A DNA Methyltransferase Inhibitor and All-trans Retinoic Acid Reduce Oral Cavity Carcinogenesis Induced by the Carcinogen 4-Nitroquinoline 1-Oxide

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Abstract The transcriptional silencing of some cell cycle inhibitors and tumor suppressors, such as p16 and retinoic acid receptor β2, by DNA hypermethylation at CpG islands is commonly found in human oral squamous carcinoma cells. We examined the effects of the DNA methyltransferase inhibitor 5-Aza-2′-deoxycytidine (5-Aza; 0.25 mg/kg body weight), all-trans retinoic acid (RA; given at 100 μg/kg body weight and 1 mg/kg body weight), and the combination of 5-Aza and the low-dose RA on murine oral cavity carcinogenesis induced by the carcinogen 4-nitroquinoline 1-oxide (4-NQO) in a mouse model. All the drug treatments were done for 15 weeks after a 10-week 4-NQO treatment. Mice in all drug treatment groups showed decreases in the average numbers of neoplastic tongue lesions. The combination of 5-Aza and RA effectively attenuated tongue lesion severity. Although all drug treatments limited the increase in the percentage of proliferating cell nuclear antigen–positive cells and the decrease in the percentage of p16-positive cells caused by the 4-NQO treatment in mouse tongue epithelial regions without visible lesions and in the neoplastic tongue lesions, the combination of 5-Aza and RA was the most effective. Collectively, our results show that the combination of a DNA demethylating drug and RA has potential as a strategy to reduce oral cavity cancer in this 4-NQO model.

Oral squamous cell carcinoma (SCC) is one of the most common cancers in the world (1). Although the cure rate for small primary tumors is high, many patients will develop second primary tumors and the long-term survival rate for this cancer is <60% (1). Two major etiologic factors in oral cavity SCC are the use of tobacco and alcohol, and malignant transformation of the oral cavity tissue is thought to be related to exposure to certain carcinogens (2). Oral cavity SCC development is a complicated, multistep process that involves genetic, epigenetic, and metabolic changes (3). About 60% to 70% of oral cavity carcinoma cases are diagnosed only after the tumors have become locally advanced (4). Therefore, in addition to treatment, prevention (i.e., a reduction in the incidence of cancer and/or the inhibition of the development of malignant lesions) of oral cancer is a very important goal.

Compared with normal cells, human cancer cells exhibit epigenetic changes, defined as the alterations of gene expression via mechanisms other than the changes of the DNA sequences of these genes. These epigenetic changes include alterations in DNA methylation status and chromatin modifications. The alterations in DNA methylation include global hypomethylation of cytosines in intergenic regions of the genome and hypermethylation of CpG islands (increases in cytosine methylation) in the promoter regions of some genes (5). These epigenetic changes, especially the hypermethylation of the gene promoter regions, occur very early during cancer development (6). The expression of some tumor suppressor genes is transcriptionally silenced by this DNA hypermethylation in their promoter regions, which is mediated by DNA methyltransferases (Dnmt; ref. 5). De novo DNA methylation and transcriptional silencing mediated by overexpression of Dnmt3b promote mouse colon carcinogenesis in vivo (7). Conversely, the deletion of Dnmt3b greatly reduced mouse intestinal tumor formation (8). Studies have shown that Dnmt inhibitors 5-Aza-2′-deoxycytidine (5-Aza) and/or zebularine suppress the development of various cancers in mouse models, including intestinal carcinogenesis in Apc (Min/+) mice (9) and prostate cancer in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice (10, 11). DNA hypermethylation of the promoters of genes that encode some cell cycle inhibitors and tumor suppressors, such as p16 and retinoic acid receptor β2 (RARβ2), is commonly seen in human oral squamous carcinoma cells (12). Therefore, Dnmts are targets for both cancer prevention and treatment (13, 14). The reversal of DNA hypermethylation by the drug 5-Azacytidine, which inhibits Dnmts, has proven to be effective in the treatment of several human cancers, including head and neck cancer (12).
However, whether the reversal of DNA hypermethylation has preventive effects on oral cavity carcinogenesis is not clear.

Retinoids, including vitamin A (retinol) and its derivatives such as all-trans retinoic acid (RA), regulate cell proliferation and differentiation (15). RA regulates gene expression by binding and activating RARs and retinoid X receptors, which heterodimerize and associate with retinoid acid response elements in the genome (16). There are three RARs and three retinoid X receptors (α, β, and γ) and each subtype has various isoforms, and the binding of RA causes a conformational change in the RAR/retinoid X receptor heterodimers that results in their dissociation from corepressor complexes and their association with coactivators (17).

Epidemiologic studies on human populations have shown that a higher retinol intake in the diet is associated with the inhibition of the progression of carcinogenesis in lung, breast, cervix, prostate, gastrointestinal tract, kidney, and oral cavity (18). Preclinical and clinical studies have shown that retinoids can be used as cancer chemopreventive and chemotherapeutic agents in human cancers, such as SCC of the head and neck (SCCHN; refs. 4, 15, 19, 20). In addition, Conway et al. (21) showed that RA treatment suppressed the formation of cancer stem cells in primary tumors. The antitumor activities of RA are carried out predominantly through RARβ2, which acts as a tumor suppressor on binding the agonist RA (17). In addition, RARβ2 gene transcription is activated through a retinoic acid response element in its promoter region on RA treatment (22). However, aberrant retinoid signaling, such as the reduction or the loss of the expression RARβ2, has been found in various human cancers, including human oral SCC cells (17). The loss of RARβ2 expression during cancer development is often associated with retinoid resistance, and the mechanism of the loss of RARβ2 expression usually involves the hypermethylation of the RARβ2 gene promoter region (23). In addition, 5-Aza has proven to be able to restore the inducibility of RARβ2 expression by RA in human SCCHN cells in which the promoter region of RARβ2 is hypermethylated (24). Collectively, these data indicate the possibility of combining RA and 5-Aza for the prevention of oral cavity carcinogenesis (Fig. 1).

Several animal models that mimic certain aspects of human oral cancer have been established, including hamster, rat, and mouse models (2, 25–27). Oral SCCs induced in mice by the carcinogen 4-nitroquinoline 1-oxide (4-NQO) added to the drinking water show similarities to human oral tumors in terms of their morphologic, histopathologic, and molecular characteristics (2, 28, 29). Therefore, this murine 4-NQO model is an excellent model to evaluate a variety of potential cancer preventive and therapeutic approaches. In this study, we examined the cancer preventive effects of RA, 5-Aza, and the combination of RA and 5-Aza on oral cavity carcinogenesis by using this mouse model.

Materials and Methods

Tumor development in the mouse oral cavity and drug treatments

Six-week-old wild-type C57BL/6 female mice were used for this study. The experiments were carried out under controlled conditions with a 12-h light/dark cycle. Animals were maintained on a normal chow (LabDiet with constant nutrition, LabDiet Co.). The mice were treated with propylene glycol (vehicle as a negative control, n = 5) or 100 μg/mL of the carcinogen 4-nitroquinoline-1-oxide (4-NQO; Sigma), made up in vehicle, in the drinking water as previously described (2) for 10 wk. Then, the mice were allowed access to the regular drinking water.

Two weeks after termination of the carcinogen treatment, mice received various drug treatments by i.p. injection: PBS (n = 10), 5-Aza (n = 10; Sigma), RA (n = 10; Sigma), 5-Aza+RA (n = 10), and high-dose RA (HDRα; n = 10). PBS (0.1 mL) was injected twice weekly, 5-Aza (250 μg/kg body weight; ref. 10) was injected i.p. twice weekly on consecutive days in 0.1 mL PBS, low-dose RA (LDRα; 100 μg/kg body weight) was injected twice weekly in 0.1 mL vegetable oil, and HDRα (1 mg/kg body weight) was injected once weekly in 0.1 mL vegetable oil. Mouse body weights and the precancerous and cancerous lesions in the oral cavities were monitored at different times for up to 15 wk or until signs of sickness or weight loss. Ten mice per group was chosen because, based on ANOVA analysis, this gives the minimum largest difference in means with 80% power at a 5% significance level in this study.

Measurement of serum retinoid levels

Before the sacrifice, mice in PBS (4-NQO/PBS), LDRA (4-NQO/LDRA), 5-Aza (4-NQO/5-Aza), the combination of RA and 5-Aza (4-NQO/5-Aza+LDRA), and HDRα (4-NQO/HDRα) treatment groups received the final drug injections. Ninety minutes later, blood samples (~100 μL each) from these mice were obtained via mouse tails. The analyses of retinoids in the mouse serum (from three mice per group) were done by high-performance liquid chromatography (HPLC) as previously reported (30, 31). Retinoids were identified by HPLC based on two criteria: an exact match of the retention times of unknown peaks with those of authentic retinoid standards and identical UV light spectra (220-400 nm) of unknowns against spectra from authentic retinoid standards during HPLC by the use of a photodiode array detector (31). The levels of retinoids were normalized to the volume of the serum measured.

Tissue dissection, lesion grade measurement, and pathologic diagnosis

The tongues of mice were dissected immediately after cervical dislocation. Gross lesions were identified and photographed, and visible cancerous lesions on the tongues were counted for the examination of multiplicity of these lesions (number of lesions per mouse) with a ×10 magnification. The severity of gross lesions on the tongues was quantified by a grading system that included 0 (no lesion), 1 (mild lesion), 2 (intermediate lesion), 3 (severe lesion), and 4 (most severe lesion) in a double-blinded manner, respectively, and the average grades from different treatment groups were used for the analyses of the tongue lesions. Mouse tongue lesions were cut in half longitudinally. Half of each tissue was fixed in freshly made 4% paraformaldehyde overnight at 4°C, embedded in paraffin, and sectioned into 5-μm sections. The other half of each tongue tissue was immediately placed in RNAlater solution (Ambion) and stored at 4°C overnight before being moved to −20°C for long-term storage. The histologic diagnosis of squamous neoplasia (four representative mice per group based on the lesion grades) was done by a pathologist (T.S.) on the H&E-stained, sectioned tissue samples. The lesions observed were classified into three types: epithelial hyperplasia, dysplasia (mild, moderate, and severe), and SCC, as described previously (2, 28).

Immunohistochemistry

Paraffin-embedded sections (from four mice per group) were deparaffinized and rehydrated, and antigen retrieval was done. The tissue sections were stained by using the M.O.M. kit (for the p16 antibody; Vector Laboratories) or the Envision + HRP (DAB+) kit [for the proliferating cell nuclear antigen (PCNA) antibody; Dako] or the Zymed SuperPicture kit [for the cyclooxygenase-2 (COX-2) antibody; Zymed Laboratories]. After quenching endogenous peroxidase with 3% H2O2, the tissue sections were blocked with the blocking reagent (from the M.O.M. kit) or 1.5% goat serum. Then, the tissue...
sections were incubated with mouse PCNA antibody (1:100; mouse monoclonal antibody; Dako), p16 antibody (1:200; mouse monoclonal antibody; Santa Cruz Biotechnology), or mouse COX-2 antibody (1:100; rabbit polyclonal antibody; Cayman Chemical), respectively, for 60 min at room temperature. The sections were then incubated with secondary antibodies [1:200, anti-mouse IgG from the M.O.M kit for p16; ready-to-use anti-rabbit IgG from the Zymed SuperPicture kit for COX-2; and ready-to-use anti-mouse IgG from the Dako Envision + HRP (DAB+) kit for mouse PCNA]. As a negative control, sections were stained without incubation with primary antibodies. Finally, signals were visualized based on a peroxidase detection mechanism, and 3,3′-diaminobenzidine (Zymed SuperPicture kit) was used as the substrate. The cells with distinct nuclear staining were regarded as positive for PCNA and p16. Four to five representative areas of each mouse tongue section were photographed and analyzed. Percentages of PCNA and p16-positive cells (labeling indices) were determined to reveal the number of these positive nuclei divided by the total epithelial cell nuclei in the areas.

**Real-time reverse transcription-PCR analysis**

Total RNA from mouse tongues was extracted and reverse transcribed to cDNA as described previously (28). Real-time PCR was done using gene-specific oligonucleotide primers, which generate cDNA fragments that cross an intron-exon boundary in the genomic DNA. Real-time PCR was done on a MyIQ real-time PCR detection system (Bio-Rad Laboratories) with an iQ SYBR Green Supermix. The conditions for the PCR were as follows: 95°C for 3 min to activate the DNA polymerase, followed by 50 cycles at 94°C for 30 s, primer annealing at 58°C for 30 s, and product extension at 72°C for 30 s. After each cycle, fluorescence was read at 84°C. 36B4 was used as a control (32). The primer sequences were as follows: mouse COX-2, 5′-TCAAAAGAAGTCGTGAAAAAGGTT-3′ (forward) and 5′-TCTACCTAGTGTTTCTGTA-3′ (reverse); mouse RARβ2, 5′-GATCTGGATTTCTACACCG-3′ (forward) and 5′-CACTGACGC-3′ (reverse); mouse c-Myc, 5′-AGAACAACCCAGCTTGAGAAA-3′ (forward) and 5′-ACACCTCCAGAAAGCCAGAGT-3′ (reverse). We used the University of California, Santa Cruz In-Silico PCR program3 to ensure that the PCR primers were not homologous to pseudogene sequences.

**Statistical analysis of the data**

The statistical analyses of the results were carried out using a one-way ANOVA test followed by a Bonferroni’s multiple comparison test, Fisher’s exact probability test, and Wilcoxon rank sum test for multiple comparisons. Differences with a P value of <0.05 were considered statistically significant.

**Results**

**Oral cavity carcinogenesis**

All of the mice survived the 10-week 4-NQO treatment, and >90% of the mice survived throughout the 15-week posttreatment period (Fig. 2A). Consistent with our previous studies (2, 28), we did not observe any gross lesions immediately after the end of the 10-week 4-NQO treatment. However, multifocal, precancerous, and cancerous lesions (papillomas and SCCs) developed during the 15-week post-4-NQO treatment period, and these lesions were primarily seen on the dorsal side of the tongue (Fig. 2B). In contrast, no visible lesions (grade 0) were detected in the control mice not treated with 4-NQO (Fig. 2B-D). The gross cancerous tongue lesion multiplicity (number of lesions per mouse) was examined. After the 4-NQO treatment, mice that received PBS injections (4-NQO/PBS) developed multiple cancerous tongue lesions, and mice in all drug treatment groups (4-NQO/5-Aza, 4-NQO/LDRA, 4-NQO/5-Aza+LDRA, and 4-NQO/HDRA) were palpable, with a statistically significant increase in the number of gross lesions (tumors and lumps) compared with the control group (4-NQO/PBS).

**Combination of RA and 5-Aza therapy**

We hypothesized that the combination of RA and 5-Aza would disrupt the 4-NQO-induced tumor development and progression. This hypothesis was based on the following findings: (a) RARβ gene expression was significantly upregulated in 4-NQO/PBS treated mice compared with control mice, whereas 5-Aza alone did not change the RARβ2 expression levels (Fig. 3A). (b) The combination of RA and 5-Aza significantly altered the expression levels of RARβ2, compared with 4-NQO/5-Aza or 4-NQO/PBS treatment alone (Fig. 3B). (c) The combination of RA and 5-Aza significantly altered the expression levels of RARβ2, compared with 4-NQO/5-Aza or 4-NQO/PBS treatment alone (Fig. 3B). (d) The combination of RA and 5-Aza significantly altered the expression levels of RARβ2, compared with 4-NQO/5-Aza or 4-NQO/PBS treatment alone (Fig. 3B). (e) The combination of RA and 5-Aza significantly altered the expression levels of RARβ2, compared with 4-NQO/5-Aza or 4-NQO/PBS treatment alone (Fig. 3B). (f) The combination of RA and 5-Aza significantly altered the expression levels of RARβ2, compared with 4-NQO/5-Aza or 4-NQO/PBS treatment alone (Fig. 3B). (g) The combination of RA and 5-Aza significantly altered the expression levels of RARβ2, compared with 4-NQO/5-Aza or 4-NQO/PBS treatment alone (Fig. 3B). (h) The combination of RA and 5-Aza significantly altered the expression levels of RARβ2, compared with 4-NQO/5-Aza or 4-NQO/PBS treatment alone (Fig. 3B).

**Statistical analysis**

The statistical analyses of the results were carried out using a one-way ANOVA test followed by a Bonferroni’s multiple comparison test, Fisher’s exact probability test, and Wilcoxon rank sum test for multiple comparisons. Differences with a P value of <0.05 were considered statistically significant.

**Discussion**

Our findings support our hypothesis that 5-Aza combined with RA therapy is effective in reducing the number of gross tongue lesions induced by 4-NQO. This is the first study to show that the combination of RA and 5-Aza can significantly reduce the number of gross tongue lesions induced by 4-NQO. This finding has important implications for the development of new therapeutic strategies for oral cavity cancer prevention.

**Conclusion**

In conclusion, our study demonstrates that the combination of RA and 5-Aza is an effective therapeutic strategy for preventing oral cavity cancer. Further studies are needed to determine the mechanism of action of this combination therapy and to evaluate its efficacy in preventing oral cavity cancer in humans.
showed statistically significant decreases in the numbers of cancerous tongue lesions (Fig. 2C). Moreover, mice in the 4-NQO/PBS group developed severe tongue lesions; 70% of the tongues in this group showed lesions more severe than grade 2, and no mice developed tongue lesions of grade 0 and 1 (Fig. 2D, PBS). Both 5-Aza treatment alone (4-NQO/5-Aza; \( P = 0.71 \)) and LDRA treatment alone (4-NQO/LDRA; \( P = 0.26 \)) showed trends of reduced incidence of the higher-grade (grade 2-4) mouse tongue lesions such that the incidence of lower-grade (grade 0-1) tongue lesions was higher as compared with the 4-NQO/PBS–treated mice (Fig. 2D, compare 5-Aza alone or LDRA alone with PBS). This occurred because these 4-NQO/PBS–treated mice primarily exhibited high-grade lesions (Fig. 2D, PBS). HDRA treatment (4-NQO/HDRA) resulted in a trend toward greater incidence of lower-grade (grade 1-2, 56%) and a lower incidence of
higher-grade (grade 2-4, 33%; \( P = 0.46 \)) tongue lesions (Fig. 2D, HDRA). Mice treated with the combination of 5-Aza+LDRA also showed a greater incidence (63%) of less severe tongue lesions of grades 0 and 1 (Fig. 2D, 5-Aza+LDRA). Statistical analyses with the Fisher’s exact probability test showed a statistically significant difference (\( P = 0.01 \)) in tongue lesion grades between the PBS group and the 5-Aza+LDRA combination group (Fig. 2D). In addition, we have previously observed precancerous and cancerous lesions in mouse esophagi at a much lower frequency (2). However, in this article, we focused on oral cavity carcinogenesis.

We also did pathologic analyses on the tongue sections from 4-NQO–treated mice. As shown in the representative pictures (Fig. 3A), the mouse tongue sections contained different stages of oral carcinogenesis, including hyperplasia, dysplasia (mild, moderate, and severe), and carcinoma (including carcinoma \textit{in situ} and invasive carcinoma), with some samples containing multiple types of lesions. Clinical studies have shown that the dysplasias with high grades have a greater probability of developing into carcinomas than the lower grades of dysplasia (33). In this study, we used a two-category system (normal/hyperplasia/mild or moderate dysplasia: low risk; severe dysplasia/carcinoma \textit{in situ} and invasive carcinoma) with the Fisher’s exact probability test showed a statistically significant difference (\( P = 0.01 \)) in tongue lesion grades between the PBS group and the 5-Aza+LDRA combination group (Fig. 2D). In addition, we have previously observed precancerous and cancerous lesions in mouse esophagi at a much lower frequency (2). However, in this article, we focused on oral cavity carcinogenesis.

To examine whether injected RA affected mouse serum retinoid levels, 90 minutes after the final drug injections, mouse serum samples were collected and the serum retinoid levels were analyzed by HPLC (three mice per group). In control mouse serum, a retinol peak was detected at the retention time of 33 minutes (Fig. 4B). The sera from mice treated with 4-NQO and subsequently treated with PBS (4-NQO/PBS), 5-Aza (4-NQO/5-Aza), LDRA (4-NQO/LDRA), the combination of 5-Aza and LDRA (4-NQO/5-Aza+LDRA), and HDRA (4-NQO/HDRA), respectively, showed similar HPLC tracings (Fig. 4C and D; data not shown). The serum retinol concentrations from these mice were not significantly different from those in the control mouse serum (Fig. 4E). Given the small volumes of sera (100 μL) collected, RA was detected only in the serum samples from the 4-NQO/HDRA group, and the serum RA concentration was 0.078 ± 0.012 μmol/L, which is close to the detection limit of HPLC quantification of RA (Fig. 4D). We did not do detailed pharmacokinetic studies on the drug RA given i.p. However, our data show that 90 minutes after an i.p. injection, RA could be detected in the sera of HDRA mice, and the serum RA concentration...
was similar to the peak plasma RA level (0.07 ± 0.009 μmol/L), which was detected between 60 and 180 minutes following a single oral administration of this drug (10 mg/kg body weight) in BALB/c nude mice (34).

**Effects of various drug treatments on RARβ2, COX-2, and c-Myc mRNA levels in mouse tongues**

Compared with normal epithelial tissues, RARβ2 mRNA levels are usually decreased or absent in SCCHNs often because of the hypermethylation of the RARβ2 promoter (17, 35). The restoration of RARβ2 has been reported to suppress the proliferation of various cancer cells in vitro and in vivo (23). The antitumor activity of RARβ2 in human esophageal cancer cells correlates with the suppression of COX-2 protein expression (36). Because 5-Aza has been shown to restore transcription of genes by reversing DNA hypermethylation at the promoter regions (24), we measured the RARβ2 mRNA levels by real-time reverse transcription-PCR in the tongues of control
(not 4-NQO–treated) and 4-NQO–treated mice that were subsequently treated with 5-Aza and/or other drugs in the post–4-NQO phase of the experiment. We observed high RARβ2 mRNA levels in all of our control mouse tongues (n = 5), and 4-NQO treatment resulted in a significant and consistent reduction in RARβ2 mRNA levels in all tongues of mice that subsequently received PBS treatment (4-NQO/PBS; n = 10; Fig. 5A). Among the mice that received the 4-NQO/5-Aza treatment (n = 10), 60% of the tongues showed higher tongue RARβ2 mRNA levels than the highest RARβ2 mRNA level detected in the tongues of mice that received only PBS injections. In the 4-NQO/LDRA group (n = 9), 56% of mice showed higher tongue RARβ2 mRNA levels than the highest RARβ2 mRNA level detected in the tongues of the PBS injection group. In the 4-NQO/5-Aza+LDRA group, 38% of mouse tongues (n = 8) showed higher RARβ2 mRNA levels than those detected in the tongues of 4-NQO/PBS–treated mice. In the 4-NQO/HDRA group (n = 9), 56% of mice showed higher tongue RARβ2 mRNA levels than the highest RARβ2 mRNA level detected in the tongues of 4-NQO/PBS–treated mice (Fig. 5A). However, the SDs were large in these assays, and we did not observe statistically significant differences in RARβ2 mRNA levels among the different drug treatment groups. The P values ranged from 0.07 to 0.90.

The COX-2 expression level is elevated in oral cavity cancers (37). During oral cavity carcinogenesis, COX-2 expression increases during the malignant transition of the oral epithelium (4). Therefore, COX-2 has been proposed as a promising molecular target for oral cancer prevention (4). We measured COX-2 mRNA levels by real-time PCR in tongues from control (not 4-NQO–treated) mice and in tongues from mice treated with 4-NQO and subsequently treated with PBS or with various drugs. In control (not 4-NQO–treated; n = 5) mouse tongues, we did not detect COX-2 mRNA expression (Fig. 5B). Carcinogen treatment resulted in COX-2 mRNA expression in all of the mouse tongues from the 4-NQO/PBS–treated group (n = 10). The 4-NQO/5-Aza–treated, 4-NQO/LDRA–treated, 4-NQO/5-Aza+LDRA–treated, and 4-NQO/HDRA–treated mouse tongues did not show statistically significant differences from the 4-NQO/PBS–treated group in COX-2 mRNA levels (Fig. 5B).

It has been reported that c-Myc transcripts are greatly overexpressed in advanced tumor stages of human SCCHN (38). We also assessed c-Myc mRNA expression in these mouse tongues. c-Myc mRNA in control (not 4-NQO–treated) mouse tongues (n = 5) was detected, and after 4-NQO treatment, 30% of mouse tongues from the 4-NQO/PBS group showed greater c-Myc mRNA levels than the control group (Fig. 5C). Among all of the different drug treatments, only the tongues from the 4-NQO/5-Aza+LDRA group showed a trend (not statistically significant) of decreased c-Myc mRNA levels. In fact, these c-Myc levels were even lower than those in the control (not 4-NQO–treated) group (Fig. 5C).

**Effects of various drugs on PCNA, p16, and COX-2 protein levels in mouse tongues**

PCNA is a protein that is expressed in the cell nuclei during the S phase of the cell cycle, and this protein is needed for DNA polymerase δ to bind DNA (39). Therefore, PCNA is a marker of cell proliferation. We measured the PCNA protein levels in mouse tongues by immunostaining. Very few nuclei...
in the tongue epithelia from the control (not 4-NQO–treated) mice showed PCNA-positive cells, and 4-NQO treatment resulted in a large increase in the percentages of PCNA-positive nuclei in both the mouse tongue epithelial regions without visible lesions and the regions with visible lesions (Fig. 6A and C). All drug treatments limited the increase in the percentages of PCNA-positive cells in these tongue regions significantly, and the combination of 5-Aza and RA had the greatest effect in terms of reducing the percentage of PCNA-positive cells (Fig. 6A, c-f, B, C, b-e, and D).

During the process of carcinogenesis, dysregulation of the cell cycle is a critical event (40). The loss of expression of p16 protein, one of the cell cycle inhibitors, has been observed in oral premalignant lesions and primary tumors of the oral cavity (41). Therefore, we examined the effects of different drug treatments on p16 protein expression in the mouse tongues. In control (not 4-NQO–treated) mouse tongues, p16 nuclear staining was observed in the epithelial basal and suprabasal layers but primarily in the basal layer (Fig. 6E, a). The carcinogen 4-NQO treatment caused a large reduction in p16 nuclear staining in the tongue lesions of the PBS treatment group (Fig. 6E, b). After 4-NQO treatment, 5-Aza treatment alone did not prevent the decrease in the percentage of p16-positive nuclei in the tongue lesions (Fig. 6E, c). However, treatments with LDRA, the combination of 5-Aza and LDRA, and HDRA limited the decrease in p16 nuclear staining in the tongue lesions (Fig. 6E, d-f, and F).

Finally, we investigated COX-2 protein expression in the mouse tongues. In control (not 4-NQO–treated) mouse tongue epithelia, we did not observe COX-2 staining, and 4-NQO treatment caused a major increase in COX-2 protein staining in the tongue lesions of the 4-NQO/PBS treatment group (Fig. 6G, a and b). After 4-NQO treatment, all drug treatments partially reduced the increase in COX-2 protein levels in mouse tongue lesions (Fig. 6G, c-f).
Discussion

Epigenetic silencing of tumor suppressor genes by the hypermethylation of their promotor regions plays an important role in many human cancers (12). Inhibition of Dnmt activities has been reported to be effective in the treatment of some human cancers, including human SCCHN (12). In this study, we show that the combination of a DNA demethylating drug (5-Aza) and RA effectively attenuates oral cavity tumorigenic progression in a mouse model of human oral cavity cancer (Figs. 2C and D and 3B).

Oral cavity tumor prevention in a mouse model

5-Aza reduced the development of prostate cancer in TRAMP mice (a prostate cancer mouse model) and delayed the progression of preexisting prostate cancer to more advanced stages (10, 11). 5-Aza also restored p16 mRNA expression in human oral SCC cells that had reduced p16 expression and restored the inducibility of RARβ2 by RA in human SCCHN cells (24, 42). Retinoids, especially RA, although regarded as candidates for cancer chemoprevention and chemotherapy, are not effective over the long term when given orally as a single drug in oral cavity cancer prevention clinical trials (17, 43). The combination of 5-Aza and RA inhibits the proliferation of human cancer cells, including SCCHN cells (24, 44). Our data suggest that the combination of 5-Aza and RA has potential for cancer prevention and treatment (Figs. 2 and 3). Consistent with these previous studies, we found that although 5-Aza or RA (low- and high-dose) treatment alone reduced the multiplicity of mouse
tongue lesions induced by 4-NQO (Fig. 2C), either drug alone did not reduce the severity of these lesions (Fig. 2D). In contrast, the combination of 5-Aza and LDRA reduced both the multiplicity and the severity of tongue lesions induced by 4-NQO (Fig. 2C and D) and potentially reduced the probability of oral carcinogenesis (Fig. 3B). Moreover, the combination of 5-Aza and RA limited the 4-NQO–induced increase in the percentage of PCNA-positive cells and the decrease in the percentage of p16-positive cells in mouse tongues (Fig. 6-A-F). Our findings indicate that the combination of 5-Aza and LDRA is an effective drug combination for the reduction of oral cancer in this murine 4-NQO model. However, although the combination of the drugs 5-Aza and LDRA significantly reduced the severity of tongue lesions, this treatment did not completely block the formation of the lesions (Figs. 2C and D and 3B). Moreover, the combination of 5-Aza and LDRA did not reduce PCNA and increase p16 labeling indices, respectively, to levels similar to those in the control (not 4-NQO–treated) mouse tongue epithelia. Our results show that the combination of 5-Aza and LDRA does not totally prevent the hyperproliferation of tongue epithelial cells and the formation of initial tongue lesions. In addition, our results show that further investigation of different doses of these drugs for the prevention of oral cancer would be important.

We also observed that mice treated with LDRA and HDRA after 4-NQO showed a smaller decrease in p16 nuclear staining in the tongue lesions as compared with mice given PBS after 4-NQO (Fig. 6E and F). During oral cavity carcinogenesis, methylation of the p16 gene promoter does not occur in all mouse tongue epithelial cells (42). It has also been reported that RARα can induce p16 protein expression in neoplastic epidermal keratinocytes (45). Therefore, RA treatment could activate p16 transcription in some cells in which this gene promoter is not methylated.

**Effects of drug treatments on RARβ2, COX-2, and c-Myc expression in the mouse tongue lesions**

The silencing of RARβ2 expression by the hypermethylation of its promoter at the early stages of head and neck cancer development has been associated with retinoid resistance (17, 23). Previous studies have shown that 5-Aza treatment inhibited the proliferation of human SCCN4 cells, induced RARβ2 mRNA expression, and restored the inducibility of RARβ2 by RA in these cells (24). In addition, the combination of 5-Aza and a histone deacetylase inhibitor, trichostatin A, improved the induction of RARβ2 mRNA expression by RA in human breast cancer cells and head and neck cancer cells (46, 47). We found that after 4-NQO treatment, all drug treatments limited the reduction in RARβ2 mRNA in mouse tongues as compared with the PBS group (Fig. 5A).

COX-2 has been considered to be a cancer chemopreventive target for human SCCN4 (4). We show here that COX-2 mRNA is not detected in the control (not 4-NQO–treated) mouse tongues and that 4-NQO treatment increases COX-2 mRNA levels (Fig. 5B), data that are consistent with previous studies (48). Mestre et al. (49) reported that RA inhibited COX-2 mRNA and protein expression in human oral squamous carcinoma cells. However, we did not observe statistically significant differences in COX-2 mRNA levels in the mouse tongues between the drug treatment groups and the PBS group (Fig. 5B). In addition, our results show a trend that all drug treatments limited the increase in COX-2 protein levels in mouse tongue lesions induced by 4-NQO (Fig. 6G), consistent with the previous reports (49).

It has been reported that the RA alone and the combination of 5-Aza and RA could suppress c-Myc mRNA expression in HL-60 myeloid leukemic cells (50). Similar to their findings, our data showed a trend that the combination of 5-Aza+LDRA decreased c-Myc mRNA levels in 4-NQO–treated mouse tongues (Fig. 5C). Taken together, our data suggest that a DNA demethylating drug could be useful for the prevention of oral cavity cancer and that the combination of a DNA demethylating drug and RA is a potentially useful approach for the prevention of oral cavity cancer in high-risk individuals.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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A DNA Methyltransferase Inhibitor and All-trans Retinoic Acid Reduce Oral Cavity Carcinogenesis Induced by the Carcinogen 4-Nitroquinoline 1-Oxide

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