Hedgehog pathway blockade with the cancer drug LDE225 disrupts taste organs and taste sensation

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Kumari A, Ermilov AN, Allen BL, Bradley RM, Dlugosz AA, Mistretta CM. Hedgehog pathway blockade with the cancer drug LDE225 disrupts taste organs and taste sensation. J Neurophysiol 113: 1034–1040, 2015. First published November 12, 2014; doi:10.1152/jn.00822.2014.—Taste sensation on the anterior tongue requires chorda tympani nerve function and connections with continuously renewing taste receptor cells. However, it is unclear which signaling pathways regulate the receptor cells to maintain chorda tympani sensation. Hedgehog (HH) signaling controls cell proliferation and differentiation in numerous tissues and is active in taste papillae and taste buds. In contrast, uncontrolled HH signaling drives tumorigenesis, including the common skin cancer, basal cell carcinoma. Systemic HH pathway inhibitors (HPIs) lead to basal cell carcinoma regression, but these drugs cause severe taste disturbances. We tested the hypothesis that taste disruption by HPIs reflects a direct requirement for HH signaling in maintaining taste organs and gustatory sensation. We show that pharmacological HH pathway blockade disrupts taste organs and chorda tympani nerve responses. We treated mice with vehicle or the HPI drug LDE225 (erismodegib, sonidegib) by oral gavage for 16 or 28 days and examined gustatory tissue and electrophysiological chorda tympani nerve responses. We tested the hypothesis that HH signaling is required for taste organ maintenance. A principal regulator of cell and tissue homeostasis is the hedgehog (HH) signaling pathway (Barakat et al. 2010). In contrast to maintaining tissues, uncontrolled HH signaling drives development of basal cell carcinoma (BCC), a common skin cancer (Teglund and Toftgard 2010). Patients with BCC are treated with HH pathway inhibitors (HPIs) that block Smoothened, a core HH pathway component (Lin and Matsui 2012). Although HPIs lead to BCC regression, patients taking these drugs report severe taste disturbance (SEKULIC et al. 2013). We suggest that HH signaling maintains neurophysiological taste function, a proposal that has not been examined.

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TASTE SENSATION ON THE ANTERIOR舌 is relayed via chorda tympani nerve fibers that connect and synapse with receptor cells in taste buds in fungiform papillae and anterior folds of the foliate papillae (Breslin and Spector 2008; Krimm and Hill 2000; Mistretta 1991). Taste buds include 50–100 differentiated cells spanning the depth of the lingual stratified squamous epithelium (Chaudhari and Roper 2010). The cells terminate apically in microvilli that are exposed to the oral environment and incorporate receptor proteins and ion channels to initiate taste transduction.

Taste cells differentiate from surrounding epithelium (Liu et al. 2013; Okubo et al. 2009) and turn over in a continuous replacement cycle with life spans from 3 to 30 days (Hamamichi et al. 2006; Perea-Martinez et al. 2013); thus they are susceptible to environmental, metabolic and pharmacological agents that affect the cell cycle. However, the regulation of taste cell differentiation and turnover and maintenance of sensation via the chorda tympani nerve are not well understood. A principal regulator of cell and tissue homeostasis is the hedgehog (HH) signaling pathway (Barakat et al. 2010). In the taste system sonic hedgehog (SHH) is localized within taste buds, positioned for paracrine signaling to HH-responding cells in perigemmal, basal epithelial and stromal cells (Liu et al. 2013). We suggest that HH signaling maintains neurophysiological taste function, a proposal that has not been examined. We suggest that the taste disturbance associated with HPI use relates to taste bud and chorda tympani nerve disturbance when HH signaling is inhibited.

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MATERIALS AND METHODS

Animals

Adult female, Gli1lacZ/+ reporter mice (Bai et al. 2002; Jackson Laboratory Strain 003081), used to monitor the expression of HH signaling, or C57BL/6 mice (Charles River) were used under National...
Institutes of Health and University of Michigan Animal Care and Use Committee approved protocols.

Experiments

Mice were treated by daily oral gavage for 2 wk (14–16 days; n = 10 LDE225-treated, 7 vehicle-treated) or 4 wk (28–29 days; 6 LDE225-treated, 6 vehicle-treated) with LDE225 (ChemieTek NVP-LDE225) dissolved in vehicle (PEG 400:5% dextrose in water) at a dose of 20 mg/kg, or vehicle alone. The dose was determined from data in Pan et al. (2010) and in preliminary studies. The oral gavage probe (22 gauge, 25 mm; Instech) by-passed all oral tissues, for direct delivery into the stomach. Treatments were 14–16 days to encompass the “average” 10 day life span of taste bud cells (Beidler and Smallman 1965), and 28–29 days to encompass about three “average” life spans. We refer to treatment times as 16 or 28 days.

After gavage, tongues were collected and prepared for tissue analysis, or electrophysiological responses were recorded from the chorda tympani nerve, and then tongues were collected.

Tissue preparation. Tongues were fixed overnight at 4°C in 4% paraformaldehyde in 0.1 M PBS, cut anterior to the intermolar eminence, bisected, and half was transferred to 70% ethanol for paraffin embedding and serial sagittal sections at 6 μm. The other half tongue was cryoprotected with 30% sucrose in PBS and frozen in O.C.T. Serial sagittal sections were cut at 10 μm and mounted for immunostaining (Liu et al. 2013).

Primary antibodies were as follows: goat anti-SHH (AF464, 0.1 μg/ml, R&D Systems); rat anti-keratin 8 (K8) (TROMA-1, 1:1,000, Developmental Studies Hybridoma Bank); rabbit anti-neurofilament-H (NB300-135, 1:1,000, Novus Biologicals); rabbit anti-P2X3 (NB100-1654, 1:2,000, Novus Biologicals). Secondary antibodies were Alexa fluor 488 or 568 (Invitrogen, 1:500). For X-Gal staining, frozen sections from Gli(+/−) reporter mice (16-day treatment) were dried, rehydrated and transferred into X-Gal solution (Liu et al. 2013).

Procedures for scanning electron microscopy were as described (Liu et al. 2013).

Electrophysiology. Mice were anesthetized with ketamine-xylazine mixture (80–100 mg/kg ketamine; 5–10 mg/kg xylazine ip) and maintained with ketamine (80–100 mg/kg). The chorda tympani was dissected, cut centrally and placed on a recording electrode, with a reference electrode nearby. Amplified neural activity was connected to an analog-to-digital converter (Cambridge Electronic Design) and passed through an integrator circuit (Shimataki et al. 2002).

Tactile and thermal stimuli consisted of stroking the anterior tongue quadrant five to seven times over about 5 s with a glass rod or applying 4°C water. Chemical stimuli were dissolved in room temperature, distilled water, and 30 ml were applied on the tongue by gravity flow at 3 ml/s: 0.1 and 0.5 M NaCl; 0.1 and 0.5 M NH₄Cl; 0.01 M HCl; 0.03 and 0.1 M citric acid; 1.0 M sucrose; 0.1 M monosodium glutamate; and 0.02 M quinine HCl. Chemicals remained on the tongue for 5 s after stimulus application, was measured as response units in millimeters. To compare chorda tympani responses, the magnitudes can be normalized or expressed as ratios relative to a standard stimulus, such as 0.5 M NH₄Cl. However, analyzing the current data in this way distorts the dramatic effects because LDE225 treatment can effectively eliminate responses to all chemical stimuli. Therefore, we used absolute measures of taste-evoked activity and plotted mean values for taste responses. Amplifier settings were unchanged across recordings.

Statistics. We used two-way ANOVA to compare differences for each papilla morphology category, with main effects of treatment or duration of treatment, and groupwise comparisons with the least significant difference approach for LDE225 vs. vehicle treatment at each duration, and for 16- vs. 28-day duration for each treatment type. The independent samples t-test, with Levene’s test for equality of variance, was used to compare the magnitude of chorda tympani responses for each chemical between treatments. For responses to cold stimuli, we used two-way ANOVA to assess main effects for treatment and duration, and least significant difference for within treatment, or duration, comparisons.

RESULTS

HH Pathway Inhibition Alters Papilla and Taste Bud Morphology and Numbers

LDE225 treatment profoundly alters fungiform papilla and taste bud morphology (Fig. 1A). Compared with vehicle treatment, there is a loss in papilla integrity to acquire a conical tip and loss of taste buds to a few cell remnants. In LDE225-treated mouse tongues, only 0–7% of papillae are typical compared with >90% in vehicle-treated mice [Fig. 1A, Typical Fungiform Papilla (FP) and Typical Taste Bud (TB); F(1,25) = 2562.2, P < 0.001; 16-day vehicle vs. LDE225, P < 0.001, 28-day vehicle vs. LDE225, P < 0.001]. Furthermore, after LDE225 gavage, 48–66% of the fungiform papillae have acquired a conical apex with thick layers of keratin compared with 2–3% with vehicle-treatment [Fig. 1A, Atypical FP and Atypical TB; F(1,25) = 192.1, P < 0.001; 16 days between treatment, P < 0.001; 28 days, P < 0.001]. Some of these atypical, hyperkeratinized papillae contain disoriented taste bud cells with or without a taste pore, but no taste buds are typical. Furthermore, in LDE225-treated mice, 27–52% of fungiform papillae had no taste buds compared with only 2–7% in vehicle-treated mice [Fig. 1A, Atypical FP and No TB; F(1,25) = 92.2, P < 0.001; 28-day between treatment, P < 0.001; 28 days, P < 0.001].

In addition, within each papilla and taste bud category (Fig. 1A), there were significant overall differences between 16- and 28-day durations [each category: F(1,25) values = 9.9, P = 0.004; F = 5.1, P = 0.033; F(1,25) = 15.7, P = 0.001]. With post hoc tests, it was apparent that the differences were for duration between LDE225-treated groups, shifting to more
extreme acquisition of atypical papillae without taste buds (P values /H110050.01 to /H110210.001).

Overall, compared with vehicle treatment, mice after LDE225 gavage have a remarkable decrease in typical papillae and taste buds. Furthermore, HPI treatment extended to 28 days compared with 16 days results in a significant increase in the Atypical FP and No TB category.

To confirm that HPI treatment reduced HH signaling in fungiform papillae, we analyzed tongues of Gli1lacZ reporter mice after 16 days of treatment. Compared with vehicle, Gli1 expressing, HH-responding, β-Gal positive cells in the papilla epithelium are eliminated after 16 days of LDE225 gavage. Stromal β-Gal positive cells remain. Dashed lines demarcate the epithelial border, and a circle denotes the TB, from surrounding perigemmal cells. C: scanning electron micrographs (SEM) for vehicle- and LDE225-treated tongues illustrates intact filiform papillae and orientation. FP are indicated with a circle. Insets represent misshapen FP with a cornified cap (asterisk) and no taste pore after LDE225 treatment. The FP in the vehicle SEM has a taste pore (arrow). Scale bars apply to all images in respective panel.

Filiform Papillae After HH Inhibition

In contrast to profound effects on fungiform papillae and taste buds, nongustatory, filiform papillae are not altered with HPI treatment. After 16 and 28 days, the stereotypic orientation of filiform papillae was intact in LDE225- compared with vehicle-treated tongues (Fig. 1C). Scanning electron microscopy also confirmed the atypical fungiform papillae with spiny cap and absence of taste pore in LDE225-treated tongues (Fig. 1C, insets).

Residual Taste Bud Cell Remnants and Papilla Innervation After HPI Treatment

In taste buds from LDE225-treated tongues, SHH was much reduced, as expected in association with a reduction in taste bud size (Fig. 2A, 16 days; 2B, 28 days, K8/SHH). Remnants of taste bud cells that remained after HPI treatment were labeled with the taste bud cell marker K8, and taste bud cells were markedly reduced in number (Fig. 2, A and B, K8/SHH insets). SHH was detected in a subset of the K8-positive taste bud cells. Because only 0–7% of papillae and taste buds were typical after 16 or 28 days of HPI treatment (Fig. 1A), there were in fact extremely few intact taste buds on the tongue.

Sustained innervation was observed in papillae of LDE225-treated tongues, comparable to that detected in vehicle-treated tongues after 16 or 28 days of treatment (Fig. 2, A and B, K8/NF). However, an apical “basket” of NF-positive innervation seen in vehicle-treated tongue is not obvious with LDE225 treatment, in association with taste bud cell reduction or loss.

Taste nerves express P2X3 receptors that are essential for synaptic transmission between afferent innervation and taste cells (Finger et al. 2005; Ishida et al. 2009). P2X3, therefore, marks afferent taste fibers. Notably, taste fibers were retained in the papilla core of LDE225- compared with vehicle-treated mice (Fig. 2, A and B, K8/P2X3). Overall, results suggest that the effect of the HPI LDE225 is principally epithelial and does not directly impair papilla and gustatory innervation. However,
further study can clarify the precise effect of HH pathway inhibition on the extent of innervation.

**Reduced Taste Nerve Responses to Chemical Stimuli After HH Pathway Inhibition**

Chorda tympani recordings after 16-day gavage in vehicle and LDE255-treated animals illustrate nerve responses (Fig. 3A). Responses were obtained to all taste stimuli in mice with vehicle treatment. Salt and acid responses were of higher magnitude than responses to sweet, bitter or umami stimuli.

Notably, responses to chemical stimuli were reduced substantially or eliminated after 16-day HPI treatment. Integrated responses to the chemical stimulus sequence from two LDE225-gavaged mice (Fig. 3A) illustrate that, although the response to each chemical was profoundly reduced in each mouse, the degree of response reduction differs. Overall, residual responses comparable to those in Fig. 3A were recorded in three animals after HPI gavage (LDE residual), whereas there were no responses from the other three animals (LDE225 no response). Residual responses typically were apparent for the higher concentrations of NaCl, NH4Cl and citric acid. Stimulation with the other taste qualities, and HCl, was ineffective.

Average responses from 16-day LDE225-treated mice (Fig. 3B) demonstrate significantly reduced responses for all chemicals ($t$ values = 3.2–5.9, $P = 0.01–0.03$), except sucrose and monosodium glutamate, where the $P$ values were marginal ($P = 0.07$, 0.11). After 28 days of LDE225 treatment, responses to taste stimuli were effectively eliminated (Fig. 3A). All comparisons of LDE225- with vehicle-treated chemical responses were highly significant (Fig. 3B; 28 days, $t$ = 3.7–11.9; $P = 0.0001–0.02$), except quinine HCl ($P = 0.18$).

Thus profound reductions or complete loss of chemical responses were observed after 16 days of HPI, and after 28 days taste responses were absent.

**HPI Treatment and Responses to Touch and Cold**

The chorda tympani responds to lingual touch and cold stimuli (Finger et al. 2005; Ogawa et al. 1968). Because responses to touch stimuli are rapidly adapting, and not sustained, summated responses are not a valid representation of the neural data. Therefore we present "raw" recordings for tactile stimulation (Fig. 4A).

Although 16 days of LDE225 treatment reduced or eliminated responses to chemicals, responses to touch and cold stimuli in the chorda tympani receptive field were not affected (Fig. 4, A and B). After 28 days of LDE225 treatment, tactile responses still were obtained from all mice (Fig. 4A). However, cold responses were lost (Fig. 4B).

There was an overall difference in treatment effect for cold responses between 16- and 28-day duration [$F(1,14) = 4.99$, $P = 0.04$]. Post hoc analysis indicated that the difference was
at the 28-day (P = 0.004), not 16-day, duration. Furthermore, for LDE225-treated mice, the difference in cold responses between 16- and 28-day durations was profound [F(1,14) = 23.2, P < 0.001]. The data indicate that alterations in chemical response magnitude did not result from a generally disrupted innervation, and that effects of HH inhibition were modality specific. Furthermore, with longer LDE225 treatment, chemical responses were no longer observed in any HPI-treated mice, and cold responses were lost.

DISCUSSION

This study is the first to demonstrate that HH signaling is a principal regulator of papilla and taste bud maintenance and neurophysiological taste sensation. We show that the HPI LDE225 profoundly affects the peripheral taste system, including altered fungiform papilla morphology, reduced taste bud numbers, and extreme reductions in or loss of chorda tympani nerve responses to all taste qualities.

BCC patients treated with HPIs experience side effects, including “taste disturbances” of decreased taste perception, loss of taste or abnormal taste (Sekulic et al. 2012; Tang et al. 2012). Observed changes in taste sensation in LDE225-treated mice can explain the alterations in taste perception in patients treated with HPIs. Although defects in taste are common in treatment with various anti-neoplastic agents, our data suggest that the taste effects with HPI treatment reflect a specific requirement for physiological HH signaling in adult taste organ structure and function that is disrupted in HPI-treated patients.

Taste Papilla and Taste Bud Effects and Innervation

SHH ligand is produced by taste bud cells for signaling in a paracrine manner to HH-responsive cells in the basal epithelial
layer, perigemmal cells, and stromal cells of the papilla (Liu et al. 2013). With HPI treatment, we now demonstrate loss of HH pathway activity (Gli1lacZ/+ expression) in HH-responding cells, primarily in the papilla epithelium. We propose that blockade of epithelial HH signaling leads to the profound alterations in taste bud structure and function.

After LDE225 treatment, fungiform papillae exhibit a strikingly atypical morphology. The apical papilla cells acquire a spinous keratin cap reminiscent of filiform papillae, supporting the specific proposition that HH signaling is required to maintain the fungiform papilla (Liu et al. 2013). Furthermore, the K8+ taste bud remnants situated under this spinous cap lack a taste pore. Although the papilla structure is not intact, NF+ and P2X3+ nerve fibers remain within the papilla core, indicating that HPI treatment affects taste organs themselves and does not grossly disrupt the innervation. Furthermore, nongustatory, filiform papillae are not obviously altered.

Chorda Tympani Responses to Taste Stimuli

In LDE225-gavaged mice chorda tympani nerve taste responses were reduced or eliminated after 16 days, and eliminated after 28 days. Logically, response reductions could relate to reduction in taste buds. The residual taste responses that we observed in one-half of the 16-day, LDE225-treated animals might be attributed to retention of some “typical” papillae and taste buds in treated tongues. Furthermore, the residual, measurable responses to NH4Cl and citric acid, in particular, could suggest that some taste cells are more gradually susceptible to HPI treatment, possibly related to variable turnover cycles of taste bud cells and response properties of specific cell types (Perea-Martinez et al. 2013).

Chorda Tympani Responses to Touch and Temperature Stimuli

In contrast to taste, responses to lingual touch and cold in the chorda tympani were not affected after 16 days of LDE225 treatment, and touch responses remained after 28 days. Presumably the tactile and thermal fibers terminate in either mechanoreceptors or thermal receptor endings, not transduced via taste bud cell receptors. Notably, chorda tympani response differences across modalities also were reported in knockout mice lacking P2X3 receptors, required in synaptic transmission between taste afferent fibers and taste bud cells (Finger et al. 2005). In these mice, although all taste responses were eliminated, responses to touch and temperature remained.

Our results suggest that mechanical and thermal modalities could be “spared” the effects of HH pathway inhibition in the taste system. However, this is complicated by reports that the chorda tympani innervates taste buds only, not surrounding papilla epithelial cells (Whitehead et al. 1985); thus tactile and thermal nerve endings presumably would be within the taste bud. It is possible that responses to touch and thermal stimuli originate from receptors adjacent to the taste bud, suggesting a broader distribution of the chorda tympani receptive field rather than only within the taste bud. Overall, the data clearly indicate that HPI treatment differentially affects oral sensations and demonstrate the importance of updated, accurate maps of taste, touch and thermal receptors in fungiform papillae.

In summary, our study establishes an essential and modality-specific requirement for HH signaling in maintaining neurophysiological taste sensation in mice, underlying the likely cause of taste disruption in HPI-treated patients. We propose that taste disturbances in HPI-treated patients derive from HH-dependent loss of taste papilla integrity and taste buds, with a specific, concomitant reduction of peripheral nerve taste responses that transmit taste sensation centrally. Our results predict that, although taste disruption in HPI-treated patients will be profound, other lingual modalities of touch and temperature may be spared.

NOTE ADDED IN PROOF

While this paper was under revision, Yang et al. reported effects on taste bud cells in the circumvallate papilla in mice treated with Vismedogib for 15 wk. The report of these findings has been added to the reference list (Yang et al. 2014).

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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