mechanism of 4-HR activity in cancer cells has yet to be elucidated. Our previous study showed that 4-HR suppressed intracellular Ca^{2+} oscillations in cultured SCC-9 cells (data not shown). It is known that Ca^{2+} regulates the activity of the TG-2 protein with GTP cooperatively (29,30). The TG-2 protein is closely related to the activation of the NF- κ B pathway in cancer (13). As a result, 4-HR decreases the TG-2 activity in a dose-dependent manner. In this study, 4-HR clearly inhibited the NF- κ B pathway. 4-HR decreased the levels of pNF- κ B and free I κ B. In the xenograft model, the cisplatin plus 4-HR combination group had a significantly increased lifespan and suppressed tumor mass growth compared to the cisplatin only group. Considering that the cisplatin plus 4-HR combination group used only half the concentration of cisplatin compared to the cisplatin only group, the combination therapy of cisplatin and 4-HR could be considered for clinical applications.

With no stimulated conditions, NF- κ B dimers bound to I κ B proteins which functions as NF- κ B inhibitors in the intracellular cytoplasm (15). However, some signals caused by cellular response to external stimuli leads to I κ B degradation (16). As a consequence of I κ B degradation, NF- κ B is released from restriction of I κ B and moves into the cellular nucleus (16). It activates the genes which regulates cell survival and proliferation (31,32). To determine which 4-HR regulates apoptosis in cancer cells through the NF- κ B pathway, we examined the NF- κ B-I κ B complex by Co-IP. The results of Co-IP experiments showed that 4-HR could increase the interaction between NF- κ B and I κ B. Next, to address whether 4-HR can functionally inhibit

the phosphorylation of NF- κ B, we introduced the 4-HR into KB cells for 10 min and pNF- κ B was significantly decreased as determined by Western blot analysis at a concentration of 10 μ g/ml of 4-HR. These results suggest that 4-HR can suppress the induction of NF- κ B in the nucleus, and then stimulate cell apoptosis by the inhibition of nucleus localization of NF- κ B.

Increased TG-2 expression correlates with tumor growth and progression in malignant tumors (33,34). NF- κ B also regulates the transcription of DNA that induces cell survival and proliferation in cancer cells (33,34). A high expression of NF- κ B proteins is related to various malignant tumors (35). In this study, the cisplatin plus 4-HR group had reduced expression of TG-2 and pNF- κ B compared to the saline and cisplatin only groups. Particularly, TG-2 and pNF- κ B expression were significantly reduced in the cisplatin plus 4-HR group compared to the saline group ($P<0.05$) (Table I). However, there was no significant difference in TG-2 and pNF- κ B expression between the saline group and the cisplatin only group ($P>0.05$). This result shows that the antitumor effect of 4-HR is correlated with the inhibition of TG-2 and pNF- κ B.

For the 4-HR and cisplatin combined therapy, the cisplatin cytotoxic antitumor effect originates from the prevention of DNA synthesis (36). To this effect, add the effect of 4-HR, which inhibits tumor growth and progression by inhibition of TG-2 and the NF- κ B pathway. These two different pharmacological pathways inhibited tumor growth and proliferation synergistically at a low dose of cisplatin. Despite the reduced dose of cisplatin, the 4-HR plus cisplatin group had better or similar antitumor effects compared with the saline group or cisplatin only groups (Fig. 2A). In addition, the mean survival time was approximately 1.6-fold longer in the cisplatin plus 4-HR group than in the other groups ($P<0.05$) (Fig. 2C). Body weight loss is related to the toxicity of the chemotherapy (37). In the comparison of the cisplatin plus 4-HR group to the cisplatin only group, there was a significant difference ($P=0.045$) (Fig. 2B). However, there was no significant difference between the cisplatin plus 4-HR group and the saline group ($P>0.05$). Therefore, cisplatin plus 4-HR combination therapy had less toxicity, similar tumor inhibition, and prolonged lifespan compared to the cisplatin only therapy.

4-HR is known to be safe and effective in topical applications for infected skin or mucosa (21). It is a phenolic compound such as other NF- κ B pathway inhibitors (19,38). It is known that there are no considerable toxicities for 4-HR. The LD50 value of 4-HR was 50 mg/kg in the case of intraperitoneal injection in mice (21). The antitumor effect of 4-HR was shown when it was administered at 62.5 or 125 mg/kg orally in rats and mice (21). In our results, intraperitoneal injection of 4-HR (2.5 mg/kg) with cisplatin (1.25 mg/kg) was given daily for treatment. For the clinical application of 4-HR, further toxicological and drug metabolism studies should be performed.

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