4-hexylresorcinol exerts antitumor effects via suppression of calcium oscillation and its antitumor effects are inhibited by calcium channel blockers

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Abstract. The bacterial dormancy-inducing factor 4-hexylresorcinol (4-HR) has been shown to have synergistic antitumor effects when used in combination with cisplatin. In the present study, 4-HR was used as a single agent in the squamous carcinoma cell line SCC-9. The results demonstrated that 4-HR suppressed SCC-9 cell proliferation compared to primary cultured gingival fibroblasts. 4-HR dose-dependently induced SCC-9 cell apoptosis as determined by caspase-3 activity, Annexin V expression, as well as by scanning and transmission electron microscopy. 4-HR inhibited intracellular calcium oscillation in both SCC-9 cells and normal human dermal fibroblasts. 4-HR-induced apoptosis was partly reversed by calcium channel blockers. Of note, 4-HR reduced the tumor mass formed by SSC-9 cell implantation in BALB/cAnNCrj-nu/nu mice and mass size reduction was also partly reversed by the concomitant application of calcium channel blockers. Collectively, our results suggest that 4-HR has strong antitumor effects by inhibiting calcium channel oscillation and inducing apoptosis.

Introduction

Resorcinol is a non-isoprenoid lipid found in a range of plant and bacterial species; they exert non-specific antioxidant and anti-mutagenic effects and regulate proliferation (1). Chemical analogs of these lipids have demonstrated anticancer effects in animal models of colon (2), lung (3) and pancreas tumor (4). 4-hexylresorcinol (4-HR) is also a chemical analog of these lipids (1).

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In microorganisms, chemical analogs of 4-HR occur in dormant cysts (5,6) and resting cyst-like cells (7,8). Exogenous administration of these auto-regulatory factors and their analogs-alkyl resorcinol induces dormancy in bacteria (9); these compounds are similarly active in eukaryotic cells such as ras-transformed fibroblasts (10). Therefore, 4-HR may also inhibit the growth of tumor cells, altering their physiological state and activity. In a previous report, 4-HR showed a preventive effect in mononuclear cell leukemia, hepatocellular neoplasm, and circulatory system tumors (11,12). Recently, combination therapy of 4-HR and cisplatin was shown to have synergistic effects in nasopharyngeal carcinoma (13). 4-HR inhibits the NF-κB pathway via the suppression of transglutaminase-2 (13,14). 4-HR also accelerates tumor differentiation by the suppression of E2F2 and E2F3 (15).

The aim of the present study was to determine the potential antitumor effects of 4-HR as a single agent on oral squamous cell carcinoma (OSCC). Additionally, the change of intracellular calcium following 4-HR application and drug interaction with calcium channel blockers were demonstrated. We used *in vitro* SCC-9 cell culture and *in vivo* xenograft model systems and assessed cell viability, intracellular calcium tracking and apoptosis.

Materials and methods

Cell cultures and MTT assay. SCC-9 was grown to confluence in Ham's F12/Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD, USA) containing 1% penicillin/streptomycin, fibroblast growth factor-2 (100 μg/ml) and 10% fetal calf serum (FCS). Primary cultured human gingival fibroblasts (PHGF) were used as controls. 4-HR (Sigma, St. Louis, MO, USA) was added to confluent cells to final concentrations of 1, 5 or 10 μg/ml.

Cell viability quantification at 48 h after 4-HR application was assessed by tetrazolium salt 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (16). Briefly, cells were incubated with MTT solution (Cell Proliferation kit I; Roche Molecular Biochemicals, Mannheim, Germany) in 6-well plates for 4 h at room temperature. Formazan crystals were solubilized overnight, and the

product was estimated by measuring absorbance at 590 nm with a Victor Multilabel counter (Perkin-Elmer Wallac GmbH, Freiburg, Germany).

Apoptosis and caspase-3/7 assays. Apoptosis was determined with the Annexin V-FITC Apoptosis Detection kit I (BD Biosciences, San Jose, CA, USA) following a 2-h incubation with 4-HR. The caspase assay was performed with a commercial kit (Caspase-Glo® 3/7 assay, Promega, Madison, WI, USA) at 30 min after 4-HR was added. Cell culture in medium lacking 4-HR was used as negative control.

Calcium tracking with confocal microscopy and calcium channel study. To monitor calcium, SCC-9 cells were treated with Fluo-4 NW calcium assay kit (Molecular Probes, Eugene, OR, USA), and then visualized using a laser scanning confocal microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) as previously described (17).

Calcium channel antagonists (blockers) were used to confirm the role of calcium in 4-HR-mediated proliferation and apoptosis. SCC-9 cells were exposed to Norvasc (0.1, 0.5 or 1 μg/ml; Pfizer Korea, Seoul, Korea), and 4-HR (10 μg/ml) was added 2 h later; control cells did not receive Norvasc. The MTT cell proliferation assay was performed 24 h after 4-HR treatment. Subsequently, SCC-9 cells were treated with calcium channel antagonists (Norvasc or diltiazem; 1 μg/ml) for 2 h before exposure to 4-HR. Diltiazem was obtained from Sigma. The caspase-3/7 assay was performed 30 min after 4-HR application.

Scanning and transmission electron microscopy. Following a 24-h incubation with 10 μg/ml 4-HR, SCC-9 cell suspension was applied to copper grids and dried in a vacuum to prepare for scanning electron microscopy (SEM). SCC-9 cells treated with 10 μg/ml cisplatin for 24 h were included as a positive control. Prior to transmission electron microscopy (TEM) analysis, cells were harvested by centrifugation at 300 x g for 10 min, and then dehydrated and fixed (18). Specimens were polymerized in Spurr (Epon 812) resin, cut on an ultratome (Leica, Uppsala, Sweden), stained with lead citrate, and mounted on copper grids. SEM and TEM were performed using a JEOL microscope (Tokyo, Japan) operating at accelerating voltage 15.0 kv and magnification x7000.

Tumor xenograft model. Male nude mice (BALB/ cAnNCrj-nu/nu) were purchased from Charles River Japan Inc. (Shin-Yokohama, Japan). Seventeen mice were subcutaneously injected with SCC-9 cells ($2.5x10^6$); all subsequently developed tumors. Commencing on the following day, two experimental groups (each group, n=6) received daily intraperitoneal injections of 4-HR (10 mg/kg of body weight) for 16 days, while the control group (n=5) received daily injections of the vehicle (normal saline). Another group of mice received daily intraperitoneal injections of 4-HR (10 mg/kg of body weight) plus diltiazem (20 mg/kg of body weight) for 16 days (19,20). Tumors were measured in two dimensions with calipers every 2 or 3 days, and tumor volumes were calculated with the following formula: volume = a x $b^2/2$, where a is the tumor measurement at its widest point and b is the measurement perpendicular to a. Tumor weight was determined at the time of sacrifice.

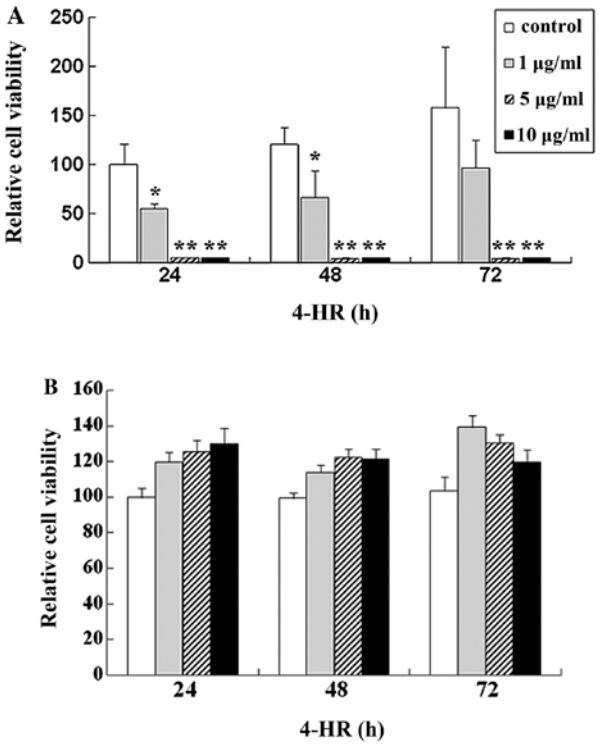

Figure 1. Effect of 4-hexylresorcinol (4-HR) on SCC-9 cell proliferation. Cells were cultured for two days after seeding and then treated with various concentrations of 4-HR. Cell proliferation was determined by MTT assay. (A) SCC-9 cells and (B) primary cultured human gingival fibroblasts demonstrated markedly different proliferation responses to 4-HR. Relative activity was based on 24-h control group. Data are expressed as the means ± SD (n=6; $^*P<0.05$, $^{**}P<0.01$).

Statistical analysis. The difference between the untreated control and the drug-treated group in each experiment was compared by independent sample t-test. Inhibitory concentration 50 (IC_{50}) was calculated at 48 h after 4-HR administration by linear regression analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

4-HR inhibits SCC-9 cell proliferation. Initially, we determined the effect of 4-HR on SCC-9 cell viability. Relative cell viability after treatment with 1, 5 and 10 μg/ml 4-HR was 55.0, 5.2 and 4.7%, respectively, compared with control (P=0.043, 0.007 and 0.007) (Fig. 1A). By contrast, 4-HR exerted only a slight effect on PHGF (Fig. 1B). IC_{50} was 2.94 μg/ml for SCC-9. However, IC_{50} of PHGF was 49.30 μg/ml.

4-HR induces apoptosis of SCC-9 cells. Subsequently, we determined whether 4-HR could induce apoptosis. Following treatment with 4-HR (10 μg/ml), SCC-9 cells were considerably smaller and rounder compared with control cells (data not shown), suggesting apoptosis. SEM and TEM examinations revealed: i) the appearance of apoptotic bodies around cells following 4-HR treatment (10 μg/ml), which was similar to cells treated with cisplatin, and ii) cleaved or fragmented nuclei with finger-like projections, which is a typical feature of apoptosis (Fig. 2A). By contrast, control cells exhibited smooth surfaces and intact intra-cytoplasmic structures and nuclei. Following

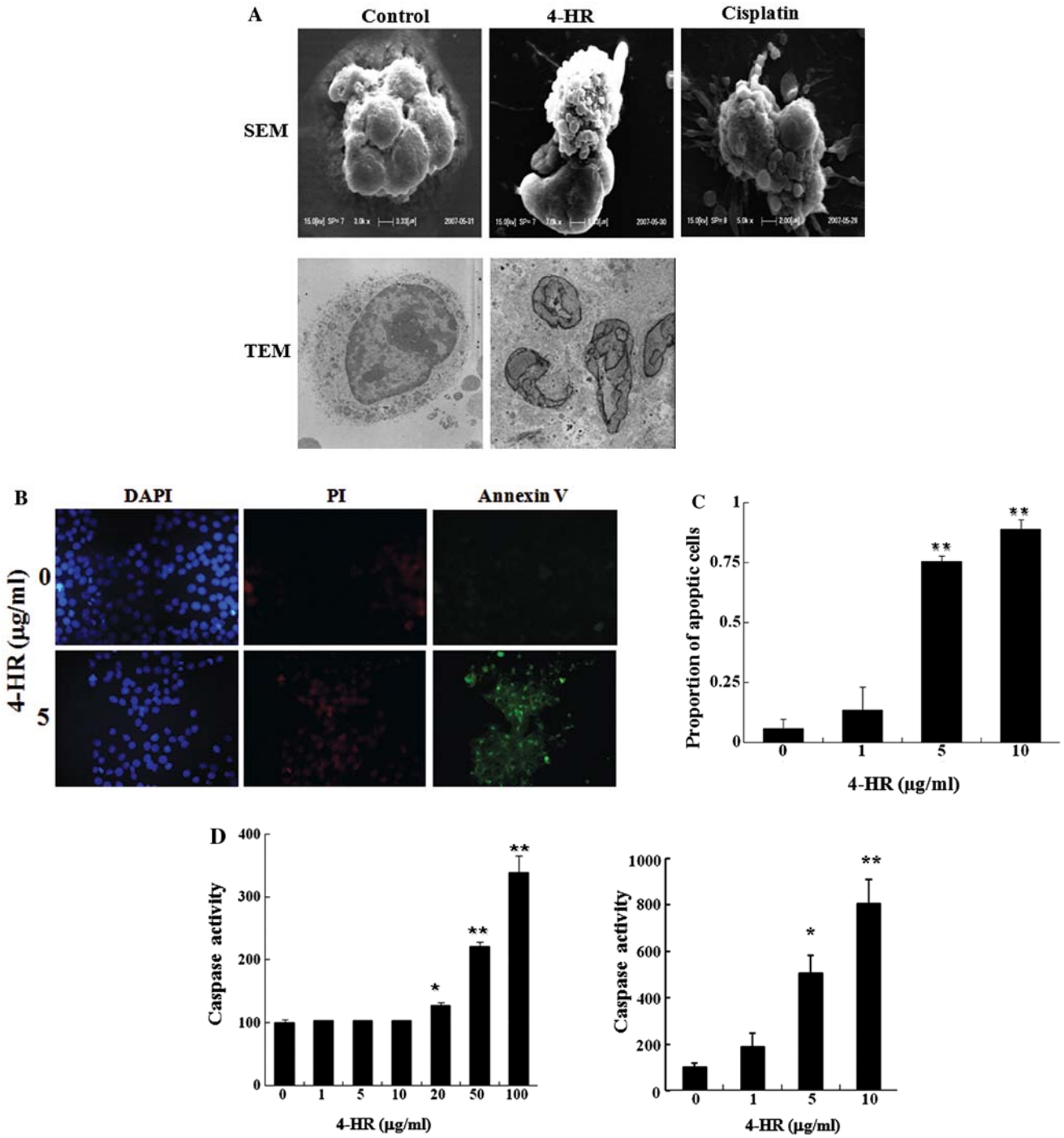

Figure 2. 4-hexylresorcinol (4-HR)-induced morphological and biochemical changes in SCC-9 cells. SCC-9 cells were treated with or without 4-HR (10 μg/ml) for 24 h. Cell morphology was visualized by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). (A) Round shape, outward lobulation, and nucleus fragmentation were observed in 4-HR-treated cells (TEM original magnification, x3000). (B) SCC-9 cells treated with 4-HR (5 μg/ml) were positive for staining with Annexin V (original magnification, x100). Propidium iodide (PI) indicates nucleus staining. (C) The proportion of apoptotic cells was significantly increased in 5 and 10 μg/ml 4-HR compared to the untreated control (**P<0.01). (D) 4-HR had a slight effect on caspase-3/7 activity in primary culture human gingival fibroblasts ≤10 μg/ml (left panel), but exerted a strong dose-dependent effect in SCC-9 cells (right panel) (*P<0.05, **P<0.01).

treatment with 4-HR (5 μg/ml), SCC-9 cells became stainable with Annexin V (Fig. 2B), most likely due to membrane changes, a primary event in apoptosis. The proportion of apoptotic cells was significantly increased in 5 and 10 μg/ml 4-HR compared to the untreated control (Fig. 2C). To confirm 4-HR-mediated apoptosis, caspase-3/7 activity was determined. Caspase-3/7 activity was dose-dependently increased by 4-HR in SCC-9 cells (Fig. 2D, right panel), while its activity was marginally increased in PHGF from 20 μg/ml of 4-HR (Fig. 2D, left panel). These results indicate that 4-HR selectively induces apoptosis of SCC-9 cells at lower concentrations than PHGF.

Differential effects of 4-HR on intracellular calcium signaling in SCC-9 cells. Calcium is known to play role in cell proliferation, differentiation and apoptosis; therefore, we assessed calcium uptake in SCC-9 cells. As shown in Fig. 3A, intra-

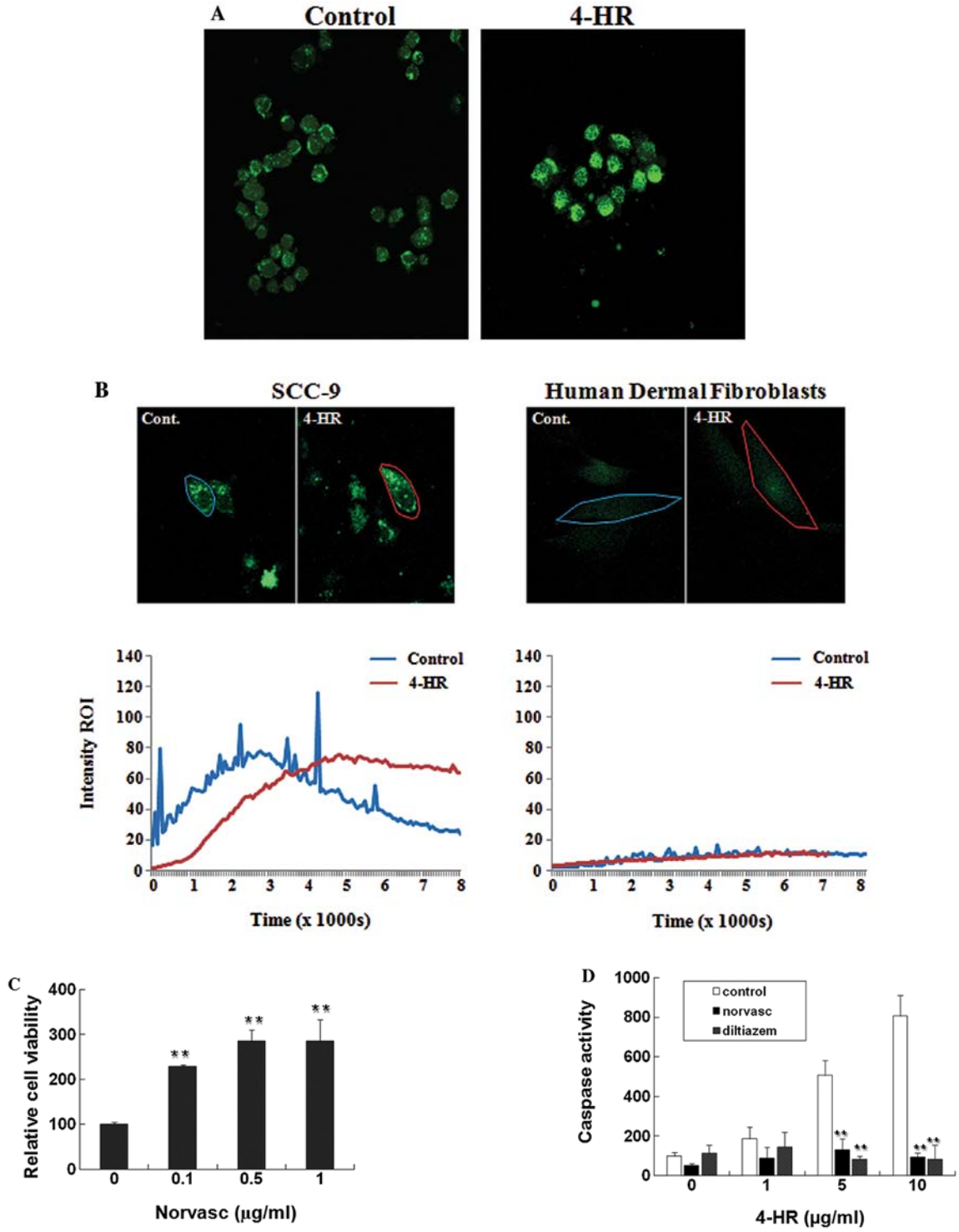

Figure 3. Essential role of intracellular calcium for 4-hexylresorcinol (4-HR)-mediated biological effects in SCC-9 cells. (A) Control and 4-HR-treated SCC-9 cells visualized by confocal microscopy after staining with calcium-sensitive Fluo-4 AM. Intracellular calcium content increased 10 min after addition of 4-HR (10 μg/ml) (original magnification, x200). (B) Differential effects of 4-HR on intracellular calcium kinetics between SCC-9 cells (left panel) and normal dermal fibroblasts (right panel). Quantification of calcium signals by image analysis and immunofluorescence staining (inserts). (C) The 4-HR-mediated antiproliferative effect was attenuated by a calcium channel blocker. Cells were pretreated with Norvasc prior to 24-h treatment with 4-HR (10 μg/ml), and then assessed by MTT assay (**P<0.01). (D) The 4-HR-mediated apoptotic effect was abrogated by calcium channel blockers. Cells were pretreated with calcium channel blockers prior to 30-min treatment with 4-HR (1 μg/ml) (**P<0.01).

cellular calcium was significantly increased in SCC-9 cells after 4-HR treatment (10 μg/ml) compared with control cells. Furthermore, increased intracellular calcium was observed specifically in SCC-9 cells, but not in normal dermal fibroblasts (Fig. 3B). In SCC-9 cells, 4-HR treatment delayed the decrease in intracellular calcium after peak concentration; however, in normal dermal fibroblasts, peak calcium levels were much lower, and 4-HR did not affect the kinetics of the calcium response. Notably, 4-HR suppressed calcium oscillation in both SCC-9 cells and normal dermal fibroblasts (Fig. 3).

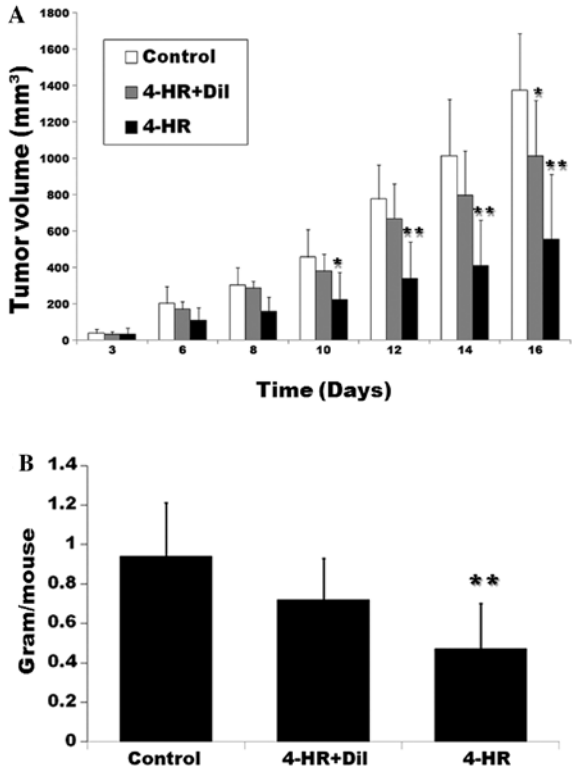

Figure 4. 4-hexylresorcinol (4-HR) inhibits tumor formation in SCC-9 cells injected into a nude mice xenograft model. (A) Mice were sacrificed at Day 16. Three representative tumor masses are shown among samples in each group (n=5 or 6). Enucleated masses were injected with saline (control), 10 mg/kg 4-HR + 20 mg/kg diltiazem (4-HR+Dil), or 10 mg/kg 4-HR (4-HR). The graph compares mean tumor size throughout the experiment (*P<0.05, **P<0.01). (B) Mass weight was measured at the time of necropsy. Average mass weight per mouse of the saline group was compared to the 4-HR only or the 4-HR + Dil group (*P<0.05, **P<0.01).

Calcium blockers were used to confirm the essential role of calcium uptake. As shown in Fig. 3C, the 4-HR anti-proliferative effect was inhibited by Norvasc (0.1-1 μg/ml; P<0.001); cellular proliferation was increased ~3-fold compared with the 4-HR control. Calcium channel blockers Norvasc and diltiazem also blocked 4-HR-mediated caspase-3/7 activity in SCC-9 cells (P<0.001 in 5 and 10 μg/ml of 4-HR; Fig. 3D). These results confirm that 4-HR-mediated effects are due, in part, to increased intracellular calcium.

4-HR reduces tumor formation in a xenograft in vivo model. Next, we tested 4-HR antitumor effects in the xenograft model. SCC-9 cells were injected subcutaneously into nude mice. In mice receiving daily 4-HR treatment (10 mg/kg) for 16 days, tumor mass was markedly smaller compared with the control group (P=0.003; Fig. 4A). In mice receiving concomitant application of 4-HR and diltiazem, tumor mass was significantly larger compared with the 4-HR group (P=0.038). There was no statistically significant difference between the control and the 4-HR + diltiazem group (P>0.05). At the time of necropsy, the average mass weight was 0.94±0.27 g/mouse in the control group (Fig. 4B), 0.72±0.21 g/mouse in the 4-HR + diltiazem group and 0.47±0.23 g/mouse in the 4-HR group. When compared to the control, the 4-HR group was statistically significantly different (P=0.007). Taken together, these results indicate that 4-HR inhibits tumor cell proliferation in mouse tumor xenografts and concomitant application of calcium channel blocker partly reverses the antitumor effect of 4-HR.

Discussion

In the present study, we demonstrated that 4-HR strongly inhibited SCC-9 cell proliferation compared with normal fibroblasts, and induced apoptosis. Furthermore, these *in vitro* effects were reproducible in xenografts after SCC-9 cell implantation in nude mice. The antitumor effect of 4-HR was partly reversed by the application of calcium channel blockers both *in vitro* and *in vivo*.

4-HR also induced the apoptosis of PHGF, although at a significantly higher concentration (>20 μg/ml; Fig. 2D), which is in accordance with previous results (21). 4-HR targets transformed cells via an unknown mechanism. Although this study focused on SCC-9 cells, we also observed the inhibitory action of 4-HR on gastric adenocarcinoma, breast cancer, lung cancer, and hepatoma cells (data not shown), suggesting that 4-HR may be applicable to other types of cancer. Selective tumor cell apoptosis is one objective in developing anticancer drugs. In the present study, we demonstrated that 4-HR stimulated apoptosis of SCC-9 cells, but not of PHGF.

4-HR increases epithelial cell differentiation in SCC-9 cells (15). The calcium is important in the epithelial cell differentiation (22). 4-HR inhibits TG-2 activity (14) and the activity of TG-2 is also calcium dependent (23). Therefore, the molecular mechanisms of the observed 4-HR-mediated effects may be partly dependent on calcium. Calcium uptake is due, in part, to upregulation of voltage-dependent calcium channels; calcium channel blockers attenuated 4-HR-mediated cellular effects include protein kinase C-α, which plays an important role in calcium-induced keratinocyte differentiation (22). The effects of 4-HR on cancer cell proliferation and differentiation may also be due to non-specific interactions with other proteins (24), changes in membrane permeability (25), and/or antioxidant and, thus, anti-mutagenic activities (26). A possible mechanism concerning the different uptake of calcium ion by 4-HR in OSCC cells and fibroblasts might be related to the glutamate receptor. The gene expression of ionotropic glutamate receptors was decreased by 4-HR application (data not shown). Glutamate receptor is related to calcium oscillation (27,28) and OSCC highly expresses glutamate receptor (29). However, the glutamate receptor-related hypothesis remains to be confirmed in further experimental studies.

The effects of 4-HR on apoptosis are dose-dependent; relatively high doses (5-10 μg/ml) of 4-HR caused the rupture of cellular membranes. This is similar to the effect of conventional anticancer drugs, which are typically toxic and induce apoptosis in cancer cells (30). However, the anti-proliferative effect of 4-HR at a low concentration (1 μg/ml) was not accompanied by cytotoxicity or apoptosis. In the previous 16 days, toxicology and carcinogenesis studies demonstrated that oral doses of 4-HR up to 500 mg/kg did not affect the survival of experimental animals (12). In the present study, the effective dose of 4-HR was significantly lower (10 mg/kg body weight) than a previous animal study (12). However, prolonged use of 4-HR causes nephropathy and osteosclerosis in humans (31) as well as in animals (11). This may be due to increased intracellular calcium concentrations and the inhibition of the NF-κB pathway (13).

Collectively, our results suggest that 4-HR has strong antitumor effects by inhibiting calcium channel oscillation and inducing apoptosis. The antitumor effects of 4-HR were partly reversed by the application of calcium channel blockers.

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