Flavones modulate respiratory epithelial innate immunity: Anti-inflammatory effects and activation of the T2R14 receptor

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This article contains supplemental Figs. S1–S6, Table S1, Raw Data from Main Text Figs. 2, 3, 6, and 7.

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 Florian rhinosinusitis is a syndrome of chronic inflammation and/or infection of the upper respiratory tract, which leads to substantial decreases in patient quality of life, creates >$8 billion in direct healthcare costs in the United States alone, and can seed lower respiratory infections and exacerbate lung diseases (1–3). CRS is also an important public health concern, as it accounts for ~20% of antibiotic prescriptions in adults in the United States (1, 4–8), making it a significant driver for the emergence of antibiotic-resistant organisms (9–15). An attractive therapeutic strategy to avoid the selective pressures for antibiotic resistance is to stimulate endogenous innate host defenses. However, this requires a better understanding of the receptors involved in activating upper airway innate immunity.

It is believed that multiple etiologies contribute to the pathogenesis of CRS, but a common phenotype of the disease is defective mucociliary clearance, the major physical defense of the conducting airways (1, 3). A thin layer of mucus secreted by airway goblet cells and submucosal glands traps inhaled pathogens and particulates. Coordinated beating of motile cilia drives transport of this mucus to the oropharynx, where it is cleared by expectoration or swallowing (16). In CRS, chronic infection and persistent inflammation is associated with stasis of sinonasal secretions, possibly through alterations of basal (17, 18) or stimulated (19) ciliary beat frequency (CBF), mucus viscosity (17, 20), or ion/liquid transport (21). Enhancing mucociliary clearance may have potential therapeutic benefit for CRS patients (1, 3).

2 The abbreviations used are: CRS, chronic rhinosinusitis; ALI, air-liquid interface; ANOVA, analysis of variance; ASL, airway surface liquid; CBF, ciliary beat frequency; CFP, cyan fluorescent protein; CKAR, C-kinase activity reporter; d-NAM, d-N6-nitroarginine methyl ester; eNOS, endothelial nitric-oxide synthase; FESS, functional endoscopic sinus surgery; GCSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; iNOS, inducible nitric-oxide synthase; L-NAM, l-N6-nitroarginine methyl ester; PMA, phorbol 12-myristate 13-acetate; TLR2, taste family type 2 receptors, or T2Rs; T2R38, taste family type 2 receptor 38; TLR4, toll-like receptor 4; TNF, tumor necrosis factor; TLR5, toll-like receptor 5; WGA, wheat germ agglutinin; WT, wild type.

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We previously showed that a bitter “taste” G-protein-coupled receptor, T2R38, is expressed in the motile cilia of cells of the upper airway (nose and sinuses) and modulates mucociliary clearance through calcium (Ca^{2+})-dependent production of nitric oxide (NO) (2, 22–27). Our data suggest that T2R38 is activated by acyl-homoserine lactone (AHL) quorum-sensing molecules secreted by Gram-negative bacteria (27), including the common airway pathogen Pseudomonas aeruginosa. When activated, T2R38-activated NO production drives an increase in CBF through protein kinase G (PKG), which phosphorylates ciliary proteins (28). This NO also directly diffuses into the airway surface liquid with antibacterial effects (27). Because NO damages the cell walls and DNA of bacteria (29, 30), NO production by the sinonasal epithelium is thought to be important for preventing infection. Supporting this, nitric-oxide synthase (NOS) polymorphisms have been associated with severe CRS (31). We found that patients homozygous for a polymorphism in the TAS2R38 gene resulting in non-functional T2R38 protein (the AVI (alanine, valine, isoleucine at positions 49, 262, and 296, respectively) polymorphism (32)) are more susceptible to Gram-negative bacterial infection (27), have a higher incidence of biofilm-forming bacteria (33), are at higher risk for CRS requiring functional endoscopic sinus surgery (FESS) (34, 35), and may have worse outcomes after FESS for CRS without nasal polyyps (36) compared with patients homozygous for the functional (PAV: proline, alanine, and valine at positions 49, 262, and 296, respectively) allele of TAS2R38. There are 25 different T2R bitter receptors in humans (22, 24, 37, 38), and other researchers have confirmed that polymorphisms in at least two TAS2R genes, TAS2R38 and TAS2R13, correlate with CRS by genome-wide association (39).

The full picture of T2R38’s role in sinonasal immunity in different patient populations is still being elucidated. A subsequent study found no correlation of TAS2R38 genotype with CRS in Italian patients (40), but this population had different clinical characteristics (e.g. more recalcitrant disease and stronger Th2 inflammatory phenotype) than our own studies. A more recent study supported a correlation between TAS2R38 genotype and CRS severity in a Polish population (41), and an Australian study concluded that AVI/AVI TAS2R38 is predictive of the presence of culturable bacteria in CRS patient sinuses (42). Moreover, a recent study reported that T2R10 and T2R14, but not T2R38, respond to some AHLs when expressed in HEK293 cells (43). The reason for this discrepancy is not yet clear, although other studies have suggested that T2R38 is indeed important for immune cell detection of AHLs (44, 45). Understanding the full range of T2Rs endogenously expressed by primary differentiated cell types as well as their oligomerization properties will help clarify the best therapeutic compounds to target these receptors to stimulate innate immune responses.

Plants produce thousands of polyphenolic flavonoids (46–49), which are of great biomedical interest, because they have effects on both eukaryotic and prokaryotic cells (47–58). Flavones are a sub-group of flavonoids that have been demonstrated to have antibacterial, antioxidant, and anti-inflammatory effects in various in vitro models (48, 51, 53, 55, 57, 59, 60). Moreover, the flavones chrysin and apigenin activate T2R14 and T2R39 (61, 62). Because of their potential antibacterial properties, and because multiple TAS2R polymorphisms have been linked to CRS (39), we sought to investigate the effects of flavones on airway epithelial cells. We previously examined a separate flavonoid class, anthocyanidins, and found that they did not activate sinonasal cilia T2Rs (58). However, anthocyanidins have a \( \geq 1 \)-log higher effective concentration (EC) for T2R activation than flavones (e.g. 250 \( \mu \)M EC for the anthocyanin cyanidin versus 8 \( \mu \)M EC for the flavone apigenin for T2R14) (61, 62). The goal of this study was to begin to investigate the potential role of flavones as a treatment for upper respiratory infections, with a special focus on their anti-inflammatory properties as well as potential reactivity with upper airway T2Rs.

**Results**

**Flavones have anti-inflammatory effects in airway epithelial cells**

Several representative flavones (backbone structure shown in Fig. 1a) were tested, including apigenin (Fig. 1b) from bee propolis (50) and wogonin (Fig. 1b) from Scutellaria baicalensis, one of the 50 fundamental herbs of traditional Chinese medicine (63). Chrysin (Fig. 1b), from Passiflora flowers (48, 51), and tangeritin (Fig. 1b), found in citrus peels (48, 51), were also used. We also used a structurally related flavanone, the backbone of which is a stereoisomer of the flavone backbone (Fig. 1c). Substituted methoxyflavonones, including 4'-fluoro-6-methoxyflavone, have been suggested to inhibit both T2R39 and T2R14 (64, 65).
We sought to determine whether flavones exhibited anti-inflammatory effects in airway epithelial cells, as reported previously in other cell types (51). The protein kinase C (PKC) pathway is utilized by many inflammatory mediators, and activation of PKC with phorbol 12-myristate 13-acetate (PMA) is frequently used to induce inflammatory effects \textit{in vitro}. PKC inhibitors are utilized as anti-inflammatory compounds in a number of diseases of chronic inflammation (66–68). Apigenin and other flavones may have PKC inhibitory activity (69, 70), suggesting they may have an anti-inflammatory role in airway cells.

We tested another important marker of inflammation, inducible nitric-oxide synthase (iNOS) (74–77). Whereas acute NO production can be antibacterial, chronic excessive NO production through transcriptional up-regulation of iNOS may exacerbate and prolong inflammation. PMA induced an up-regulation of iNOS in A549 alveolar-derived airway cells (Fig. 2b) that was likewise reduced with individual flavone compounds (10 μM each; Fig. 2b). As a control for the specificity of iNOS as an inflammatory marker, we examined changes in
endothelial NOS (eNOS), typically not up-regulated during inflammation. Immunofluorescence for eNOS exhibited a perinuclear Golgi-like localization, as reported previously (78, 79).

We also directly quantified cytokines released from air-liquid interface (ALI) cultures of primary human sinonasal cells. ALIs from primary cells differentiate into ciliated and goblet cells mimicking the in vivo epithelium (24, 27, 80, 81). Primary sinonasal ALIs were stimulated as above with PMA on the apical side in the presence or absence of apigenin or chrysin, e, IL-8 release (pg/ml) in response to pycocyanin in 16HBE, Calu-3, and primary sinonasal ALI cultures (n = 5 ALIs for each condition). Significance was determined by one-way ANOVA, Tukey-Kramer, or Bonferroni post-test; *, p < 0.05; **, p < 0.01 for all graphs, and for D, #, p < 0.01 versus pycocyanin alone. Data values from bar graphs are reported in supplemental Material.

Figure 3. Flavones reduce cytokine release from 16HBE14o, Calu-3, and primary sinonasal ALI cultures. a, release of IL-8, GCSF, and GMCSF from primary sinonasal ALIs assayed by ELISA. b, traces of CKAR FRET/CFP emission ratio from transfected 16HBE cells (n = 6–8 experiments for each condition) under control conditions or pre-treated with apigenin for 1 h prior to experiment. Cells were stimulated with PKC-activating PMA (10 μM) followed by the global PKC inhibitor Gö6983 (10 μM). c and d, similar experiments performed with 16HBE ALIs (c) and primary sinonasal ALIs (d) stimulated with TNFα (10 ng/ml; 24 h) in the presence or absence of apigenin or chrysin. e, IL-8 release (pg/ml) in response to pycocyanin in 16HBE, Calu-3, and primary sinonasal ALI cultures (n = 5 ALIs for each condition). Significance was determined by one-way ANOVA, Tukey-Kramer, or Bonferroni post-test; *, p < 0.05; **, p < 0.01 for all graphs, and for D, #, p < 0.01 versus pycocyanin alone. Data values from bar graphs are reported in supplemental Material.

We likewise examined IL-8 secretion in both 16HBE ALIs (Fig. 3c) and primary sinonasal ALIs (Fig. 3d) in response to tumor necrosis factor α (TNFα) stimulation. The flavonol quercetin was reported to reduce TNFα-induced IL-8 secretion in 16HBE cells through a phosphatidylinositol 3-kinase (PI3K)-dependent pathway (84). We found that TNFα induced IL-8 secretion ~10-fold in both 16HBE (Fig. 3c) and primary sino-
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Figure 4. Immunofluorescence co-localization of endogenous T2R38 and T2R14 in sinonasal cilia. a, upper panels show a representative confocal immunofluorescence image of a primary sinonasal ALI culture and co-localization of the motile cilia marker β-tubulin IV (β-TubIV, green) with T2R38 (cyan) and T2R14 (magenta). Detection of T2R39 was not observed (lower panels). Orthogonal slice of T2R14 and β-tubulin IV is shown at top right. b, T2R14 immunofluorescence was observed in non-permeabilized cells using an antibody recognizing an extracellular epitope, supporting surface localization; β-tubulin IV staining was conducted after subsequent second fixation and permeabilization. Images are representative of results observed with ALI cultures from at least three individual patients.

Flavones activate T2R14 expressed in sinonasal epithelial cell cilia

The above data suggest that flavones modify sinonasal epithelial inflammation-induced up-regulation of Muc5AC, iNOS, and cytokines. As mentioned above, apigenin and chrysin may also activate T2R14 and T2R39 (61), whereas tangeritin may activate T2R38 and T2R46 but not T2R39 (99). We hypothesized that flavones may activate one or more of these T2Rs located in the upper respiratory epithelium (22, 25). The molecular structures of flavones were examined using BitterX, an open-access platform for predicting whether a molecule is bitter as well as predicting which T2R receptors are likely to respond (100). BitterX identified T2R14 and T2R39 as having the highest probabilities of being activated by all flavone molecules examined here (supplemental Table 1).

We examined endogenous T2R14 and T2R39 expression in primary sinonasal ALIs with anti-C-terminal antibodies validated against cross-reactivity by heterologous expression of T2R14 and T2R39 in AS49 cells (supplemental Fig. 1). Using these antibodies, we detected endogenous expression of T2R14 but not T2R39 in primary sinonasal ALIs (Fig. 4a). T2R14 co-localized with the motile cilia marker βTubulin IV and T2R38 (Fig. 4a), previously demonstrated to be localized to sinonasal cilia (27). Surface localization of T2R14 was confirmed by staining un-permeabilized cells with an antibody raised against an epitope (amino acids 213–262) containing an extracellular loop between transmembrane helix 6 and 7 (Fig. 4b). The co-localization observed between T2R38 and T2R14 was highly distinct from the pattern observed with T2R38 or T2R14 and T2R4. T2R4 localized only to the distal tips of cilia (supplemental Fig. 2, a and b). This distal localization of T2R4 was previously observed in bronchial cilia (101), and it may be related to previous HEK293T heterologous expression data suggesting that recombinant T2R4 does not oligomerize with T2R14 or T2R38, whereas T2R14 and T2R38 can oligomerize with each other (102, 103). T2R14 and T2R38 co-localization was similar to that observed with T2R16 (supplemental Fig. 2, c and d).

We tested the ability of the flavone compounds to activate recombinant T2Rs4, -5, -14, -38, -39, and -16 by expressing these T2Rs in HEK293T cells with a Ca2+-activating Gα chimera, Gα16-gust44, a Gαq fused to the last 44 amino acids of gustducin that couples to T2Rs, a commonly used method to test T2R activation (104). HEK293T cells expressing T2R14 + Gα16-gust44, but not Gα16-gust44 alone, exhibited robust intracellular Ca2+ ([Ca2+]i) responses to all flavones tested (supplemental Fig. 3). We found no responses to apigenin in cells transfected with T2R4, -5, 16, or -38, despite robust responses to known cognate ligands (supplemental Fig. 4). T2R39 did respond to apigenin (supplemental Fig. 4), as predicted.

Because endogenous T2R14 and T2R38 appeared to strongly co-localize, and because the primary antibodies used here were both raised against the C termini (Fig. 5a), we examined whether endogenously expressed T2R38 and T2R14 interacted closely enough to observe Förster (also known as fluorescence) resonance energy transfer (FRET; schematic shown in Fig. 5b) in primary sinonasal ALIs. FRET is a highly quantitative measure of co-localization. Although T2Rs have been previously proposed to oligomerize, presumably as dimers (102, 103), these studies have been done in heterologous overexpression HEK293 cell systems, and thus results must be interpreted with...
with AF-conjugated Fab fragments. We noted a significant T2R14 FRET signal in the AF647 emission channel when T2R38 AF555 was excited using a 559-nm laser (Fig. 5c). Similar T2R38 FRET signals were observed when T2R14 was labeled with AF555 and T2R38 was labeled with AF647. When the acceptor fluorophore was bleached using a 647 laser, donor fluorescence increased (Fig. 5, d–f). Quantification of the relative increase in donor fluorescence after acceptor bleaching allows calculation of the FRET efficiency (E). E was measured to be 15 ± 3% when T2R38 was the donor and T2R14 was the acceptor, 17 ± 1% when T2R14 was the donor and T2R38 was the acceptor, and 16 ± 2% when the two conditions were pooled. R0 is the distance at which the E is 50% (51 Å for AF555 and AF647); FRET efficiency decreases to the 6th power as distance (R) increases: R = R0(1 − E/E)1/6. Thus, with 0.16 E, R = 51 Å(1 − 0.16)/0.16)1/6 = 67 Å. Although we cannot conclude that the two proteins form oligomers per se from our data due to the sizes of the antibodies involved in our measurements, similar antibody-based FRET measurements have been used to suggest a “close association” of endogenously expressed proteins (105). These quantitative FRET measurements demonstrate a close co-localization of T2Rs to a higher resolution (~7 nm) than yet achievable by most current super-resolution light microscopy techniques (106).

With the knowledge that T2R14 is endogenously expressed in primary sinonasal ciliated epithelial cells and the knowledge that this receptor is activated by flavones, we sought to determine whether flavones activate T2R14-dependent Ca2+-driven nitric oxide (NO) responses in these cells, as demonstrated previously with T2R38 (27). Apigenin and chrysin activated low-level Ca2+ responses (Fig. 6, a–c) identical to what was previously observed with stimulation of sinonasal cilia-localized T2R38 (27). Although these [Ca2+]i changes are small, it is important to note that these are measurements of global [Ca2+]i; localized responses at or near the apical membrane and/or within cilia could be much higher (107). Importantly, the ECs observed here fit with ECs previously determined for these compounds for heterologously expressed T2R14 (8 μM for apigenin and 63 μM for chrysin (61, 62)). These responses were also blocked in the presence of 4′-fluoro-6-methoxyflavanone (Fig. 6, d and e), a previously identified T2R14 inhibitor (64, 65). We found that 4′-fluoro-6-methoxyflavanone inhibited apigenin-induced T2R14 [Ca2+]i responses at 50 μM but had activating effects at higher concentrations, suggesting that 4′-fluoro-6-methoxyflavanone may act as a partial agonist rather than a competitive antagonist (supplemental Fig. 5). Nonetheless, this inhibition in primary cells supports that these responses are mediated by T2R14. No difference in [Ca2+]i responses was observed when cultures from T2R38 PAV/PAV (homozygous taster) or AVI/AVI (homozygous non-taster) individuals were compared, suggesting that, despite close co-localization and possible heterodimerization, the non-functional AVI T2R38 does not adversely affect T2R14 Ca2+ signaling in cilia (supplemental Fig. 6, a, b, and e). Supporting this, we found no inhibition of apigenin-induced Ca2+ responses with the T2R38 inhibitor probenecid (1 mM) (108) and no inhibition of phenylthiocarbamide-induced Ca2+ responses in PAV/PAV cultures with 4′-fluoro-6-methoxyflavanone (supplemental Fig. 6, c–e).

Figure 5. Demonstration of a close co-localization of endogenously expressed T2R38 and T2R14 in sinonasal cilia by FRET. a, antibody labeling FRET strategy. b, diagram of basic principle of FRET. c, x-y plane images (top) and x-z plane image (orthogonal view; bottom) of T2R14-AF647 FRET upon T2R38-AF555 excitation. d and e, images showing T2R14 and T2R38 immunofluorescence signals before (d) and after (e) acceptor photobleaching (via 647 laser and tornado bleaching function in Olympus Fluoview; photobleached area shown in yellow circle). f, left panel shows ratio image showing increase in donor fluorescence after acceptor bleaching. Right panel shows relative change in donor emission under two conditions (T2R14 acceptor + T2R38 donor, as shown, and T2R38 acceptor + T2R14 donor) as well as pooled data. n.s., not significantly different.
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Activation of T2R14 in sinonasal cilia results in NO production and increases ciliary beat frequency

As observed with T2R38, flavone/T2R14-induced Ca^{2+} responses were associated with increases in fluorescence of DAF-FM, which reacts with intracellular reactive nitrogen species (Fig. 7a). DAF-FM fluorescence increases reflected NO production, as they were blocked by the nitric-oxide synthase (NOS) inhibitor L-NAME but not by its inactive analogue D-NAME (Fig. 7b). These responses were blocked by the phospholipase C inhibitor U73122 but not by its inactive analogue U73343 (Fig. 7c). Like flavone-induced Ca^{2+} signals, flavone induced NO production was also inhibited by 4′-fluoro-6-methoxyflavanone (Fig. 7d). These results are summarized in Fig. 7e.

Abolishment of Ca^{2+} signaling by pre-loading cells with a Ca^{2+} chelator (10 μM BAPTA-AM for 30 min) followed by stimulation with apigenin or chrysin under extracellular Ca^{2+}-free conditions (Ca^{2+}-free solution containing 2 mM EGTA) likewise abolished NO production (Fig. 7g). We also tested a structurally unrelated T2R14 agonist, niflumic acid (NFA; 50 μM; EC_{50} ~ 5 μM for T2R14) (109), which also activated NO production (Fig. 7g).

The constant and coordinated beating of airway motile cilia drives mucociliary clearance (1, 3, 110, 111). Because NO is known to increase ciliary beating through activation of guanylyl cyclase and production of cGMP (27, 28), we measured CBF during stimulation with apigenin or chrysin. Both compounds increased CBF (Fig. 8a; n = 6–8 ALIs from at least three patients per condition). The increases in CBF were dependent on NO production, as they were blocked by L-NAME but not by D-NAME (Fig. 8b).

Discussion

This study first demonstrates that flavones have anti-inflammatory effects on Muc5AC and iNOS up-regulation as well cytokine secretion in airway epithelial cells. This supports prior studies that apigenin, chrysin, and wogonin inhibit Muc5AC up-regulation in NCI-H292 bronchial cancer cells in response to epidermal growth factor (91, 112, 113) or TNFα (114, 115). Our data suggest this occurs partly through direct inhibition of kinase C rather than upstream or downstream mechanisms, as they were directly able to block PKC activity in response to PMA. We also demonstrate relevance of prior findings from cell lines to primary well differentiated airway epithelial cells. We postulate that flavones may be useful potential anti-inflammatory mediators in airway diseases, as has also been suggested for the related, but structurally different, flavonol quercetin (84).

Second, we demonstrate that flavones activate T2R14 in primary sinonasal epithelial cell cilia. T2R14 is also expressed in bronchial cilia (101) and airway smooth muscle cells (116). This is the first demonstration of T2R14 in sinonasal cilia, and the finding that T2R14 closely co-localizes (<7 nm) with T2R38 is important and novel. These studies were facilitated by the commercial availability of valid and specific antibodies directed at moieties on the same side of the plasma membrane. Although we cannot directly determine whether these T2Rs oligomerize by the methods used here due to the size of the labeled antibodies, this study is one of the first to demonstrate such a close interaction with endogenously expressed T2Rs. Previous studies of T2R interactions have been carried out in heterologous expression HEK293 models (102, 103). Results from such models, where overexpression may induce interactions that do not normally occur at physiological protein levels, must be interpreted with caution and must be verified in more differentiated in vitro models and/or tissues. Future studies in airway epithelial cells may help to clarify the oligomerization properties of the subset of T2Rs endogenously expressed in motile cilia, shedding important light into the cell biology of these receptors. Although T2R38 is required for AHL detection in sinonasal epithelial cells (27) and immune cells (44, 45), another study has suggested T2R10 responds to AHLS in a heterologous expression system (43). Oligomerization has substantial functional consequences for many G-protein-coupled receptors.
A better understanding of the oligomerization of endogenous T2Rs, both with other T2Rs as well as with other receptor classes (116), may help explain some such discrepancies that exist in the literature.

Activation of T2R14 results in similar Ca\(^{2+}\)/H\(_{11001}\)-dependent NO production and ciliary beat increases to those observed during T2R38 stimulation (27). These CBF increases were smaller than those observed with ATP but were sustained and were similar to elevations previously observed with T2R38 stimulation (27) that may have clinical relevance (27, 34–36, 39, 41, 42, 119).

Quercetin was previously shown to increase CBF in primary human sinonasal cells (120), and our data suggest this activation may at least partly occur through activation of T2R14. Interestingly, T2R14 is activated by several other compounds that have been used clinically, including NFA, the antihistamine diphenhydramine, the non-steroidal anti-inflammatory drug flufenamic acid, and quercetin (62). NFA is commonly used experimentally at high micromolar-to-low millimolar concentrations as a Ca\(^{2+}\)/H\(_{11001}\)-activated Cl\(^{-}\)channel blocker (121–126). NFA was shown to activate Ca\(^{2+}\)/H\(_{11001}\) release from intracellular stores in rat pulmonary artery smooth muscle cells (127); as bronchial smooth muscles express T2R14 (116, 128), arterial

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Immune responses in patients to treat airway infections without the use of antibiotics (2, 22, 25–27, 129). However, there is a high frequency of non-functional (non-taster) T2R38 polymorphisms. In Caucasians, ~25% of the population is homozygous for the AVI non-functional T2R38 (27, 32, 130–132). These patients would be expected to be completely non-responsive to T2R38-directed therapeutics, and we have previously confirmed that ALIs grown from AVI/AVI patients do not respond to T2R38 agonists (27). The observation that T2R14 activates a highly similar set of responses from both T2R38 PAV/PAV and AVI/AVI sinonasal cells suggests that T2R14 may be a viable therapeutic target to activate innate immune pathways in T2R38 AVI/AVI patients. However, polymorphisms in TASS2R14 also exist (133), although their effects are not as well studied. Future work is needed to understand how known TASS2R14 polymorphisms affect T2R14 function. Additionally, identifying the full gamut of sinonasal cilia taste receptors will allow elucidation of agonist mixtures that activate multiple sinonasal T2Rs to achieve maximum possible benefit in the largest patient population possible. T2R agonists such as flavones that have additional beneficial non-T2R-dependent effects (e.g. PKC inhibition) may increase efficacy of this therapeutic strategy.

Experimental procedures

Reagents and solutions

Unless indicated, all reagents and solutions used were as described previously (23, 24, 27, 80, 81). Fura-2-AM, DAF-FM-DA, and BAPTA-AM were from Invitrogen. Flavones, phorbol 12-myristate 13-acetate (PMA), 1- and d-N6-nitroarginine methyl ester (L-NAME and d-NAME), ionomycin, U73122, and U73343 were from Cayman (Ann Arbor, MI), and 4'-fluoro-6-methoxyflavone (2-(4-fluorophenyl)-6-methoxychrooman-4-one) was purchased from VitaScreen, LLC (Urbana-Champaign, IL). Anti-T2R38 (ab130503; rabbit polyclonal), anti-β-tubulin IV (ab11315; mouse monoclonal), anti-T2R16 (ab75106 rabbit polyclonal), and anti-Muc5AC (ab3649; mouse monoclonal) primary antibodies were from Abcam (Cambridge, MA). Anti-T2R14 (PA5-39710; rabbit polyclonal) and T2R39 (PA5-39711; rabbit polyclonal) primary antibodies were from Thermo Fisher Scientific (Waltham, MA). Anti-α-tubulin (12G10; mouse monoclonal) antibody was from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City). Anti-iNOS (sc-654; rabbit polyclonal) and T2R4 (T13, sc169494; goat polyclonal) were from Santa Cruz Biotechnology. Anti-eNOS antibody (NB-300-605; rabbit polyclonal) was from Novus (Littleton, CO). Anti-T2R14, amino acids 229–278 (LS-C120957; rabbit polyclonal), was from (LSBio, Seattle WA). ELISAs for GCSF and GMCSF were from PeproTech (Rocky Hill, NJ); ELISA for IL-8 was from Thermo Fisher Scientific. ELISAs were carried out according to the manufacturers’ instructions. Unless indicated below, all other reagents were from Sigma. Stock solutions of flavones were made at 100 or 120 mM in DMSO; working solutions contained ≤0.1% DMSO and were made immediately before use. CKAR expression vector was obtained from Alexandre Newton (University of California San Diego) via Addgene (Cambridge, MA; plasmid no. 14860).

Culture of human cell lines

A549 alveolar carcinoma-derived cells were obtained from ATCC (Manassas, VA) and cultured in Ham’s F-12K (Kaighn’s) medium (Gibco/Thermo Fisher Scientific) plus 10% fetal bovine serum (FBS) and 1× penicillin/streptomycin. A549 cells were used at passage 20–25. 16HBE14o−, an SV40-immortalized ciliated bronchial epithelial cell line (134–136), was obtained from Dr. D. Gruenert (Department of Otolaryngology, University of California at San Francisco) and cultured in minimal essential media (MEM) with Earle’s salts (MEM: Gibco/Thermo Fisher Scientific) plus 10% FBS and 1× penicillin/streptomycin. Cells were used within 20 passages of receipt. Calu-3 bronchial adenocarcinoma cells were obtained from ATCC and grown in MEM plus 10% FBS and 1× penicillin/streptomycin.

For ALI cultures, cells were seeded onto collagen-coated ThinCert cell culture inserts (0.4-μm pore size, transparent PET membrane, Greiner Bio-One, Germany) in 12-well plates (1.1-cm² surface area). After ~3–5 days of culture and confirmation of confluence and establishment of transepithelial electrical barrier (~300 ohms·cm²), cultures were exposed to air on the apical side by removal of apical media and washing three times with PBS. Cultures were fed from the basolateral side for at least 2 weeks before use to allow for full differentiation. For cytokine release studies, ALI cultures were stimulated on the apical side only (to simulate mucosal exposure) with PMA, TNFα, or pyocyanin dissolved in sterile PBS. Cytokines were quantified from the basolateral medium.

HEK293T cells (ATCC) were cultured in high-glucose DMEM (Gibco). Transfection of A549 and HEK293T cells for heterologous expression experiments was carried out with Lipofectamine 2000 according to the manufacturer’s instructions. Sequences of human TASS2R4, TASS2R5, TASS2R14, TASS2R16, TASS2R38 (PAV functional isoform), and TASS2R39 were cloned into pcDNA3.1 vectors containing the first 45 amino acids of the rat type 3 somatostatin receptor at the N terminus to enhance membrane expression, as described previously (103, 137). Each T2R was co-transfected with pcDNA3.1 containing Goα16-gust44, a chimeric Go protein containing the last 44 amino acids of gustducin, as described previously (137). Cells were used 24–48 h after transfection. Transfection of 16HBE cells with the protein kinase C (PKC) FRET-reporter construct CKAR (82, 83) was performed with Lipofectamine 3000; cells were used 24 h after transfection.


**Generation of primary sinonasal ALI cultures**

All experimental protocols were carried out in accordance with the University of Pennsylvania School of Medicine guidelines regarding use of residual clinical material in research. Patients undergoing sinonasal surgery were recruited from the Department of Otorhinolaryngology at the University of Pennsylvania with full IRB approval (#800614), and written informed consent was obtained for all participating patients in accordance with the United States Department of Health and Human Services code of federal regulation Title 45 CFR 46.116. Exclusion criteria included a history of systemic diseases (e.g. Wegener’s granulomatosis, sarcoidosis, and cystic fibrosis), immunodeficiencies, or use of antibiotics, oral corticosteroids, or anti-biologics (e.g. Xolair) within 1 month of surgery. Human sinonasal epithelial cells were enzymatically dissociated and grown to confluence in proliferation medium (DMEM/Ham’s F-12 plus BEBM; Clonetics, Cambrex, East, NJ) for 7 days as described previously (27, 138). Confluent cells were dissociated and seeded on porous polyester membranes coated with BSA, type I bovine collagen, and fibronectin in cell culture inserts in LHC basal medium (Invitrogen). Culture medium was removed from the upper compartment, and basolateral media were changed to differentiation medium (1:1 DMEM/BEBM) containing human epidermal growth factor (0.5 ng/ml), epinephrine (5 ng/ml), BPE (0.13 mg/ml), hydrocortisone (0.5 ng/ml), insulin (5 ng/ml), triiodothyronine (6.5 ng/ml), and transferrin (0.5 ng/ml), supplemented with 100 units/ml penicillin, 100 g/ml streptomycin, 0.1% retinoic acid, and NuSerum (BD Biosciences) as described previously (27, 138).

**Live cell imaging of intracellular Ca²⁺, reactive nitrogen species production, and PKC activation**

[Ca²⁺], and reactive nitrogen species were imaged in primary sinonasal ALIs using the Fura-2 and DAF-FM, respectively, as described previously (23, 24, 27, 139). ALI cultures were loaded with Fura-2 AM (5 μM applied apically) for 90 min followed by washing and 20 min of incubation in the dark. Cultures were similarly loaded with 10 μM DAF-FM diacetate for 90 min in the presence of 5 μM cPTIO, followed by washing to remove unloaded DAF-FM and cPTIO and incubation for 15 min prior to imaging. Imaging was performed using an Olympus IX-83 microscope (10 × 0.4 NA PlanApo objective, Olympus Life Sciences, Tokyo, Japan) equipped with a fluorescence xenon lamp (Sutter Lambda LS, Sutter Instruments, Novato, CA), excitation and emission filter wheels (Sutter Instruments), and a 16-bit Hamamatsu Orca Flash 4.0 sCMOS camera. Images were acquired using MetaFluor (Molecular Devices, Sunnyvale, CA). Dual excitation of fura-2 was carried out with 340/11-nm and 380/11-nm bandpass excitation filters, 510 long pass dichroic mirror, and 560/80 nm emission filter (set 79002-ET, Chroma Technologies, Rockingham, VT). Excitation of DAF-FM was carried out with 470/40-nm excitation filter, 495 long pass dichroic, and 525/40-nm emission filter (Chroma 49002-ET). For fura-2 [Ca²⁺], measurement in submerged HEK293T cells, cells grown on coverslips were loaded with 2 μM fura-2 AM for 30 min in Hanks’ balanced salt solution plus 10 mM HEPES, followed by washing and imaging with a ×30 (1.05 NA) UPLSAPO silicone immersion objective (Olympus) using MetaFluor.

Calibration of fura-2 fluorescence from primary nasal cells to [Ca²⁺], was carried out as described previously (123, 124, 126) using the method of Grynkiewicz et al. (140) and R⁰₟/min R⁰ₙ max, and β values empirically determined for this specific imaging system using live cells with 0-Ca²⁺ (1 mM EGTA) and high Ca²⁺ (10 μM) solutions containing 10 μg/ml ionomycin and thapsigargin. DAF-FM measurements utilized raw fluorescence values to compare experiments performed under identical conditions with identical microscope settings.

Imaging of CKAR (82, 83) was carried out on submerged cells grown on chamber slides as described previously (80) using a dual CFP and YFP filter set (89002-ET; Chroma Technologies, Rockingham, VT) with dual 430/24- and 500/20-nm excitation filters, dual 470/24- and 535/30-nm emission filters, and multipass beam splitter. Images were acquired at 10-s intervals, and background-subtracted ratios were calculated using MetaFluor.

**Measurement of CBF**

Whole-field CBF was measured using the Sisson-Ammons Video Analysis system (141) as described previously (27, 81, 139) at 28–30 °C. Cultures were imaged using at 100 frames/s using a Leica DM-IL microscope (×20/0.8 NA objective) with Hoffman modulation contrast (Leica IMC). Experiments utilized Dulbecco’s PBS (1.8 mM Ca²⁺) on the apical side and HEPES-buffered Hanks’ balanced salt solution supplemented with 1× MEM vitamins and amino acids on the basolateral side.

**Immunofluorescence microscopy**

Immunofluorescence was carried out as described previously (27), with modifications outlined below. ALI cultures were fixed in 4% formaldehyde for 20 min at room temperature, followed by blocking and permeabilization in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), 5% normal donkey serum, 0.2% saponin, and 0.3% Triton X-100 for 1 h at 4 °C. A549 or 16HBE cells were fixed in 4% formaldehyde for 20 min at room temperature, followed by blocking and permeabilization in PBS containing 1% BSA, 5% normal donkey serum, 0.2% saponin, and 0.1% Triton X-100 for 30 min at 4 °C. Primary antibody incubation (1:100 for anti-T2R antibodies and 1:250 for tubulin antibodies) were carried out at 4 °C overnight. AlexaFluor-labeled donkey anti-mouse or rabbit secondary antibody incubation (1:1000) was carried out for 2 h at 4 °C. Transwell filters were removed from the plastic mounting ring and mounted with Fluoroshield with DAPI (Abcam). For direct labeling of primary antibodies to co-localize T2R38 and T2R14, Zenon antibody labeling kits (Invitrogen/Molecular Probes/Thermo Fisher Scientific) were used according to the manufacturer’s instructions. Images of ALIs were taken on an Olympus Fluoview confocal system with IX-81 microscope and ×100 (1.4 NA) objective. Images of A549 and 16HBE cells were taken in wide field with a ×60 (1.4 NA oil) objective on an Olympus IX-83 inverted microscope running Metamorph. Images were analyzed using Metamorph, Fluoview software, and/or FIJI (142)/ImageJ (W. Rasband, Research Services Branch, National Institute of Mental Health, Bethesda).
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**Data analysis and statistics**

One-way ANOVA was performed in GraphPad Prism with appropriate post-tests as indicated; $p < 0.05$ was considered statistically significant. For comparisons of all samples within a data set, Tukey-Kramer post-test was used. For preselected pairwise comparisons, Bonferroni post-test was performed. For comparisons to a single control group, one-way ANOVA with Dunnett’s post-test was used. All other data analysis was performed in Excel. For all figures, one asterisk (*) indicates Dunnett’s post-test was used. All other data analysis was performed in Excel. For all figures, one asterisk (*) indicates $p < 0.05$ and two asterisks or pound signs (** or ##) indicate $p < 0.01$, respectively; “n.s.” indicates no statistical significance. All data are presented as mean ± S.E.

**Author contributions**—B. M. H. and R. J. L. conceived the study and wrote the paper. B. M. H., D. B. M., J. R. F., and R. J. L. performed experiments and analyzed/interpreted data. B. C., L. J. D., N. D. A., J. N. P., and D. W. K. recruited patients, aided with tissue procurement and primary human cell culture, and maintained clinical databases and records. C. J. M., D. R. R., and P. J. contributed critical reagents and expertise for heterologous expression (P. J.) and patient sample genotyping (C. J. M. and D. R. R.). All authors have reviewed and approved the final version of the manuscript.

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