Recovery of taste organs and sensory function after severe loss from Hedgehog/Smoothened inhibition with cancer drug sonidegib

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Significance

Hedgehog pathway-inhibitor drugs effectively treat basal cell carcinoma, a common skin cancer. However, many patients taking such drugs report severe taste disturbances that impair their quality of life. To understand the biology behind these adverse effects, we studied the consequences of Hedgehog pathway inhibition on taste organs and neural sensation in mice. Taste bud progenitor-cell proliferation and differentiation were altered, resulting in taste bud loss. Nerve responses to lingual taste stimuli were also eliminated, while responses to touch and cold stimuli remained. After stopping Hedgehog pathway inhibition, taste buds and sensory responses recovered. This study advances our understanding of Hedgehog signaling in taste homeostasis and the reported taste recovery after clinical treatments with Hedgehog pathway-inhibiting drugs.

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SMO is the core signal transduction component of HH signaling (Fig. 1A) (17, 18). In HH signaling, pathway activity is repressed in the absence of ligand via the transmembrane receptor PTCH, which represses the pivotal HH signaling protein SMO (19–21). HH binding to PTCH1 blocks its inhibition of SMO; downstream signaling is then initiated, resulting in the modulation of GLI transcription factors (GLI1, GLI2, GLI3) and leading to the transcription of target genes, including Gli1 and Pch1 (Fig. 1A). In the tongue, HH pathway components are in distinct compartments of adult taste papillae and TB (Fig. 1B), including actively proliferating TB progenitor cells (8, 13). The secreted SHH ligand in epithelium is produced principally within TB cells (13, 22), whereas the HH targets, Pch1 and Gli1, are expressed within periglomerular cells around the TB, in basal cells of the stratified epithelium that lines the papilla core, and in stromal or connective tissue cells of the papilla core (Fig. 1B) (11, 13). Paracrine signaling from SHH in TB cells to surrounding epithelial and connective tissue cells has been proposed (13, 23).

Sonidegib and other HPI drugs block the HH pathway at SMO and are effective in treating patients with advanced BCC (3, 16, 24–26). However, these patients experience dysgeusia and ageusia that compromise treatment adherence and quality of life (4, 5, 27). Therefore, in patients treated with sonidegib who experience dysgeusia, it was important first to determine the temporal aspects of HH/SMO signaling inhibition in mice gavaged with sonidegib for 5–36 d. We quantified effects by characterizing FP and TB morphology and the recovery of taste organs and sensation. Our data provide insight into the regenerative biology and clinical consequences in patients treated with sonidegib who experience dysgeusia.

### Results

#### Treatment with HPI Drug Sonidegib Alters FP Taste-Organ Morphology Within 10 D.

Before testing recovery from HPI drug treatment, it was important first to determine the temporal aspects of HH/SMO signaling inhibition in mice gavaged with sonidegib for 5–36 d. We quantified effects by characterizing FP and TB morphology as category I (typical FP/TB), II (atypical FP/TB), or III (atypical FP/no TB) (Fig. 1C). Compared with vehicle-treated mice, there were no differences in FP/TB categories after 5 d of sonidegib gavage (Fig. 1D). However, after just 10 d of sonidegib treatment the proportion of category I FP (typical FP/TB) FP was reduced by half, and category II FP (atypical FP/TB) had increased several fold to 40% of all FP, compared with ∼2% in vehicle treatment. After 16 d of sonidegib gavage only ∼1% of all FP were category I (typical FP/TB) FP; category II (atypical FP/TB) FP comprised almost 70%, and category III (atypical FP/no TB) FP accounted for ∼30% of all FP (Fig. 1D). With 28 d of sonidegib gavage, category I (typical FP/TB) were essentially eliminated, replicating our prior results (7); notably, after 36 d, virtually all FP were category III, atypical FP/no TB (Fig. 1D). Therefore, in a detailed time course, HPI drug duration of 10–16 d eliminated typical FP and TB, whereas 5 d was not effective, and in progressive effects by 36 d only atypical papillae that lack TB (category III) remained on the tongue. (Statistics for data in Fig. 1D are given in Fig. S1A.)

Importantly, we quantified all FP numbers in a standard 600-μm region of vehicle- and sonidegib-treated tongues and found no reduction in papilla density across treatments (Fig. S1B). That is, FP were not eradicated, and we were not categorizing fewer FP at long treatment durations. Rather, these duration-specific results suggest that HPI drug effects are related to the disruption of physiologic cell renewal in taste organs.
To further understand the progression of TB loss in FP, we used immunostaining to test whether all TB cell types were susceptible to sonidegib treatment or whether specific taste cell types (type 1: NTPDase2+/p63+, type 2: PLCG2+/p63+, type 3: SNAP25+/p63+) were lost in a particular sequence or time course or were retained. Whereas cell types 2 and 3 were essentially eliminated in TB after 28 d, type 1 cells were not completely lost until 36 d of sonidegib gavage (Fig. 1F). The type 1 cells are most numerous in the TB and have a long turnover time, up to 24 d (29–31), which could account for their longer retention after HPI treatment. However, turnover for no TB cell type was resistant to the blockade of SMO, the HH signaling effector targeted by the Smo inhibitor (Fig. 1C). This early decrease in the number of Ki67+ TB progenitors in the FP was sustained after 16 and 28 d of drug treatment (Fig. 2A and B). Alterations in proliferating cell numbers were not observed in basal or perigemmal regions of FP epithelial cells (Fig. 2A and B). Note that perigemmal counts were made only within category I and category II FP, which have TB or TB remnants, because there were no TB in type III FP (Fig. 2A, 28 d and Fig. 2B). Notably, there was no reduction in Ki67+ cells in the basal cells of filiform papillae (Fig. 2A and B).

We did not observe differences in the extent or distribution of cell death markers [TUNEL assay, Cleaved caspase 3 (CC3) antibody] in FP or filiform papillae of vehicle- or sonidegib-treated tongues (Fig. S2A and B). That is, accelerated or altered cell death did not contribute to TB cell loss in HPI.

**Cell proliferation and cell death.** To test whether HPI altered turnover in lingual epithelium, we studied cell proliferation within FP and the nontaste filiform papillae. In FP we designated three regions, basal, apical, and perigemmal zones of FP basal cells (Fig. 24, vehicle), and quantified Ki67+ cells for proliferation. Cells in all these regions include TB progenitors (13, 32). In basal cells of the apical FP wall there were substantial reductions in Ki67+ cells as early as 3–5 d of HPI treatment (Fig. 2 A and B), that is even before the FP/TB organs had noticeable morphological disruption (Fig. 1D). This early decrease in the number of Ki67+ TB progenitors in the FP was sustained after 16 and 28 d of drug treatment (Fig. 2A and B). Alterations in proliferating cell numbers were not observed in basal or perigemmal regions of FP epithelial cells (Fig. 2A and B). Note that perigemmal counts were made only within category I and category II FP, which have TB or TB remnants, because there were no TB in type III FP (Fig. 2A, 28 d and Fig. 2B). Notably, there was no reduction in Ki67+ cells in the basal cells of filiform papillae (Fig. 2A and B).

To establish that the effects observed in sonidegib-treated mice reflected the blockade of SMO, the HH signaling effector targeted by the Smo inhibitor, we generated mice to conditionally (doxycycline-regulated) delete *Smo* globally (R26Rcre;E10371, referred to as *Kumari et al.* PNAS)
“cSmoKO” mice) or in the epithelium (K5rtTA;tetO-cre;Smo<sup>fl/fl</sup>, referred to as “cSmoKO-epi” mice), diagrammed in Fig. 3A. In cSmoKO mice, the category I FP (typical FP/TB) were reduced to less than 10% of all FP after 16 d of Smo deletion (Fig. 3B). With epithelial deletion of Smo, in cSmoKO-epi mice, there were no effects at d 5 after gene deletion, but after 16 d only 15% of FP were category I (typical FP/TB) (Fig. 3B). After 24 d, more than 40% of all FP were category III (atypical FP/no TB), which is comparable to the 16-d effects in cSmoKO mice. Therefore the major target cell population on which sonidegib acts to alter FP and TB is likely to be epithelial. Statistical analyses for the data in Fig. 3B are in Fig. S3A. The FP density did not alter with time in either Smo deletion model (Fig. S3B).

Overall the data from two cSmoKO models are similar in time course, extent, and effects after sonidegib treatment. Further, when comparing sonidegib and Smo-deletion experiments a similar phenotypic was seen in category III FP (atypical FP/no TB) (Fig. S3C, H&E). That is, a heavily cornified, conical papilla apex was observed with a discernible cell collection where the K8<sup>+</sup> taste cells had been located. As with sonidegib treatment, in the category III papilla after Smo deletion, K5<sup>+</sup> cells occupied the conical papilla apex (Fig. S3C, K5/K8). With HPI, therefore, K5<sup>+</sup> cells replace differentiated cells of the apical FP that typically include TB cells and accumulate as suprabasal, stratified epithelial cells.

**After Smo Deletion, SHH<sup>+</sup> and K8<sup>+</sup> Cells Are Reduced in FP, and HH-Responding Cells Are Eliminated from the Epithelium but Remain in the Connective Tissue Core.** Associated with the loss of FP cells (using K8 as a taste-cell marker), the SHH<sup>+</sup> FP cells also were reduced in cSmoKO and cSmoKO-epi mice (Fig. 4A, SHH/K8). Furthermore, Gli1lacZ<sup>+</sup>-positive HH-responding cells were not retained in the FP epithelium, in either the perigemmal cells or basal cells of the FP epithelial wall (Fig. 4A, Gli1lacZ<sup>+</sup>). In the papilla core, however, HH-responding cells remained but were reduced in number after global Smo deletion compared with controls. The remaining HH-responding cells in the FP core of cSmoKO mice might relate to noncanonical signaling; alternatively, the efficiency of Smo deletion in stromal cells might be less than in epithelial cells.

As HHI signaling activity was eliminated in the epithelium, there was an apparent decrease in K67<sup>+</sup> proliferating cells in the basal cell layer of the apical papilla wall but not in the basal region (Fig. 4B, Ecad/Ki67). This demonstrates a disruption in the supply of a subset of TB cell progenitors.

**After Smo Deletion, Nerves Remain Within the FP Connective Tissue Core.** Whereas TB cells were absent after Smo deletion in both cSmoKO and cSmoKO-epi mice, innervation remained within the FP core from the lingual nerve that typically innervates the FP walls and perigemmal regions (neurofilament, NF<sup>+</sup>, nerve fibers) and from the chorda tympani nerve that typically innervates TB cells (P2X3<sup>+</sup> taste nerve fibers) (Fig. 4C, K8/NF and K8/P2X3). In addition, X-Gal staining for Gli1lacZ<sup>+</sup>-positive cells plus NF immunofluorescence demonstrates a close physical association of nerves and HH-responding cells in the FP connective tissue core in control and cSmoKO mice (Fig. 4D), suggesting potential interactions between the papilla innervation and stromal cells. Furthermore, the results demonstrate that nerve fibers sustained within the FP are not able to maintain TB in the context of HPI effects in lingual epithelium.

**HPI Leads to Decreased TB in CV on Posterior Tongue.** Fig. 3. Smo deletion alters FP morphology and reduces TB. (A) Diagrams showing the conditional deletion of Smo from all tissues (cSmoKO) or from epithelial cells and progeny (cSmoKO-epi). The gray/shaded region on the cSmoKO-epi diagram indicates normal Smo expression. (B) Percentage of category I (typical FP/TB), category II (atypical FP/TB), and category III (atypical PP/TB) FP in cSmoKO or cSmoKO-epi mice. Bars are mean ± SEM. Numbers of tongues are in parentheses. Brackets indicate significant differences (two-way ANOVA with Tukey’s HSD post hoc tests). *p < 0.001 for control vs. cSmoKO or cSmoKO-epi. Complete F and P values are given in Fig. S3A.

**Recovery of FP and TB Cells and Molecular Phenotypes When Sonidegib Treatment Is Discontinued.** To test potential FP taste-organ restoration after HPI, we gavaged mice with sonidegib for 16 d and then discontinued treatment and maintained mice for recovery periods of 7, 14, or 21 d and for 3, 5, or 9 mo (Fig. 6A). A recovery period of 7 d was not sufficient to restore papillae to category I morphology (typical FP/TB); rather most papillae were atypical, in
category II or III (Fig. 6B). Remarkably, however, 14 d were sufficient to restore about 50% of all papillae to category I morphology, and only 5% or fewer of all FP were category II FP (atypical FP/TB). About 40% of all papillae were category III (atypical FP/no TB).

We used prolonged recovery times to determine whether an increased proportion of FP was restored to category I after several months. Notably, the proportions of typical FP/TB (category I) and atypical FP/no TB (category III) did not change from 14 d through 9 mo of recovery (Fig. 6B). This suggests that a proportion of FP is not resilient and cannot recover after HPI drug treatment, whereas another FP subset recovers and maintains TB after sonidegib treatment is discontinued. Details of FP/TB morphology during treatment and the recovery transition period from 7 to 14 d after stopping sonidegib treatment, and after 9 mo of recovery are seen with H&E staining in Fig. S5A. The category III (atypical FP/no TB) phenotype persists throughout treatment and recovery. This category, which loses all TB cells, apparently cannot recover from HPI effects, whereas category II FP (atypical FP/TB), which retain TB cell remnants, presumably can recover.

Statistical analysis for FP/TB categories across recovery in Fig. 6B is presented in Fig. S6A, and additional data are included to demonstrate that FP numbers per tissue region do not differ across sonidegib treatment and recovery periods (Fig. S6B).

To test whether all taste cell types in the FP/TB recover after discontinuing sonidegib treatment or whether some types were resistant to recovery, we used immunostaining and quantified cell types. As noted, all cell types were eliminated after the HPI drug (Fig. 1D), but when treatment was discontinued the three cell types were restored, with similar time courses, after 14 d in category I (typical FP/TB) and category II (atypical FP/no TB) FP to the values seen in vehicle-treated FP (Fig. S6C). Category III FP (atypical FP/no TB) were not quantified because no TB were present.

During recovery from HPI treatment, which occurs between 7 and 14 d after stopping sonidegib gavage, there was an increase in SHH+ and K8+ cells in the reconstituted taste buds (Fig. 6C, SHH/K8), associated with recovery of Gli1lacZ+ cells in the perigemmal region and FP epithelial walls (Fig. 6C, Gli1lacZ). A gradual restoration of Gli1lacZ+ cells was apparent in the varied expression patterns seen at 7 d recovery, when HH-responding cells were observed in perigemmal regions only or partially patterned in perigemmal cells and papilla walls (Fig. 6C, 7 d recovery and inset). Overall, the HH ligand and responding cells returned to FP/TB in concert at 14 d after drug cessation.

Recovery of TB in the CV. TB in the posterior tongue CV papillae were decreased after 16 d of sonidegib treatment (Fig. 5). When sonidegib gavage was discontinued, there was no restoration of TB with pores after 7 d (Fig. S5B), but there was a trend toward increased numbers at 14 d. By 3–9 mo of recovery the numbers of TB with pores were not different from the numbers in vehicle-treated mice. Therefore, there was recovery from HPI effects in the CV as well as in the FP.

Innervation in the FP. Innervation, shown with NF+ and TB-specific P2X3+ fibers, was retained within the FP core during sonidegib treatment and also was obvious and robust during recovery from HPI (Fig. 7A and B). The nerve fibers were observed even in category III papillae (atypical FP/no TB) that have no taste buds (Fig. S7A) and in FP after prolonged HPI for 36 d (Fig. S7B). There were no detectable differences in the extent of innervation in papillae treated with vehicle or the HPI drug (Fig. S7C). However, nerves alone could not maintain or restore TB in FP with epithelial effects of HH/SMO inhibition.

We found that HH-responding cells and nerve fibers remain within the FP connective core after HPI and continue in recovery periods (Figs. 6C and 7A and B). Nerve fibers and neurons in the dorsal root ganglion are a source of SHH in signaling to Gli1-expressing cells in the hair follicle (35). To determine whether fibers in the FP and their soma express SHH, we studied geniculate ganglion (GG) and trigeminal ganglion (TG) neurons in Shh reporter mice. SHH expression has been reported in TG neurons (36), and we confirmed that Shh+ fibers were observed even in category III papillae (atypical FP/no TB) that have no taste buds (Fig. 7C). Importantly, we further observed Shh expression within GG neurons and Shh+ fibers within the tongue and FP and projecting into TB (Fig. 7C). To test whether SHH+ neurons were altered after HH pathway inhibition, we used immunoreactions for SHH in ganglia after vehicle or sonidegib treatment and quantified SHH+ neurons with NeuN coexpression in the GG. No differences were detected (Fig. 7D). Although not quantified, similar results were seen in the TG (Fig. 7E). This indicates that a GG ganglion source of SHH ligand remains during pathway inhibition, although the lingual epithelial SHH associated with TB cells is eliminated. The GG- and TG-derived SHH sources could potentially maintain HH signaling in papilla stroma via secretion from nerves.

Responses to Chemical Stimuli from the Chorda Tympani Nerve Recover After Sonidegib Treatment Is Stopped, and Tactile and Cold Responses Are Maintained Throughout. To determine early sensory effects after HPI and to test whether taste sensation is restored in concert with taste-organ recovery, we recorded from the chorda tympani nerve that innervates TB in FP and stimulated the tongue with chemicals including salts, acids, sucrose, and quinine to elicit taste responses. Compared with vehicle treatment, after 10 d of sonidegib treatment the taste responses were fully maintained (Fig. 8). Therefore, although 10 d of HPI drug treatment effectively reduced the proportion of category I (typical FP/TB) FP to about 60% of the proportion in vehicle treatment (Fig. 1D), neurophysiological responses were not altered. However, after 16 d of sonidegib, responses to chemicals
were essentially eliminated, replicating our prior data for 16-d treatment (7), and category I (typical FP/TB) papillae were reduced to less than 3% of all FP (Fig. 1D).

We further tested whether neural responses could be restored after HPI that had effectively eliminated intact FP taste organs. When sonidegib treatment was stopped after 16 d, a recovery period of 7 d was not sufficient to restore chorda tympani nerve responses to lingual taste stimuli (Fig. 8). Nor is this recovery period sufficient for restoring category I (typical FP/TB) papillae on the tongue in large proportion (Fig. 6B). In contrast, after 14 d recovery and continuing through 9 mo, chorda tympani nerve responses to taste stimuli were fully restored. However, during this period the category I (typical FP/TB) papillae were ∼55% and category III (atypical FP/no TB) taste organs were ∼40% of all papillae (Fig. 6B). Therefore, normal chorda tympani nerve responses can be obtained with only about 55% of intact FP taste organs recovered. (Quantified analysis for data in Fig. 8 is presented in Fig. S8A.)

**Modality-specific effects.** Importantly, we also stimulated the anterior tongue with tactile (stroking with a wooden rod) and cold (water at 4 °C) stimuli. Although HPI for 16 d can eliminate chorda tympani nerve taste responses, responses to tactile and cold modalities were retained (Fig. 8), replicating our prior data (7). This particularly noteworthy result demonstrates that HH differentially regulates oral sensory modalities and suggests that the receptor organs for touch and cold are not within the TB cells per se.

The tactile and cold responses were sustained throughout all the recovery periods, from 7 d through 9 mo (Fig. 8). We have observed Shh+ nerve fibers in ShhCre reporter mice that extend in apical FP outside the bounds of TB cells (Fig. 7C) and propose that these might represent fibers that respond to touch and/or cold FP stimulation or fibers that connect to epithelial receptors for tactile and temperature.

**Response/concentration series and chloride salt stimuli.** We challenged the taste organs and neurophysiological properties of the chorda tympani nerve with ability to generate graded responses to a series of NaCl (from 0.05–1.0 M) during HPI and with recovery. After sonidegib treatment for 16 d, nerve responses were essentially not discernible, except as very small responses to 0.5 and 1.0 M NaCl (Fig. S9A). A 7-d recovery period after sonidegib treatment was not sufficient for restoration of responses. However, after 14 d
graded responses to lingual stimulation with increasing concentrations of NaCl were apparent (Fig. S9A). The timing of recovery, between 7 and 14 d, matches that for responses to a broad range of taste stimuli (Fig. 8). Further, response/concentration functions demonstrated typical curves after 14 d of recovery and through 9 mo (Fig. S9B).

We also used a series of 0.5 M chloride salts, which have distinctive "tastes" and receptor mechanisms, after HPI and with recovery. After sonidegib treatment the chorda tympani nerve did not respond to stimulation with Mg^{2+}, Ca^{2+}, K^{+}, NH_{4}^{+}, or Na^{+} chloride salts (Fig. S8B). However, recovery in responses to values comparable to vehicle treatment was apparent after sonidegib had been discontinued for 14 d.

Therefore, chorda tympani nerve responses to a range of chemical taste qualities, a concentration series of NaCl, and high-concentration disparate chloride salts were eliminated by HPI but recovered by 2 wk after treatment was discontinued. On the other hand, responses to lingual stimulation with touch or temperature modalities were not affected by treatment with the HPI drug sonidegib.

Discussion

We tested the potential for restoration of taste homeostasis after HH signaling deregulation and show that taste organs and sensory responses can recover after severe loss from HH/SMO inhibition with the cancer drug sonidegib. Indeed, after elimination of TB and of chorda tympani nerve responses to taste stimuli, there is a remarkable restoration of TB in taste organs and full recovery of taste nerve responses. TB elimination based on the deregulation of essential HH signaling controls for cell proliferation and differentiation and loss of all TB cell types is rapid, within 2 wk. Whereas nerves remain during HH/SMO inhibition and recovery, they are not able to sustain or restore TB in the context of selective taste papilla epithelial effects. However, when sonidegib treatment is stopped, the taste organs are restored, and neural taste sensation returns to typical response patterns within 2 wk. Although restoration of a full complement of TB numbers is not achieved, the recovery of chorda tympani nerve responses can be supported by about 55% of all FP taste buds. Redundancy in the taste periphery has long been noted (37, 38) as being crucial for life-essential sensations but has not previously recognized as important in recovery from HPI drug effects. We suggest that the reversible taste disruption in HPI-treated patients is, therefore, a specific, directed effect reflecting the physiologic requirement for HH/SMO signaling in taste-organ homeostasis.

Whereas HH signaling is required for TB maintenance and restoration, it is not clear why some papillae can recover but others cannot after HPI is stopped. We suggest that as long as some TB cells remain, and some HH-responding cells are in the epithelium (in category II, atypical FP/TB papillae), there is potential for TB reconstitution. However, if TB are eradicated along with putative TB stem cells, then there is no TB restoration even up to 9 mo after stopping treatment. This places TB stem cells within the TB. In fact, basal cells of the TB have been indicated as stem/progenitor cells (13, 22, 30). In addition, after

Fig. 6. Cessation of sonidegib treatment results in the recovery of FP/TB morphology, SHH ligand, and HH-responding cells within 14 d and continuing up to 9 mo. (A) Time line for studying recovery from the effects of 16 d sonidegib treatment. (B) Percentage of category I (typical FP/TB), category II (atypical FP/TB), and category III (atypical FP/no TB) FP after 16 d of sonidegib treatment followed by recovery periods of 7, 14, or 21 d and 3, 5, or 9 mo. Examples of FP/TB morphology are shown in Fig. S5A. Bars are mean ± SEM. Numbers in parentheses are number of mice analyzed. Brackets denote significant differences for treatment durations (two-way ANOVA with Tukey’s HSD post hoc tests); ***P ≤ 0.001 for vehicle vs. sonidegib treatments. Complete F and P values are given in Fig. S6A. (C) Antibody detection of SHH (red) and K8 (green, Inset) for TB cells and X-Gal staining (blue) for Gli1lacZ after 16 d of sonidegib treatment and recovery for 7, 14, or 21 d. The Gli1lacZ Inset at 7 d recovery is included to illustrate the variability in the recovery phenotype at 7 d. Dotted lines demarcate the basal lamina. (Scale bar: 50 μm.) (Magnification: C, Lower, Inset, 0.6×.)
pathway inhibition we observed the loss of HH-respondering cells in FP walls, known regions of TB progenitors (13, 32); thus, another proposed progenitor/stem cell population is eliminated. We believe there is no source of TB stem/progenitor cells and that these are regulated by HH signaling (8, 11).

HH Pathway Regulation of Proliferation and Differentiation. With complementary pharmacologic and genetic approaches to inhibit SMO-dependent HH signaling, we found consistent phenotypes across models. There were rapid effects on TB loss in both ectoderm-derived anterior tongue FP taste organs and endoderm-derived posterior tongue CV taste organs, but there were no discernible effects on the nontaste filiform papillae. Thus, HH/SMO signaling is an essential and selective regulator of gustatory epithelia and TB that turn over constantly in dynamic papilla organs. Cell cycles of the three TB cell types range from 3 to 30 days (9, 10, 31), with an average TB cell life span of 10 d (9). TB elimination began after 10 d and derived from the loss of all three taste-cell types. Further, effects were also of FP as distinct from filiform papilla morphology. HH/SMO signaling is a principal regulator not only of TB maintenance but also of TB differentiation, as previously reported (7, 8) and as observed here after sonidegib treatment and identification of Shh expression in constitutive ShhCre;R26RFP mice and after tamoxifen (TAM) administration in ShhCreER;R26RFP mice. In both GG and TG, all cell bodies expressed Shh. Anterior tongue TB include Shh+ cells and their progeny. Arrows denote Shh+ nerve fibers in the anterior tongue, FP core, and surrounding the TB reaching into the apical epithelium. Dotted lines demarcate the basal lamina. (Scale bars: 50 μm.)

The acquisition of a conical apex in the FP that accompanies the loss of TB cells is characteristic of reduced HH pathway activity, as previously reported (7, 8) and as observed here after sonidegib administration or Smo deletion. This reproducible phenotype with HH signaling suppression or inhibition is reminiscent of an acquired filiform papilla-like form observed in FP after prolonged periods of denervation (42). A key feature in all these experimental models is the loss of TB accompanied by the loss of HH-resonding epithelial cells. This indicates that HH signaling is a principal regulator not only of TB maintenance but also of FP as distinct from filiform papilla morphology.

Functional Effects of HPI on Lingual Sensation. The rapid effects of HPI on taste organs were associated with loss of neurophysiological taste responses. Here we found that the altered neural taste responses to stimuli representing all taste qualities relate directly to the loss of all taste-cell types. Further, effects were observed even though the TB were challenged with stimulation across high concentrations of varied chloride salts that might have elicited nonspecific effects. Interestingly, however, typical taste nerve responses were still obtained after 10 d of drug HPI, although only about 50% of FP were category I (typical FP/TB) at this time point. Whereas chorda tympani nerve taste responses were absent after 16 d of sonidegib gavage, it is notable that nerve responses to lingual
tactile or cold stimuli were not altered. Previously we suggested that the receptors for responses to tactile and cold stimuli must not be TB cells per se, because these have been eliminated, and we indicated that mechanoreceptor or thermal receptor endings might be adjacent to TB (7). Now we have shown nerve endings next to TB that are Shh⁺. Studies of receptive fields of GG neurons (soma of chorda tympani nerve fibers) have demonstratedFP that respond only to tactile or temperature stimuli and not taste (43). This suggests that there are GG/nerve fiber subsets, possibly with end organs that are specifically somatosensory. HH signaling presumably has different roles in maintaining epithelial sense organ specializations vs. receptor nerve endings.

**FP Innervation and HH Signaling.** Whereas TB are dependent on an intact innervation (14), the retention of nerves within FP in the face of TB elimination indicates that nerves alone are not sufficient to maintain TB in an epithelium that is devoid of SHH ligand and HH-responding cells. Similarly, nerves in the CV labeled with NF immunostaining are retained in the papilla core but apparently are not able to sustain the full complement of TB. Notably these results substantiate our data after the suppression of HH/SHH signaling (11).

Here we have shown physical association between papilla innervation and Gli1lacZ-positive HH-responding cells during genetic HPI in both FP and CV stroma. This affords an opportunity for nerve interactions with HH-responding stromal cells in the papilla, not previously proposed. SHH secreted from dorsal root ganglion nerve fibers activates signaling via Gli1HH-responding cells in touch domes (44) and maintains homeostasis in the hair follicle (35). Because we have observed SHH protein expression in neurons of the GG and TG that include the soma for nerves innervating FP and have observed Shh⁺ expression in fibers within the FP, we propose interactions between SHH⁺ nerve fibers and Gli1lacZ-positive stromal cells in maintaining the FP structure. Furthermore, Shh⁺ fibers contact basal lamina components of the FP and remaining epithelial cells; these could participate in signaling interactions within a taste-organ niche (8).

SHH likely has varied roles in taste-organ development and maintenance. SHH is a morphogen in developing FP placodes (45) but might also serve as an axon-guidance molecule or chemotactic signal via noncanonical signaling (46, 47) to direct growing nerves specifically into the developing papillae (48). In the adult taste organ, HH signaling is required for epithelial cell proliferation and differentiation in FP and TB homeostasis. We suggest that SHH, retrogradely secreted from nerve fibers in the FP core, signals to HH-responding cells and tissue elements in the connective tissue.

**CV Papillae and HPI.** In our current experiments, we did not record from the glossopharyngeal nerve that innervates taste buds in the CV. Because we noted TB loss in the CV after 16 d of HPI drug treatment and the recovery of about 80% of TB after 14 d, we predict that any loss of posterior tongue sensation would recover also. In mice treated with the HPI drug vismodegib for 15 wk (49), statistically significant but biologically small effects were seen on TB cell numbers in the CV, whereas we found a substantial loss of TB (up to about half of control values) after 4 wk of sonidegib gavage. The only other reports of altered HH signaling on TB in the CV are from HH/GLI suppression in mice, where TB were substantially reduced/lost (11). Here we observed effects in TB of both the FP and CV, that is, in two major lingual taste regions. Although there were no reported behavioral effects in two bottle preference/avoidance tests for sucrose or denatonium benzoate in vismodegib-treated mice (49), we predict behavioral effects after HH/SMO signaling inhibition.

**Implications for Adverse Taste-Disturbance Effects in Patients Treated with HPI Drugs.** This work is at the confluence of HH/SHH signaling as a principal regulator of taste organs and taste sensation, deregulated HH signaling in basal cell carcinoma (BCC), and BCC treatment with drugs that inhibit HH signaling with associated severe taste disruption (4, 5, 26, 28, 50) that can alter patient quality of life (27). The demonstrated duration-dependent effects on TB in the FP and CV and the profound loss of chorda tympani nerve responses to all taste stimuli are the biological bases for severe taste disturbances in patients. We found substantial effects in anterior and posterior tongue TB in both the FP and CV, which have different innervation and chemical response profiles (51). Effects in patients taking HPI drugs therefore are likely to be wide ranging across taste chemical stimuli and oral regions, and the reported aguesia in >20% of patients (5) is not unexpected. Further, based on our neurophysiological data, it is not likely that even very strong chemicals would elicit responses in patients during treatment with HPI drugs. However, our modality-specific results support consideration of overall lingual sensation, and patient diets focused on the texture and temperature characteristics of nutrients could temper the loss of taste sensation in overall flavor of meals. Notably, a majority of taste organs and TB regain cell integrity, and taste nerve responses rapidly return to normal patterns in the post-HPI drug recovery period in mice. Therefore, the reported recovery from taste disturbances after treatment is discontinued in patients (5) is related directly to the recovery of taste organs and sensory responses. Importantly, management of

Kumari et al.
reported decreased food and fluid intake in patients should address flavor perception. We are investigating responses to gustatory and olfactory stimuli in patients treated with HPI drugs to learn about possible contrasting effects in taste and smell.

Materials and Methods

Animals. Animal use and care procedures were performed according to the guidelines of the National Institutes of Health and approved protocols of the University of Michigan Institutional Animal Care and Use Committee. C57BL/6 mice were treated for 3–36 d by daily oral gavage with sonidegib [NVP-LDE225E dichlorophosphate salt; Chemietek catalog no. CT-LDE225] dissolved in vehicle, PEG 400/5% dextrose in water (75:25 vol/vol), at a dose of 20 mg/kg, or vehicle alone. Sacrifice or determination of shh-GFP expression was done after Cunningham et al. (7). For recovery experiments, mice were treated with sonidegib or vehicle for 16 d; then the drug was discontinued, and animals were maintained for 7, 14, or 21 d or for 3, 5, or 9 mo in standard housing. Mouse genotypes and sources are given in SI Materials and Methods.

Tissue Analyses. Tissues were collected and prepared for tissue analysis, or recordings were made from the chorda tympani nerve and then tongues were digested. Tissue processing, immunohistochemistry and neuropathological protocols, and data and quantification analyses are described in SI Materials and Methods.

Statistics. Animal numbers and statistical tests are included in the graphs and figure legends. See SI Materials and Methods.

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