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Aldehyde dehydrogenase 3A1 activation prevents radiation-induced xerostomia by protecting salivary stem cells from toxic aldehydes

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Xerostomia (dry mouth) is the most common side effect of radiation therapy in patients with head and neck cancer and causes difficulty speaking and swallowing. Since aldehyde dehydrogenase 3A1 (ALDH3A1) is highly expressed in mouse salivary stem/progenitor cells (SSPCs), we sought to determine the role of ALDH3A1 in SSPCs using genetic loss-of-function and pharmacological gain-of-function studies. Using DarkZone dye to measure intracellular aldehydes, we observed higher aldehyde accumulation in irradiated Aldh3a1−/− adult murine salisphere cells and in situ in whole murine embryonic salivary glands enriched in SSPCs compared with wild-type glands. To identify a safe ALDH3A1 activator for potential clinical testing, we screened a traditional Chinese medicine library and isolated β-limonene, commonly used as a food-flavoring agent, as a single constituent activator. ALDH3A1 activation by β-limonene significantly reduced aldehyde accumulation in SSPCs and whole embryonic glands, increased sphere-forming ability, decreased apoptosis, and improved submandibular gland structure and function in vivo after radiation. A phase 0 study in patients with salivary gland tumors showed effective delivery of β-limonene into human salivary glands following daily oral dosing. Given its safety and bioavailability, β-limonene may be a good clinical candidate for mitigating xerostomia in patients with head and neck cancer receiving radiation therapy.

Significance

Radiation therapy for head and neck cancer often leads to dry mouth, a debilitating condition that affects speaking, swallowing, and other functions related to quality of life. Since salivary functional recovery after radiation is largely dependent on the number of surviving salivary stem/progenitor cells (SSPCs), we reasoned that protection of SSPCs from injury is critical for mitigating dry mouth. Following radiation, SSPCs accumulate toxic aldehydes that damage DNA, proteins, and lipids, leading to cell death. Here, we identified β-limonene as an activator of aldehyde dehydrogenase 3A1 (ALDH3A1) with a favorable safety profile for clinical use. ALDH3A1 activation decreases aldehyde accumulation in SSPCs, increases sphere-forming ability, reduces apoptosis, and preserves salivary gland structure and function following radiation without reducing the anticancer effects.

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The authors declare no conflict of interest.

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levels and increasing SSCP survival without reducing the anticancer benefit of radiation treatment.

**Results**

**Loss of ALDH3A1 Leads to Increased Aldehyde Levels in SSCPs After Radiation and Accelerates Hyposalivation.** To determine the role of ALDH3A1 in aldehyde clearance in both adult and embryonic murine SSCPs and whether ALDH3A1 is required for aldehyde removal. Dissociated salivary spheres (sali spheres) enriched in SSCPs were cultured from adult WT and Aldh3a1−/− murine salispheres 2 h after IR, measured as median fluorescence intensity of DarkZone dye by FACs (n = 2–6; bars indicate SEM; *P < 0.05). The experiment was repeated in SI Appendix, Fig. S1A. (B, Left) Representative images of WT (Left) and Aldh3a1−/− (Right) E13.5 mouse SMGs after 24 h in culture, treated with DarkZone dye, 3 h after IR, in brightfield (Upper Row) and with a florescence filter (Lower Row). (Scale bars: 50 μm.) (Right) Quantification of DarkZone dye fluorescence intensity of embryonic SMGs, normalized to WT (n = 6; bars indicate SEM; *P < 0.05). (C) Pilocarpine-induced saliva production collected in female C57BL/6J WT and Aldh3a1−/− mice at baseline and 1, 2, 4, 6, and 8 wk after 15-Gy IR (single dose) (n = 8–11; bars indicate SEM; *P < 0.05; **P < 0.01; ***P < 0.001).

**Identification of δ-limonene, an Activator of ALDH3A1.** To determine if ALDH3A1 activation is sufficient to protect salivary glands from IR, we screened for a safe and specific ALDH3A1 activator using a library of 135 traditional Chinese medicine (TCM) extracts (Sun Ten Pharmaceutical Co.). Because TCM extracts have a long history of human use, we reasoned that identified activators would have a higher likelihood of being safe for clinical use. Seven extracts increased ALDH3A1 activity (see list in SI Appendix, Fig. S3). HPLC fractionation of these extracts and NMR characterization of the fractions identified several single-molecule constituents that activate ALDH3A1 in a dose-dependent manner (Fig. 24). All identified active constituents were monoterpenes, which are commonly found in plant essential oils and may explain the high hit rate observed in TCM plant extracts. Of these, we identified δ-limonene as the active component present in three extracts (Citrus reticulata, Nelumbo nucifera, and Anemarrhena asphodeloides) with the lowest EC50 (~14 μM) and a maximal activity of ~4.6 (Fig. 24). δ-limonene occurs naturally in citrus fruit oils and bears the Food and Drug Administration designation of “generally recognized as safe” (as a food-flavoring agent) under the Code of Federal Regulations Title 21. δ-limonene has an estimated maximum tolerated dose of 8 g·m−2·d−1 (~15 g/d) (34) and no known risk of mutagenicity, carcinogenicity, or nephrotoxicity in humans (35). Given its potency and favorable safety profile, it is a good candidate for clinical investigation.

To characterize δ-limonene’s enzymatic activity, we observed that both δ-limonene and Alda-89 increase the catalytic activity of ALDH3A1 toward small aldehydes, such as acetaldehyde and propionaldehyde, but not toward aromatic or long-chain aldehydes (Fig. 2B). Furthermore, δ-limonene appears ALDH3A1-specific and does not increase the activity of the highly homologous ALDH family members ALDH1A1, ALDH2, ALDH3A2, ALDH4A1, ALDH5A1, or ALDH7A1 (Fig. 2C). To confirm the specificity of δ-limonene for ALDH3A1, we measured the effect of δ-limonene on ALDH activity in WT and Aldh3a1−/− salispheres lysates and observed that α-d-limonene increases the ALDH activity of WT lysate by ~30% but not that of Aldh3a1−/− lysate, which exhibited lower basal activity (Fig. 2D). δ-limonene may increase the catalytic activity of ALDH3A1 by reducing the size of the catalytic tunnel, thus increasing the number of productive interactions between the substrate and the catalytic Glu333 while simultaneously protecting Cys253 from addition and inactivation by the substrate (Fig. 2E, Left). This effect is similar to observations for ALDH2 and Alda-1, an activator of ALDH2 (36). The selectivity of δ-limonene for ALDH3A1, relative to ALDH2, may be due to the size of the catalytic tunnel of these enzymes, δ-limonene fits in the catalytic tunnel of ALDH3A1 without blocking the catalytically critical Glu333 (Fig. 2E, Lower Left), whereas in ALDH2, access to this catalytic glutamate (Glu329 in ALDH2) appears hindered (Fig. 2E, Lower Right).

**ALDH3A1 Activation with δ-limonene Reduces Aldehyde Load, Improves Sphere Growth, and Mitigates Hyposalivation in Vivo After Radiation.** Based on the above observations, we hypothesized that ALDH3A1 activation with δ-limonene would reduce IR-induced aldehyde levels in SSCPs. Using DarkZone, we observed that δ-limonene treatment of irradiated WT salispheres decreased the aldehyde load to nearly nonirradiated levels compared with vehicle control (Fig. 3A). In contrast, post-IR aldehyde levels in δ-limonene-treated and nontreated Aldh3a1−/− salispheres were not statistically different (SI Appendix, Fig. S4). Furthermore, δ-limonene treatment of irradiated ex vivo E13.5 SMGs also reduced saliva production in WT and Aldh3a1−/− mice before and after 15-Gy IR (Fig. 1C and SI Appendix, Fig. S2). Aldh3a1−/− mice exhibited decreased saliva production after IR compared with WT mice, suggesting that ALDH3A1 is required, in part, to protect SMG function after IR.
To determine whether d-limonene protects SSPCs after radiation, we measured the ability of dissociated SMG cells to form salispheres after IR. Compared with cells from mice that received no treatment, dissociated SMG cells from d-limonene–treated mice 24 h after 15-Gy IR demonstrated an approximately twofold increase in sphere-forming ability (Fig. 3C), and cells from d-limonene–treated mice 20 wk after 30-Gy IR demonstrated an approximately 30-fold increase in sphere-forming ability (Fig. 3D). These data suggest that d-limonene improves both short- and long-term SSPC survival after IR.

We next determined whether d-limonene can protect salivary gland structure and function after IR in vivo in mice. After collection of baseline saliva, the treatment group received d-limonene daily in chow starting 1 wk before IR. Measurement of d-limonene levels by GC-MS showed that oral d-limonene treatment for 2 wk led to drug levels in murine SMGs of ~7,000 ng/g (mean 7.0 ± 1.0 × 10^3 ng/g; SEM; n = 5). Mice were irradiated with 15 Gy, and, in a second experiment, with 30 Gy (6 Gy/d). In both experiments, d-limonene–treated mice had significantly more saliva production after IR than nontreated mice (Fig. 3E and SI Appendix, Fig. S5 A and B). Eight weeks after 30-Gy IR, Periodic acid Schiff (PAS) staining for acinar cells showed that d-limonene–treated SMGs maintained ~90% preservation of the acinar area (relative to nonirradiated glands) compared with <30% for the irradiated control group (SI Appendix, Fig. S5C). In a third experiment, we irradiated mice with 30 Gy, began daily d-limonene treatment starting 24 h after the final IR dose, and collected saliva up to 20 wk after IR. The treatment group again sustained significantly higher saliva levels than the nontreated group (Fig. 3F and SI Appendix, Fig. SSD). Consistent with these data, PAS staining of SMGs 20 wk after 30-Gy IR showed greater than three times more acinar cell preservation with d-limonene treatment compared with the nontreated group (Fig. 3G). d-limonene did not increase saliva production in nonirradiated mice (SI Appendix, Fig. S5E).

To learn whether d-limonene affects tumor growth or reduces the radiation effect on cancer, we implanted SCID mice with either SAS [human papillomavirus (HPV)-negative] or SCC90 (HPV-positive) HNC squamous cell carcinoma cells s.c. and treated them with or without d-limonene and with or without IR to the tumor site. d-limonene treatment did not promote tumor growth (SI Appendix, Fig. S6 A and C) or lessen the antitumor effects of IR (SI Appendix, Fig. S6 B and D).

**ALDH3A1 Activation Reduces Apoptosis in SMGs.** To determine how ALDH3A1 activation and reduced aldehyde levels protect salivary gland structure and function after IR, we performed RNA-sequencing (RNA-seq) studies on EpCAM+ cells enriched in SSPCs (33) isolated from WT and Aldh3a1+− murine SMGs 2 wk after 30-Gy IR. These data showed an increase in apoptotic-related gene expression after IR, which was exacerbated in Aldh3a1+− cells (Fig. 4A, Left) but suppressed with d-limonene treatment (Fig. 4A, Right). To determine the role of ALDH3A1 on early and late apoptosis in SSPCs, we stained dissociated murine EpCAM+ cells isolated from WT and Aldh3a1+− SMGs with annexin V (an apoptosis marker) and propidium iodide (PI) and observed that Aldh3a1+− cells had approximately 2.2-fold more early and late apoptotic cells than the vehicle-treated control (Fig. 4B). We then tested the effect of d-limonene on apoptosis in irradiated WT EpCAM+ cells and observed ~60% fewer early and late apoptotic cells compared with the irradiated vehicle-treated control (Fig. 4C). Furthermore, we stained murine SMGs removed immediately after 30-Gy IR (6 Gy/5 d) with cleaved caspase-3, a marker of apoptosis activation. Irradiated SMGs had a greater than fivefold increase in apoptotic cells compared with nonirradiated SMGs, and d-limonene treatment in chow reduced apoptosis to nearly nonirradiated levels (Fig. 4D). RNA-seq of irradiated EpCAM+ cells isolated from WT mice 2 wk after 30-Gy IR to the SMG also showed a correlation between d-limonene treatment and increased

aldehyde levels by approximately fourfold compared with vehicle control, to nearly basal levels (Fig. 3B). These data indicate that d-limonene treatment reduces aldehydes after IR in both adult and embryonic SSPCs.
expression of genes related to glutathione metabolism, which reduces oxidative stress, and decreased expression of immune response-related genes (SI Appendix, Fig. S7 A and B).

**Distribution of Oral D-limonene to Human Salivary Glands.** To assess the feasibility of D-limonene as an oral therapy for radiation-induced xerostomia, we initiated a phase 0 study to determine whether oral D-limonene is distributed to human salivary glands. Patients scheduled to undergo surgical removal of a salivary gland tumor were given 2 g/d oral D-limonene (1 g twice daily) for 2 wk immediately before their scheduled surgery. Saliva and plasma were collected at baseline and on the day of surgery, and normal salivary tissue was collected at surgery. D-limonene was measured in plasma, saliva, and gland tissue by GC-MS. Data from four patients showed that D-limonene was present at high levels in the salivary gland, measuring on average ~2,000 ng/g (Fig. 5A and SI Appendix, Table S1A). This is within the same order of magnitude as drug levels measured in murine salivary glands after 2 wk of 10% D-limonene delivered in chow (~7,000 ng/g), suggesting that this dose may be sufficient to achieve a clinical benefit. D-limonene levels were lower in saliva and blood, possibly due to the compound’s hydrophobic properties (Fig. 5B and C and SI Appendix, Table S1 B and C).

**Discussion**

Adult SSPCs are thought to enable the repair of damaged salivary glands and thereby mitigate xerostomia after radiation therapy in patients with HNC (37). Using a combination of loss- and gain-of-function studies, we demonstrated that IR increases aldehyde production in SSPCs, which likely contributes to their injury and death after IR. Furthermore, we showed that ALDH3A1 increases aldehyde clearance in both adult salivary cells and embryonic SMGs. ALDH3A1 activation with D-limonene further enhanced aldehyde metabolism in SSPCs beyond normal basal function, reduced apoptosis, and increased survival after IR, as evidenced by increased sphere-forming ability after IR in treated mice. Importantly, D-limonene reduced hyposalivation and improved acinar survival in vivo after IR. Although we cannot rule out the possibility that the effect of D-limonene in vivo could be mediated by additional pathways or could originate from other cell types, the combined data presented here show that ALDH3A1 plays an important role in protecting SSPCs from IR-induced injury by increasing aldehyde scavenging.

D-limonene is a major component in citrus peel oil and is a common food flavoring. Clinical studies evaluating D-limonene’s activity in patients with refractory solid tumors (34) or those with early breast cancer undergoing surgery (38) suggest that D-limonene has an acceptable safety profile for cancer patients at clinically relevant doses. Our preclinical studies indicated that ALDH3A1 activation with D-limonene did not promote tumor growth or protect tumors from radiation in vivo. Similarly, higher ALDH3A1 protein levels in human HNC tumors were not associated with worse prognosis (30). We therefore proceeded with a phase 0 human study and observed that D-limonene concentrates at high levels in human salivary glands. Further investigation is needed to determine whether these levels are sufficient to protect human SSPCs from radiation damage.
Materials and Methods

See SI Appendix, Supplemental Methods for experimental details.

Animals. C57BL/6J and SCID mice were purchased from Jackson Laboratories, and C57BL/6J Aldh3a1−/− mice were obtained from the laboratory of Vasilis Vasiliou at Yale School of Public Health, New Haven, CT (39). Timed pregnant CD-1/ICR mice were purchased from Envigo. The Administrative Panel on Laboratory Animal Care at Stanford University and the Institutional Animal Care and Use Committee at the University of California, San Francisco approved all animal protocols.

DarkZone Dye Aldehyde Assay in Murine Salispheres and ex Vivo E13.5 Whole SMGs. Primary salivary gland cells were grown into spheres as previously described (40). Dissociated spheres were incubated with 20 μM DarkZone dye and 10 mM 2,4-dimethoxyaniline catalyst and were analyzed by FACS (32). E13.5 whole SMGs were manually dissected and cultured ex vivo for 24 h (41) and were incubated with DarkZone dye. Average fluorescence intensity was quantified using ImageJ (NIH). See SI Appendix, Supplemental Methods for details.

ALDH Enzymatic Assay. ALDH isozyme activity was measured spectrophotometrically (29) using 5 μg/mL of recombinant protein and 50 mM sodium pyrophosphate buffer (pH 7.4) in the presence of 2.5 mM NAD⁺ and 10 mM substrate or with a fluorescence-coupled assay using 1 U/mL diaphorase and 0.1 mM resazurin (42). See SI Appendix, Supplemental Methods for details.

Docking of α-limonene. α-limonene was prepared for docking using LigPrep (Schrödinger) and was docked to both ALDH2 and ALDH3A1 using their respective generated grids (the Alda-1 binding site for ALDH2 and the

Fig. 4. α-limonene reduces apoptosis. (A, Left) Heatmap of the apoptotic RNA signature of WT and Aldh3a1−/− EpCAM⁺ cells from murine SMGs 2 wk after 30-Gy IR (6 Gy/d). (Right) Heatmap of the apoptotic RNA signature of WT SMG EpCAM⁺ cells 2 wk after 30-Gy IR (6 Gy/d) isolated from mice treated or not treated with daily 10% α-limonene starting 7 d before IR. (B) Percentage of early (annexin V⁺PI⁻) and late (annexin V⁺PI⁺) apoptotic nonirradiated EpCAM⁺ WT and Aldh3a1−/− SMG cells by FACS analysis (n = 3; bars indicate SEM; *P < 0.05). (C, Left) Percentage of early and late apoptotic WT EpCAM⁺ SMG cells treated with 100 μM α-limonene or vehicle (PEG-400) control 18 h after 4-Gy IR by FACS analysis (n = 5–6; bars indicate SEM; ***P < 0.01; ****P < 0.001). (Center) FACS dot-plot for control vehicle-treated cells. (Right) FACS dot-plot for α-limonene-treated cells. (D, Left) Representative images of cleaved caspase-3 (red) and DAPI (blue) staining of fixed SMGs removed immediately after the final fraction of 30-Gy IR (6 Gy/d). The treatment group began 10% α-limonene 7 d before IR. (Scale bars: 50 μm.) (Right) The number of cleaved caspase-3⁺ cells (red) per field blindly counted (n = 6–7 SMGs, 18 images; bars indicate SEM; ****P < 0.001).

Fig. 5. Phase 0 human study of α-limonene drug distribution in four patients. (A) α-limonene levels in human salivary gland tissue collected after 2 wk of 2 g/d oral α-limonene measured by GC-MS (n = 4 tissue samples per patient; bars indicate SEM). (B) α-limonene levels in human plasma measured by GC-MS at baseline and after 2 wk of α-limonene treatment (n = 2 samples per patient per time point; the fourth patient was unable to provide plasma). (C) α-limonene levels in human saliva were measured by GC-MS at baseline and after 2 wk of α-limonene treatment (n = 1 sample per patient per time point).
analogue site for ALDH3A1). See SI Appendix, Supplemental Methods for details.

**Stimulated Saliva Collection.** Female C57BL/6J mice (9 to 11 wk old) were irradiated to the SMG. Pilocarpine (1.5 mg/kg) was delivered s.c., and saliva was collected for 15 min (14). See SI Appendix, Supplemental Methods for details.

**PAS Staining and Acinar Quantification.** Mouse SMGs were paraffin-embedded and stained with PAS (0.5%). Ten images per group were collected at random. The acinar area was quantified by RT Image software (43). See SI Appendix, Supplemental Methods for details.

**RNA-Seq and Analysis.** Sequencing data were generated on an Illumina HiSeq 4000 system. Genes that showed differential expression between groups were selected and analyzed using MetaCore (GeneGo). See SI Appendix, Supplemental Methods for details.

** Annexin V/PI Apoptosis Assay.** Dissociated EpCAM+ salivary cells were stained with annexin V and PI and were analyzed by FACS. See SI Appendix, Supplemental Methods for details.

**Cleaved Caspase-3 Staining.** Mouse SMGs were paraffin-embedded and stained with caspase-3 rabbit antibody (1:200; Cell Signaling) and DAPI. Three random images were taken from each gland and were quantified by counting cleaved caspase-3+ cells per field. See SI Appendix, Supplemental Methods for details.

**Phase 0 Study in HNC Patients.** This study was approved by Stanford University Institutional Review Board, and written informed consent was obtained. n-iminone levels were measured with GC-MS with perilyl aldehyde as an internal standard (38). See SI Appendix, Supplemental Methods.

**Statistical Summary.** Data consisting of measurements collected at multiple time points were analyzed in a linear mixed-effects model to account for within-mouse correlation. Data that were measured at a single time point were analyzed in an ANOVA model. Post hoc testing of multiple pairwise comparisons was done using either a Tukey adjustment when comparing all possible pairs or a Dunnett’s adjustment when comparing all groups with a single control group. A significance cutoff of 0.05 was used.

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