Impact of Short-term 1,25-Dihydroxyvitamin D3 on the Chemopreventive Efficacy of Erlotinib against Oral Cancer


Abstract

Activation of the epidermal growth factor receptor (EGFR) pathway is an early event in head and neck carcinogenesis. As a result, targeting EGFR for chemoprevention of head and neck squamous cell carcinomas (HNSCC) has received considerable attention. In the present study, we examined the impact of 1,25(OH)2D3, the active metabolite of the nutritional supplement vitamin D, on the chemopreventive efficacy of the EGFR inhibitor, erlotinib, against HNSCC. Experimental studies were conducted in patient-derived xenografts (PDX) and the 4-nitroquinoline-1-oxide (4NQO) carcinogen-induced model of HNSCC. Short-term treatment (4 weeks) of PDX-bearing mice with 1,25(OH)2D3 and erlotinib resulted in significant inhibition of tumor growth. Noninvasive MRI enabled longitudinal monitoring of disease progression in the 4NQO model with 100% of control animals showing evidence of neoplastic lesions by 24 weeks. Among the experimental groups, animals treated with the combination regimen showed the greatest reduction in tumor incidence and volume (P < 0.05). Combination treatment was well tolerated and was not associated with any significant change in body weight. Histopathologic assessment revealed a significant reduction in the degree of dysplasia with combination treatment. Immunoblot analysis of whole tongue extracts showed downregulation of phospho-EGFR and phospho-Akt with the combination regimen. These results highlight the potential of 1,25(OH)2D3 to augment the efficacy of erlotinib against HNSCC. Further optimization of schedule and sequence of this combination regimen along with investigation into the activity of less calcemic analogues or dietary vitamin D is essential to fully realize the potential of this approach.

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Introduction

The epidermal growth factor receptor (EGFR), a membrane-bound receptor that belongs to the Erb-B/HER family of receptors, is overexpressed in approximately 80% to 90% of head and neck squamous cell carcinomas (HNSCC; refs. 1, 2). Encouraging results from preclinical studies led to the clinical evaluation and subsequent approval of cetuximab, a monoclonal antibody against EGFR, for clinical use in HNSCC (3, 4). Another EGFR-targeted tyrosine kinase inhibitor, erlotinib, is currently undergoing clinical evaluation in the treatment of HNSCC (5).

Activation of the EGFR is considered to be an early event in the genesis of HNSCC (6, 7). Increased production of EGFR mRNA has been reported in histologically normal mucosa of patients at risk for primary or secondary head and neck cancers possibly due to “field cancerization” (6). Taoudi Benkekrout and colleagues have shown that EGFR expression and gene copy number can predict the progression of premalignant lesions to oral SCC (7). These observations have led to the investigation of EGFR as a target for chemoprevention. Erlotinib is currently undergoing clinical evaluation for its chemopreventive potential in head and neck cancer patients (8, 9). Novel combination approaches to augment the chemopreventive efficacy of erlotinib are also under investigation (10, 11). In this regard, natural compounds or nutritional supplements with favorable safety profiles are attractive for use as potential “bio-adjuvants” for cancer prevention (10, 11). In the present study, we examined the impact of 1,25-dihydroxyvitamin D3 [1,25(OH)2D3], the active metabolite of the nutritional supplement vitamin D, as a bio-adjuvant, on the chemopreventive efficacy of erlotinib against HNSCC.

Vitamin D is a secosteroid pro-hormone that regulates calcium and phosphorus metabolism in vivo through its actions on the kidneys, intestines, and bone (12). Epidemiologic studies have suggested that vitamin D could serve as a potential risk-modifying factor in cancer (13, 14). Vitamin D can be obtained from the diet or synthesized from 7-dehydrocholesterol in the skin following UV light exposure. It is initially transported to the liver where it is hydroxylated to 25-hydroxyvitamin D3 and then further hydrolyzed in the kidneys to 1,25(OH)2D3 (12). Preclinical studies have shown that 1,25(OH)2D3 can inhibit the growth and proliferation of SCC cells at nanomolar concentrations.
The antitumor activity of 1,25(OH)2D3 has been attributed to a spectrum of mechanisms ranging from cell-cycle arrest to induction of apoptosis and inhibition of angiogenesis (16–18). Activation of the EGFR results in downstream activation of PI3K-AKT signaling that is involved in cell survival, proliferation, and angiogenesis (19). 1,25(OH)2D3 has been shown to decrease phosphorylation of Erk and Akt that is necessary for their activation (20). On the basis of this knowledge, we hypothesized that targeting these interacting pathways using 1,25(OH)2D3 in combination with erlotinib could be an effective strategy to prevent HNSCC. To test this hypothesis, experimental studies were carried out in patient-derived xenografts (PDX) and a carcinogen-induced model of HNSCC. Noninvasive imaging in combination with histologic and molecular analyses was performed to examine the safety and efficacy of this combination strategy against HNSCC.

**Materials and Methods**

**Tumor models**

Experimental studies were carried out using patient-derived HNSCC xenografts established in SCID mice (Laboratory Animal Resource, RPCI) and C57Bl/6 mice (National Cancer Institute, Rockville, MD) exposed to the carcinogen, 4-nitroquinoline-1-oxide (4NQO; Sigma) in drinking water. Animals were housed in micro-isolator cages in a laminar flow unit in air conditioned and light controlled rooms (12-hour cycles). We have previously described the establishment of PDX models of HNSCC (21). Briefly, xenografts were established in SCID mice by subcutaneous surgical transplantation of tumor pieces under transient inhalational anesthesia (2%–3% Isoflurane). The carcinogen 4NQO was dissolved in propylene glycol at 50 mg/mL and added to the drinking water at a final concentration of 100 µg/mL. Animals were administered 4NQO in their drinking water for 14 weeks. The water was changed ad libitum every 7 to 10 days. Regular autoclaved water was provided following completion of the 14-week period of carcinogen exposure. Control animals received autoclaved water at all times. The protocol for carcinogen exposure was selected based on published reports that demonstrated consistent formation of lesions that progress through various stages of carcinogenesis in this model (22, 23). All experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at RPCI (Buffalo, NY).

**Study design**

**PDX-HNSCC study.** The schematic for the study in the PDX model is shown in Fig. 1A. One week postimplantation, female SCID mice (~20 g body weight) bearing subcutaneous HNSCC xenografts were randomized into one of the four groups: control, erlotinib alone, 1,25(OH)2D3 alone, or combination (n = 6–10 per group). Animals received erlotinib (Selleck Chemicals) at a dose of 25 mg/kg (p.o. 5 days a week). 1,25(OH)2D3 (Hoffmann-La Roche, Inc.) was reconstituted in 100% ethyl alcohol and PBS and administered at a dose of 0.1 µg (i.p. 3 days/week, MWF for 4 weeks). Tumor growth kinetics and change in body weight were measured over a 30-day period.

**4NQO model.** The study design for studies in the 4NQO model is shown in Fig. 1B. Following a 14-week exposure to 4NQO, animals were randomized to control, erlotinib, 1,25(OH)2D3, or combination arms (n = 12–13 per arm). Drug dosage and treatment duration was the same as the PDX study. Longitudinal MRI examination was performed at week 14 (following carcinogen exposure and before start of treatment), week 18 (following 4 weeks of treatment), and at weeks 22, 24, and 26 following completion of treatment. Naive animals (n = 3) exposed to regular drinking water also underwent MRI examination at weeks 0 and 26. Animals were monitored for tumor growth by visual examination of the oral cavity under white light once every 2 days throughout the duration of the study. Histopathologic assessment was carried out at week 26 (n = 7–9 per group). Systemic toxicity was assessed by body weight measurements obtained thrice weekly during and after intervention. Animals were humanely euthanized when sustained loss of body weight (~20% of initial body weight) was noted. Immunoblot analysis was carried out in a subset of animals (n = 3 per group) at weeks 18 and 26.

**Tumor growth measurements**

For animals bearing subcutaneous HNSCC xenografts, caliper measurements were made to calculate tumor volume (mm3) using the formula, \( V = \frac{1}{2}a \times b \times c \), where \( a \) is the longest axis, and \( W \) is perpendicular to \( a \). Measurements were made two to three times a week and plotted as change in tumor volume (fractional tumor volume). Animals were euthanized when tumor burden reached the threshold for euthanasia as per institutional protocols.

**In vivo imaging**

Experimental MRI examinations were performed using a 4.7T/33-cm horizontal bore magnet (GE NMR Instruments) incorporating AVANCE digital electronics (Bruker Biospec with Paravision 3.0.2; Bruker Medical Inc.). Animals were anesthetized using 2.5% Isoflurane (Benson Medical Industries, Markham) before and during imaging. The mice were secured in a form-fitted, MR compatible sled (Dazai Research Instruments) equipped with temperature and respiratory monitoring sensors. The sled, along with a phantom containing 0.15 mmol/L gadopentetate dimeglumine (Gd-DTPA; Magnevist, Berlex Laboratories), was positioned inside the scanner using a plastic carrier tube. Animal body temperature was maintained at 37°C during imaging using an air heater system (SA Instruments Inc.), and automatic temperature feedback was initiated through thermocouples in the sled, in conjunction with computer software supplied with the heater. Axial multislice, T2-weighted spin echo images incorporating RARE (rapid acquisition with relaxation enhancement) encoding were acquired for each mouse using the following parameters: matrix size 256 × 192, TE/TR = 41/2500 ms, slice thickness 1.1 mm, field of view (FOV): 3.2 × 3.2 cm, number of slices = 20. Following MR image acquisition, raw image sets were transferred to a processing workstation and converted into Analyze format (AnalyzeDirect, version 7.0). A region of interest (ROI) was traced around the entire tumor and saved as an object map. Tumor volume was calculated by measuring the cross-sectional area on each slice containing the tumor and multiplying the sum by slice thickness.

**Immunostaining and histopathologic assessment**

Whole tongue specimens were fixed in 10% neutral-buffered formalin (Sigma) for immunostaining and histology. Immunostaining of whole tongue sections for the vitamin D receptor was performed using the antibody against the vitamin D receptor (product code SC-7918; Santa Cruz Biotechnology, Inc.). Following antigen retrieval, slides were incubated with the primary antibody (1:100) overnight at 4°C. For immunohistochemical staining, slides were washed in 3% hydrogen peroxide and 1% normal rabbit serum for 15 min each in between steps. Slides were then incubated with the primary antibody (1:100) for 1 h at room temperature. After the staining procedure, slides were washed in 0.05% Triton X-100 in 0.1 M phosphate buffer (pH 7.4) for 5 min. The secondary antibody was then added and incubated for 30 min at room temperature. Finally, slides were incubated with diaminobenzidine (DAB; Vector Laboratories) for 5 min and mounted.

**Histologic**

Histologic sections were stained with hematoxylin and eosin (H&E) (product code 53F-0003; BioCare Medical, Inc.). Histologic sections were also stained with antibodies against Ki-67 (product code sc-56 at 1:100; Santa Cruz Biotechnology, Inc.), p21 (product code 9215 at 1:200; Cell Signaling Technology, Inc.), and VDR (product code SC-7918 at 1:100; Santa Cruz Biotechnology, Inc.). For immunohistochemical staining, slides were prepared as described above, and slides were incubated with the appropriate secondary antibody for 30 min at room temperature. Finally, slides were incubated with diaminobenzidine (DAB; Vector Laboratories) for 5 min and mounted.
VDR) was performed using the monoclonal antibody 9A7 (MA1-710; Thermo Fisher Scientific Pierce Antibody Products) as described previously (24). Paraffin-embedded sections cut at 4 μm were placed on charged slides, and dried at 60°C for 1 hour. Deparaffinized slides were loaded on a Dako autostainer (Dakocytomation) for immunostaining (24). Slides were counterstained with harris hematoxylin (Poly Scientific). Slides were scanned and digitized using the Scanscope XT system (Aperio) and images were captured using the ImageScope software. Hematoxylin and eosin-stained sections of the tongue were examined by a board-certified pathologist with a clinical practice focus in head and neck cancer, blinded to therapeutic arm, imaging findings and outcome. Histologic assessment of hyperkeratosis (thickened keratinized layer), dysplasia grade (reported as worst grade identified per specimen; architectural disarray, increased nuclear to cytoplasmic ratio, hyperchromatic nuclei, increased or abnormal mitotic figures), and invasive carcinoma (frank invasion into the connective tissue stroma) was conducted as described previously (25).

Immunoblotting
Expression levels of EGFR, vitamin D receptor (VDR), and phosphorylated forms of EGFR and Akt in tissues were measured by immunoblotting of whole tongue extracts using primary antibodies specific for these markers. Western blot analyses were performed using the following primary antibodies: polyclonal rabbit anti-EGFR (1005; Santa Cruz), monoclonal rat anti-VDR (clone: 9A7; Thermo Fisher Scientific), monoclonal rabbit anti-phospho-EGFR (phospho Y1092; abcam), polyclonal rabbit anti-phospho-AKT1 (phospho S473; abcam). Anti-rabbit and anti-mouse horseradish-peroxidase-conjugated secondary antibodies (GE Healthcare) were used and blots were developed using enhanced chemiluminescence. Actin (I-19; Santa Cruz) was used in each experiment as a loading control. To assess difference in protein expression, Western blot films were analyzed using the UN-SCAN-IT program (Silk Scientific, Inc.). Equal sized ROIs were fitted around each expression band to measure total pixels. Using the total pixels acquired from the blot and the total pixels acquired from the corresponding actin blot, a
normalized expression value was achieved [(antibody total pixels/actin total pixels)/average control tissue expression].

Statistical considerations
All measured values were reported as the mean ± SEM. All statistical analyses were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software; www.graphpad.com). Tumor-doubling times (PDX model) were calculated from change in tumor volume (fractional tumor volume) over time using an exponential growth equation. Disease progression was defined as time to reach three times initial tumor volume in the PDX model. Kaplan–Meier survival curves of progression-free survival were generated from the change in fractional tumor volume and differences in survival analyzed using the log-rank test. Differences in tumor volumes calculated from MRI were analyzed using two-tailed unpaired t test. Differences in the incidence of lesions on MRI and histology were analyzed using a two-tailed Fisher’s exact test. Differences in protein expression between groups were analyzed using a two-tailed t test. P values of <0.05 were considered statistically significant.

Results
Efficacy of combination treatment on PDX-HNSCC tumor growth
We examined the efficacy of our combination regimen against patient tumor-derived HNSCC xenografts. Mice bearing subcutaneous tumors were given erlotinib alone, 1,25(OH)2D3 alone, or the combination for 4 weeks. Figure 2 shows the change in tumor volumes of individual animals in control (Fig. 2A), erlotinib alone (Fig. 2B), 1,25(OH)2D3 alone (Fig. 2C), and combination groups (Fig. 2D). As single agents, erlotinib and 1,25(OH)2D3 had minimal effect on tumor growth. However, the combination treatment suppressed tumor growth and resulted in a significant increase (P < 0.05) in tumor doubling time (Fig. 2E) compared to controls and single-agent treatment. Kaplan–Meier survival curves (Fig. 2F) generated from change in tumor volume showed a significant survival benefit with combination treatment compared with controls and erlotinib or 1,25(OH)2D3 alone.

Vitamin D receptor expression in the 4NQO model
The effects of 1,25(OH)2D3 are mediated through its interactions with the VDR. Therefore, before examining the efficacy of the combination regimen, we performed immunostaining for VDR on tongue sections obtained from naïve and 4NQO exposed mice. The panel of images shown in Fig. 3 represents photomicrographs of VDR-stained sections of tongues obtained from naïve (Fig. 3A) and 4NQO-treated mice (Fig. 3B and C). Corresponding H&E-stained sections of naïve (Fig. 3D) and 4NQO-treated tongues are also shown (Fig. 3E and F). Increased expression of VDR was seen in the 4NQO-exposed tongue (Fig. 3B), particularly in regions exhibiting hyperplastic and dysplastic changes (Fig. 3E), compared with the normal epithelium of naïve tongues (Fig. 3A and D). Higher expression of VDR was also observed in regions of the 4NQO-treated tongue with invasive squamous cell carcinoma (Fig. 3C and F) compared with the naïve untreated tongue (Fig. 3A and D).

Noninvasive imaging of oral carcinogenesis in vivo
We utilized noninvasive MRI to monitor oral carcinogenesis in the 4NQO model. Figure 4B shows axial T2-weighted MR images of a 4NQO-treated animal at 14, 18, and 26 weeks. Compared with naïve controls (Fig. 4A), exposure to 4NQO (Fig. 4B) resulted in morphologic changes in the oral cavity that included thickening of the tongue, particularly in the lateral borders (outlined in white) suggestive of hyperplasia, as well as development of exophytic lesions on the dorsal surface (Fig. 4B, arrow). Figure 4C shows MR images along with corresponding in vivo and ex vivo digital images at the end point of the study (week 26), as well as whole tongue H&E sections of the naïve and 4NQO-treated tongue. Consistent with the imaging data, histologic assessment of the 4NQO-treated tongue confirmed dysplasia and focal invasive squamous cell carcinoma (Fig. 4C) at week 26.

Radiologic and pathologic response assessment
We examined the impact of our preventive intervention on tumor incidence in the 4NQO model using MRI. T2-weighted MRI was performed at week 26 in animals (n = 12–13 per group) from all four experimental cohorts as outlined in Fig. 1B. As shown in Fig. 4D, 100% of the animals in the control group exposed to the carcinogen 4NQO showed presence of tumor on MRI at 26 weeks. As a single agent, 1,25(OH)2D3 had minimal effect on tumor incidence, while erlotinib alone resulted in a 33% reduction in tumor incidence. Only animals exposed to the combination of erlotinib and 1,25(OH)2D3 showed the greatest and statistically significant reduction in tumor incidence on MRI (~40%, P < 0.05). Difference in the incidence of visible lesions on MRI between erlotinib alone and combination groups was not statistically significant. Longitudinal MRI was performed in a subset of animals (n = 5–6 per group; Fig. 5). MRI exam revealed onset of tumor growth in the control arm as early as week 15. At this time point, T2-weighted MRI revealed the presence of a large tumor (~220 mm3) on the dorsum of the tongue with extensive infiltration into surrounding muscle and bone in 1 of 6 animals in the control group (Supplementary Fig. S1). Figure 5A shows longitudinal T2-weighted MR images from an animal in each group. At week 18 (following completion of 4 weeks of treatment), 4 out of the remaining 5 animals in the control group and 3 of 5 animals in the 1,25(OH)2D3 alone group showed evidence of tumor growth on MRI. Although 1 of 5 animals treated with erlotinib alone showed presence of tumor, none of the animals (0/5) in the combination group showed evidence of tumor growth on MRI. By week 24, 100% of the animals in the control group showed evidence of tumor growth on MRI. Individual tumor volumes of animals in all groups are shown in Supplementary Fig. S2 and digitized ex vivo images of tongues from animals are shown in Supplementary Fig. S3. One tumor in the control group and one tumor in the 1,25(OH)2D3 were identified as statistical outliers (Supplementary Fig. S2) and were excluded from the analysis. Mean tumor volumes between the control and 1,25(OH)2D3 alone groups were comparable (Fig. 5B). Animals in the combination group exhibited the lowest tumor volume among all the groups (P < 0.05 compared with controls; Fig. 5B).

Histopathologic assessment of whole tongue tissues was performed to determine the effects of the preventive intervention on preneoplastic and neoplastic lesions. Control tongues (7/7) showed moderate-severe dysplasia on histologic examination (Fig. 5C and Supplementary Fig. S4). Treatment with 1,25(OH)2D3 or erlotinib led to minimal reduction in the degree of dysplasia (Fig. 5C). In comparison, combination treatment resulted in a significant (P < 0.05) reduction in the severity of
dysplasia with 5 of 9 (56%) animals exhibiting mild dysplasia. Control tongues exhibited highest incidence of invasive SCC (71.4%), whereas single-agent treatment with 1,25(OH)2D3 and erlotinib was associated with an SCC incidence of approximately 38% and 63%, respectively. Animals treated with the combination showed the greatest reduction in the incidence of invasive SCC (33%; P < 0.05 compared with controls). Body weight measurements were obtained during the entire course of the study as a measure of safety and tolerability of the treatments. No significant changes in body weight were seen among any of the groups throughout 4NQO exposure and treatment with erlotinib, 1,25(OH)2D3 or the combination (Fig. 5D).

Mechanisms of interaction
To investigate the potential mechanisms of interaction between the two agents, we examined the effects of erlotinib and 1,25(OH)2D3 on the levels of EGFR, phosphorylated EGFR and AKT, and VDR following completion of treatment (week 18) and 8 weeks following cessation of treatment (week 26). Treatment with 1,25(OH)2D3 resulted in increased EGFR (Fig. 6A) compared with erlotinib treatment without any effect on p-EGFR levels (Fig. 6A). Combination treatment was associated with a significant increase in EGFR but was associated with a significant reduction in p-EGFR at week 18 (Fig. 6A) and week 26 (Fig. 6B). Notably, immunoblotting of whole

Figure 2.
Efficacy of 1,25(OH)2D3 in combination with erlotinib in a PDX model of HNSCC. Change in tumor volumes of individual animals in (A) control (n = 10), (B) erlotinib only (n = 6), (C) 1,25(OH)2D3 only (n = 10), and (D) combination treatment (n = 6) arms. E, bar graph shows doubling times of tumors in all groups calculated from the growth curves. F, Kaplan-Meier survival curves of animals in all four groups showing a significant survival benefit in animals treated with the combination compared with controls and either monotherapy. *, P < 0.05.
tongue extracts revealed a significant reduction in p-EGFR and p-AKT following combination treatment compared with either erlotinib alone ($P < 0.05$) or 1,25(OH)$_2$D$_3$ alone at week 26 (Fig. 6B). VDR levels were lower among all treatment groups compared with controls at week 18 (Fig. 6A). At week 26, combination treatment resulted in a significantly lower VDR expression compared with single-agent erlotinib treatment (Fig. 6B).

**Figure 3.**
VDR expression in the 4NQO model. Immunostained sections of naive (A) and 4NQO exposed tongues (B and C) at week 26 for the VDR. Corresponding H&E stained sections of the naive tongue (D), 4NQO-treated tongue (E and F) are also shown. Magnification bars, 100 μm.

**Discussion**

Head and neck cancers are locoregionally aggressive tumors that result in debilitating functional and esthetic sequelae in patients (26). Identification of novel preventive strategies could have a major impact on quality of life and survival of these patients. An extensive body of literature exists on the activity of 1,25(OH)$_2$D$_3$ against breast, prostate, colon, and
skin cancers (16, 27 and references within). 1,25(OH)₂D₃ has been shown to inhibit growth of oral SCC cell lines in vitro at physiologic and supraphysiologic concentrations (28). Meier and colleagues have shown that administration of 1,25(OH)₂D₃ inhibits carcinogenesis in the hamster buccal pouch model (29).

In the present study, we investigated the efficacy of short-term 1,25(OH)₂D₃ treatment in combination with the EGFR inhibitor erlotinib against HNSCC. Results of studies conducted in PDX-bearing mice revealed significant inhibition of tumor growth with combination treatment.

To investigate the impact of this combination approach against the entire spectrum of carcinogenesis, we conducted preclinical studies in a well-validated carcinogen-induced model of HNSCC. These studies showed that combination treatment was well tolerated and was not associated with any significant change in body weight. Pathologic assessment revealed a marked reduction in the severity of dysplasia with combination treatment compared with either monotherapy.

Histopathologic confirmation of biopsy specimens remains the gold standard for detection of oral cancer in patients. However, the invasive nature of the technique precludes serial assessment of carcinogenesis in vivo. To overcome this limitation, we used a noninvasive imaging approach based on MRI to longitudinally characterize oral carcinogenesis in the 4NQO model. MRI offers excellent soft tissue contrast and good spatial resolution without the use of ionizing radiation or radioactive tracers. Among the experimental groups, animals treated with the combination regimen showed the greatest reduction in tumor incidence and volume. Although the combination group exhibited the lowest incidence of visible lesions on MRI and histologic examination, the reduction in tumor incidence with 4 weeks of combination treatment was not significantly different compared with single-agent erlotinib. These observations suggest that 1,25(OH)₂D₃ has...
Figure 5. Chemopreventive efficacy and toxicity of erlotinib-1,25(OH)$_2$D$_3$ combination treatment in the 4NQO model. A, panel of images represent axial T2-weighted MR images of a mouse from each experimental group weeks 18, 22, and 26. Following 14 weeks of exposure to 4NQO, animals were randomized into either control (no treatment), erlotinib alone (25 mg/kg p.o, 5 days/week for 4 weeks), 1,25(OH)$_2$D$_3$ (0.1 mg i.p, 3 days/week for 4 weeks) or combination arms. Arrows point to location of tumor on the tongue. B, tumor volumes calculated from multislice T2-weighted images at different times are shown (n = 5 per group). C, incidence of preneoplastic (dysplasia) and neoplastic lesions in animals from all four experimental groups based on histologic assessment. Tongues were also obtained from animals exposed to drinking water only (Group 5; n = 3). D, body weight measurements obtained over the 26-week period in animals from all four experimental groups (n = 5 per group).

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<tr>
<th>Group</th>
<th>Treatment</th>
<th>Mild dysplasia (%)</th>
<th>Moderate–severe dysplasia (%)</th>
<th>SCC (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>4NQO only</td>
<td>0/7</td>
<td>7/7 (100)</td>
<td>5/7 (71.4)</td>
</tr>
<tr>
<td>2</td>
<td>4NQO + erlotinib</td>
<td>2/8 (25)</td>
<td>6/8 (75)</td>
<td>3/8 (37.5)</td>
</tr>
<tr>
<td>3</td>
<td>4NQO + 1,25(OH)$_2$D$_3$</td>
<td>1/8 (12.5)</td>
<td>7/8 (87.5)</td>
<td>5/8 (62.5)</td>
</tr>
<tr>
<td>4</td>
<td>4NQO + erlotinib + 1,25(OH)$_2$D$_3$</td>
<td>5/9 (56)*</td>
<td>4/9 (44)*</td>
<td>3/9 (33.3)</td>
</tr>
<tr>
<td>5</td>
<td>Drinking water only</td>
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*P < 0.05 compared with group 1 (Fisher exact probability test)
a stronger impact on disease progression rather than incidence of invasive cancer. However, investigation into the efficacy of this combination regimen over a longer duration of treatment (10–12 weeks) is needed to better understand this issue.

The biologic activity of 1,25(OH)₂D₃ is mediated through the VDR, a high affinity, low-capacity receptor protein located primarily in the nucleus that acts as a ligand-dependent transcription factor and binds to vitamin D response elements leading to activation or repression of target genes (16, 17). The VDR is found in the intestinal tract, kidneys, bone, and in several cancer types, including breast, prostate, colon, and lung cancers (24, 30). In our study, we observed increased VDR staining in carcinogen-exposed tongues compared with naïve tongue tissue. This observation is consistent with a recent report by Yuan and colleagues, in which

Figure 6. Immunoblot analysis of molecular changes in response to combination treatment. Expression levels of EGFR, p-EGFR, p-AKT, and VDR in whole tongue extracts (n = 3 per group) obtained from untreated control animals and animals treated with erlotinib alone, 1,25(OH)₂D₃ alone, or combination at week 18 (A; 24 hours post completion of 4-week treatment regimen) and at week 26 (B) are shown. *, P < 0.05.
increased VDR immunoreactivity was seen in human OSCC compared with normal oral mucosa (31). In the same study, increased VDR immunostaining was also observed in murine OSCC lesions compared with normal oral mucosa in mice treated with 4NQO. Increased expression of VDR has also been reported in human precancerous and oral SCCs in patients (32).

To determine the potential mechanism of interaction between 1,25(OH)2D3 and erlotinib, we examined the effects of the two agents on EGFR and VDR pathways. We observed an increase in EGFR following treatment with 1,25(OH)2D3. This is consistent with a previous observation by Desprez and colleagues in breast cancer (33). Interestingly, reduction in EGFR expression has also been reported in breast cancer cell lines (34). Tong and colleagues have shown that growth regulation of colon cancer cells by EGF and 1,25(OH)2D3 is mediated by mutual modulation of receptor expression. Specifically, treatment with 1,25(OH)2D3 sensitized ligand-occupied EGFR while EGF suppressed VDR expression in Caco-2 colon adenocarcinoma cells (35). In our study, Western blot analyses revealed a significant reduction in p-EGFR expression following combination treatment compared with control and single-agent treatment. This downregulation of p-EGFR expression with 1,25(OH)2D3 appeared to be sustained even after cessation of treatment (week 26 corresponds to 8 weeks post cessation of treatment). Similarly, we observed a down regulation of VDR following treatment at week 18 compared with controls. Although this reduction was not statistically significant, at week 26, a significant reduction in VDR was seen with combination treatment compared with erlotinib alone. Segaret and colleagues have previously shown that VDR expression in keratinocytes is restricted to actively cycling cells (36). The observed reduction in VDR following combination treatment could therefore reflect the underlying decrease in cell proliferation. Marked downregulation of p-AKT was also observed with the combination of 1,25(OH)2D3 and erlotinib compared with controls. Consistent with this observation, Cordero and colleagues have shown that treatment with 1,25(OH)2D3 inhibits ligand-dependent phosphorylation of EGFR and downregulated EGFR growth signaling in human epidermoid cancer cells (37). Collectively, these observations suggest the interaction between EGFR and VDR signaling pathways is complex and the nature of cross-talk varies between different cancer cell types. In addition, Lathers and colleagues have shown that administration of 1,25(OH)2D3 can improve the immune competence of HNSCC patients by reducing the presence of immunosuppressive CD34+ cells (38). Treatment of HNSCC patients with 1,25(OH)2D3 for 3 weeks before surgery has also been shown to stimulate immune cell infiltration into tumors and delay the time to recurrence (39). Given these results, investigation into the potential immunologic and anti-inflammatory effects of 1,25(OH)2D3 would also provide valuable mechanistic insight. Although the mechanism of interaction between the two agents requires further investigation, our results indicate sustained inhibition of multiple signaling pathways downstream of EGFR with combination treatment.

Successful clinical implementation of any combination approach requires demonstration of safety and tolerability since a critical requirement of any preventive intervention is to “first do no harm” (40). In this regard, several clinical trials have demonstrated that administration of 1,25(OH)2D3 is safe and feasible (41, 42). The ease of administration of 1,25(OH)2D3 along with its diverse biologic effects strongly supports its usefulness as a chemopreventive agent. In our study, 1,25(OH)2D3 was administered at a dose of 0.1 μg (i.p.) 3 days/week (MWF) for 4 weeks. Pharmacokinetic analysis in mice has shown that the peak plasma concentration at this dose is 12 ng/ml (43), a concentration that can be safely achieved in humans. On the other hand, erlotinib is an orally active drug that can be taken by patients without a visit to the chemo infusion clinic and the risk of infusion-related anaphylactic reactions. Published preclinical studies of erlotinib have reported efficacy at doses of 50 mg/kg or higher in mice (44, 45). We observed evidence of tumor growth inhibition with erlotinib at a dose of 25 mg/kg (5 days/week for 4 weeks). On the basis of the body surface area normalization method (46), this corresponds to a daily dose of approximately 122 mg for a human. However, a recent study by Rosenthal and colleagues has highlighted poor tolerance of long-term erlotinib at 150 mg (daily) as an adjuvant in head and neck cancer patients who had undergone surgery (9). Of the 31 patients enrolled in the study, only 8 completed the 12-month course of erlotinib therapy given at 150 mg (5 days per week). Discontinuation of treatment as well as dose reductions occurred due to intolerance to prolonged therapy. Although we did not observe any evidence of toxicity in our studies, future studies should therefore investigate the efficacy and toxicity of long-term erlotinib treatment at lower doses.

We recognize the limitations of our study. Given the primary goal of the work was to examine the impact of short-term combination treatment with 1,25(OH)2D3 and erlotinib, we utilized a predefined end point of 26 weeks for assessment (based on published literature). As a result, the studies were not powered to examine the impact of long-term treatment on survival (time to sacrifice). Second, our studies were conducted using a single schedule of administration of both agents. The effects of varying treatment schedule and/or duration on efficacy therefore remain to be addressed. Given the cross-talk between the EGFR and VDR pathways, it would be reasonable to speculate that optimizing the dose, sequence, duration of exposure, and timing of intervention (preneoplastic vs. early disease vs. established disease) with both agents may be a critical determinant of efficacy. Finally, the potential risk of hypercalcemia with 1,25(OH)2D3 complicates its clinical translation. To overcome this, future studies should investigate the potential of less calcemic analogues of vitamin D against HNSCC. Dietary vitamin D could also be examined as a safe approach to drive local tissue production of 1,25(OH)2D3. The ease of dietary supplementation makes it particularly attractive for clinical translation. We have begun to address some of these questions in our laboratory and will report our findings in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.D. Bothwell, T. Shaurova, C.S. Johnson, M. Seshadri

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.D. Bothwell, T. Shaurova, M. Merzianu, C.S. Johnson, M. Seshadri

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