

In vitro effects of anthocyanidins on sinonasal epithelial nitric oxide production and bacterial physiology

Benjamin M. Hariri, B.S.,¹ Sakeena J. Payne, M.D.,² Bei Chen, M.D.,¹ Corrine Mansfield, B.S.,³ Laurel J. Doghramji, R.N.,¹ Nithin D. Adappa, M.D.,¹ James N. Palmer, M.D.,¹ David W. Kennedy, M.D.,¹ Masha Y. Niv, Ph.D.,⁴ and Robert J. Lee, Ph.D.^{1,5}

ABSTRACT

Background: T2R bitter taste receptors play a crucial role in sinonasal innate immunity by upregulating mucociliary clearance and nitric oxide (NO) production in response to bitter gram-negative quorum-sensing molecules in the airway surface liquid. Previous studies showed that phytochemical flavonoid metabolites, known as anthocyanidins, taste bitter and have antibacterial effects. Our objectives were to examine the effects of anthocyanidins on NO production by human sinonasal epithelial cells and ciliary beat frequency, and their impact on common sinonasal pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Methods: Ciliary beat frequency and NO production were measured by using digital imaging of differentiated air-liquid interface cultures prepared from primary human cells isolated from residual surgical material. Plate-based assays were used to determine the effects of anthocyanidins on bacterial swimming and swarming motility. Biofilm formation and planktonic growth were also assessed.

Results: Anthocyanidin compounds triggered epithelial cells to produce NO but not through T2R receptors. However, anthocyanidins did not impact ciliary beat frequency. Furthermore, they did not reduce biofilm formation or planktonic growth of *P. aeruginosa*. In *S. aureus*, they did not reduce planktonic growth, and only one compound had minimal antibiofilm effects. The anthocyanidin delphinidin and anthocyanin keracyanin were found to promote bacterial swimming, whereas anthocyanidin cyanidin and flavonoid myricetin did not. No compounds that were tested inhibited bacterial swarming.

Conclusion: Results of this study indicated that, although anthocyanidins may elicit an innate immune NO response from human cells, they do not cause an increase in ciliary beating and they may also cause a pathogenicity-enhancing effect in *P. aeruginosa*. Additional studies are necessary to understand how this would affect the use of anthocyanidins as therapeutics. This study emphasized the usefulness of *in vitro* screening of candidate compounds against multiple parameters of both epithelial and bacterial physiologies to prioritize candidates for *in vivo* therapeutic testing.

(Am J Rhinol Allergy 30, 261–268, 2016; doi: 10.2500/ajra.2016.30.4331)

Chronic rhinosinusitis (CRS)^{1,2} affects more than one in eight adults in the United States^{1,3} and accounts for >\$8 billion in direct health care costs^{3–5} and >\$20 billion dollars in total economic burden⁶ annually. CRS is a syndrome of complex etiologies that involve chronic infection and/or persistent inflammation of the paranasal sinuses, often linked to ineffective sinonasal mucociliary clearance⁷ and/or chronic bacterial infection and biofilm formation.⁸ Patients with CRS who require surgery report worse quality-of-life scores for physical pain and social functioning than those patients with chronic obstructive pulmonary disease, congestive heart failure, or angina.⁹

CRS accounts for one in five adult antibiotic prescriptions in the United States,^{10,11} a modality that is becoming less effective with the emergence of antibiotic-resistant microorganisms. Nonantibiotic topical CRS therapeutics are needed. Bitter taste receptors (T2Rs) are emerging potential therapeutic targets.^{12–16} Originally identified on the tongue, T2Rs are also located throughout the sinonasal cavity,^{12–16}

where they stimulate nitric oxide (NO) production^{17–19} or antimicrobial peptide secretion.²⁰ Moreover, a nonfunctional (“nontaster”) polymorphism in the *TAS2R38* gene encoding T2R38 is an independent risk factor for CRS^{17,18,21} and predicts surgical outcome in non-polypoid CRS.²² Because T2Rs regulate important host defense responses in the sinonasal cavity, bitter compounds may be useful as topical therapeutics to stimulate endogenous immune responses.

Anthocyanidins are a class of naturally occurring plant flavonoids found in red berries and other fruits, with antioxidant activity *in vitro*.²³ Moreover, anthocyanidins can activate some T2R receptors^{24,25} and have been linked to endothelial NO production.²⁶ Because NO is an important component of sinonasal innate immunity,^{19,27,28} we hypothesized that anthocyanidins may activate sinonasal T2Rs to generate NO. In addition, anthocyanidin-rich plant extracts have antibacterial properties against *Staphylococcus aureus*,^{29,30} *Escherichia coli*,^{29,31} *Corynebacterium diphtheria*,³² *Moraxella catarrhalis*,³² *Bacillus cereus*,³³ and *Pseudomonas aeruginosa*.³⁴ However, other studies have indicated that anthocyanidins do not have antibacterial effects against *B. cereus*,³⁴ *E. coli*,^{34,35} or *P. aeruginosa*,³⁰ or that anthocyanidins only reduce inflammation.³⁶ The interactions between anthocyanidins and bacteria are complex,^{37–39} but interest in anthocyanidins has increased with the correlation between consumption of grapes and grape products (e.g., wine) with lower incidences of cancers and inflammatory diseases.⁴⁰

We hypothesized that anthocyanidins may be potential natural topical therapeutics for CRS, either as T2R agonists or direct antimicrobials. Because plant compounds can have multiple effects on airway epithelial cells,⁴¹ we sought to investigate the effects of various representative anthocyanidins on primary human epithelial cells and common sinonasal pathogens, *P. aeruginosa* and *S. aureus*. In addition to microbiologic assays, we used optical imaging of air-liquid interface (ALI) cultures of primary human sinonasal cells isolated from residual surgical material, a state-of-the-art *in vitro* model for testing epithelial cell responses to novel compounds. Analysis of the results of this study emphasizes the

From the ¹Department of Otorhinolaryngology—Head and Neck Surgery, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania, ²Division of Otolaryngology, Department of Surgery, Pennsylvania State University Milton S. Eshelman Medical Center, Hershey, Pennsylvania, ³Monell Chemical Senses Center, Philadelphia, Pennsylvania, ⁴The Institute of Biochemistry, Food and Nutrition, Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Israel, and ⁵Department of Physiology, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania

This study was supported by National Institutes of Health grant 1R03DC013862 to R.J. Lee as well as a grant from the Department of Otorhinolaryngology at the University of Pennsylvania to R.J. Lee

N.D. Adappa and J.N. Palmer are consultants for Acclarent. The remaining authors have no conflicts of interest pertaining to this article

Address correspondence to Robert J. Lee, Ph.D., Department of Otorhinolaryngology—Head and Neck Surgery, University of Pennsylvania Medical Center, 5th Floor Ravid Building, 3400 Spruce Street, Philadelphia, PA 19104

E-mail address: rjl@mail.med.upenn.edu

Copyright © 2016, OceanSide Publications, Inc., U.S.A.

importance of testing multiple parameters of epithelial cell responses when screening candidate compounds *in vitro*, which may help to identify and prioritize the best compounds for subsequent *in vivo* testing.

METHODS

Solutions and Reagents

Anthocyanidins (Fig. 1) were obtained from Cayman Chemical (Ann Arbor, MI). Stock solutions of chemicals were dissolved in dimethyl sulfoxide before dissolving in Dulbecco's phosphate-buffered saline (PBS), with the highest working concentrations reflecting no more than 0.1% dimethyl sulfoxide, a level that does not affect sinonasal ALIs.^{19,20,42,43} In the experiments, PBS was primarily used on the apical side and Hank's Balanced Salt Solution (which contained 10 mM HEPES and Minimal Essential Medium amino acids) basolaterally, as described elsewhere.^{19,20,42–44} 4-Amino-5-methylamino-2',7'-difluorescein (DAF-FM) was from Life Technologies, Inc. (Carlsbad, CA). All other reagents were from Sigma-Aldrich (St. Louis, MO).

Sinonasal ALI Cultures

ALIs were prepared as described elsewhere.^{19,20,43–46} Patients were recruited from the Department of Otorhinolaryngology—Head and Neck Surgery, University of Pennsylvania, with full institutional review board approval (protocol 800614, "Pathogenesis of Chronic Rhinosinusitis"). Informed consent was obtained during the preoperative clinic visit or in the preoperative waiting room. The criterion for patient selection was those patients undergoing sinonasal surgery. Criteria for exclusion were a history of systemic diseases (Wegener's granulomatosis, cystic fibrosis, sarcoid, immunodeficiencies) and the use of antibiotics, oral corticosteroids, or antiangiogenics (*e.g.*, omalizumab) within 1 month of surgery.

ALIs were established from sinonasal epithelial cells enzymatically dissociated from residual surgical material and grown in proliferation medium that consisted of Dulbecco's Modified Eagle's Medium (Invitrogen/Life Technologies, Inc.)/Nutrient Mixture F-12 Ham and bronchial epithelial basal medium (Lonza Group, Basel, Switzerland) for 7 days. Cells were then trypsinized and seeded on Transwell cell culture inserts ($6-7 \times 10^4$ cells per membrane; Corning Inc., Corning, NY) coated with bovine serum albumin, bovine collagen, and fibronectin. Five days later, the culture medium was removed from the upper compartment and the epithelium was allowed to differentiate in medium that consisted of 1:1 Dulbecco's Modified Eagle's Medium and bronchial epithelial basal medium (Lonza Group) with human epidermal growth factor, epinephrine, bovine pituitary extract, hydrocortisone, insulin, triiodothyronine, and transferrin, with penicillin, streptomycin, retinoic acid, and fetal bovine serum in the basal compartment.

Low-Light-Level Live-Cell Fluorescence Imaging of Intracellular NO Production

Cells were loaded with DAF-FM as described elsewhere^{19,20,43,44,47} by incubation in 10 μ M DAF-FM diacetate and 5 μ M 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide, a cell-permeant NO scavenger to prevent NO production during loading. After 30 minutes, cultures were washed with PBS to remove 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide and unloaded DAF-FM, followed by 15 minutes of incubation to allow for dye retention. Images were acquired on an IX-81 microscope ($\times 10$, 0.3 NA UPlanFLN objective; Olympus, Tokyo, Japan) at 5-second intervals by using a 488 laser and FluoView confocal system (Olympus). Because magnitudes of fluorescence changes were used to approximate NO production, care was taken to follow the loading protocol strictly to normalize dye loading, and microscope and/or software settings

were identical for each experiment. No gain, offset, or gamma alterations were used.

High-Speed Digital Imaging of Ciliary Beat Frequency

Ciliary beat frequency (CBF) was performed as described elsewhere.^{19,20,43,44,46,48} Images of beating cilia from mature cultures were visualized by using a $\times 20$ objective on an inverted microscope (DM IL; Leica Microsystems, Inc., Wetzlar, Germany) with a model A602f Basler area scan high-speed monochromatic digital video camera (Basler, AG, Ahrensburg, Germany) at 100 frames/s. Video images were analyzed with the Sisson-Ammons Video Analysis (SAVA) system version 2.1 (Ammons Engineering, Clio, MI). Experiments were performed at $\sim 28^\circ\text{C}$. Whole-field analysis was performed, with each measured point representing a single cilium. The reported frequencies represent the arithmetic means of these values.

Biofilm Formation Assays

Crystal violet biofilm assays were carried out as described elsewhere.^{49–51} Briefly, 100 μ L of 0.5 McFarland *P. aeruginosa* (PAO1 or ATCC 27853) or *S. aureus* (methicillin-resistant strain M2⁵²) cultures in 50% lysogeny broth (LB) were added to a 96-well plate and grown for 48 hours statically at 37°C . Media were changed after 24 hours. The plates were washed two to three times with water, then stained with 1% crystal violet for 30–60 minutes, followed by a second wash. Stained biofilms were solubilized in crystal violet with 30% acetic acid and read in a spectrophotometer plate reader at ~ 550

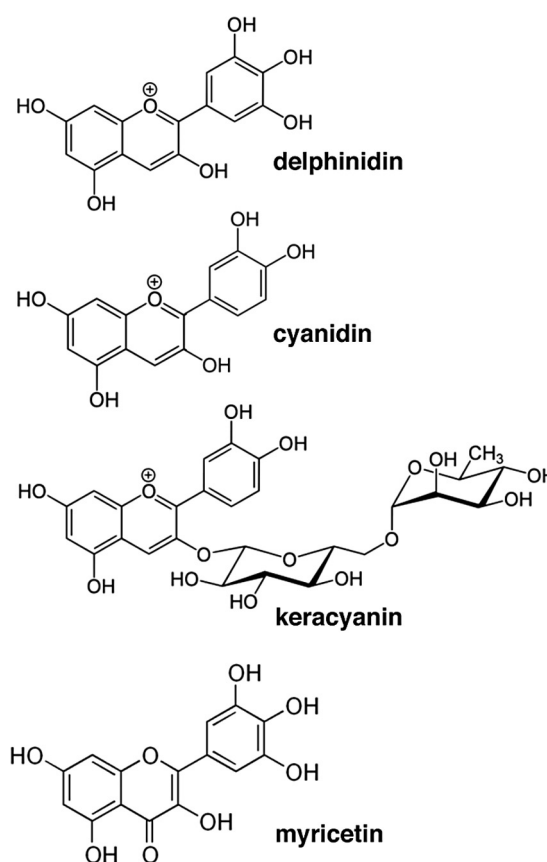


Figure 1. Compounds tested in this study. Two anthocyanidins, delphinidin and cyanidin, were tested. Keracyanin, the glycosylated anthocyanin derivative of cyanidin, also was examined. The non-anthocyanidin flavonoid myricetin was used as a control.

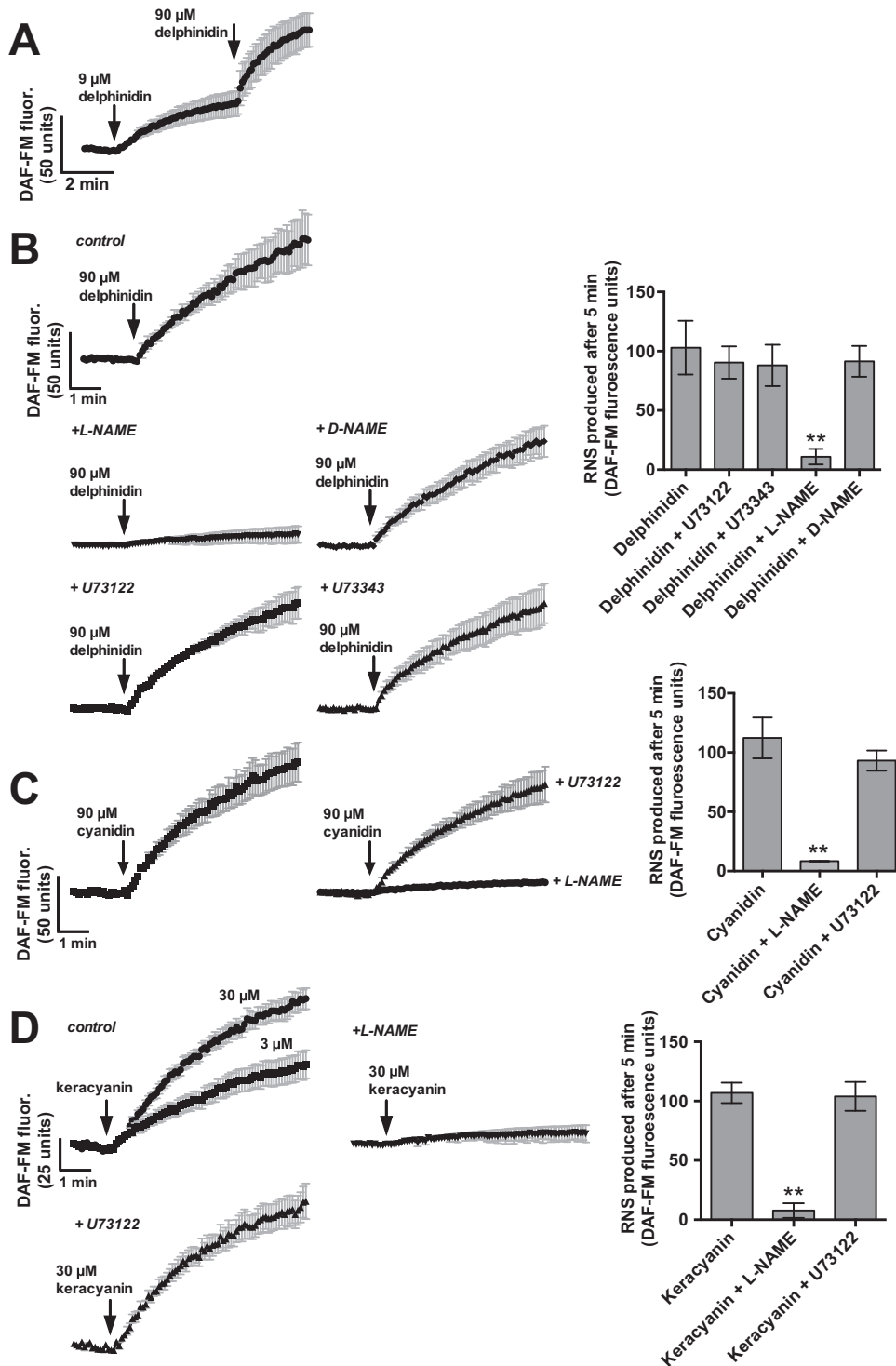


Figure 2. Anthocyanins and anthocyanidins can stimulate sinonasal epithelial nitric oxide (NO) production. (A) Average traces of 4-amino-5-methylamino-2',7'-difluorescein (DAF-FM) fluorescence ($n = 6$ ALI cultures from three patients), showing increases in reactive nitrogen species (RNS) production in response to 9 and 90 μM delphinidin, an anthocyanidin. (B) Pharmacology of the delphinidin-induced response. DAF-FM fluorescence increases in response to 90 μM delphinidin (103 ± 23 units; $n = 3$ cultures from three patients) were inhibited in the presence of L-N^G-nitroarginine methyl ester (L-NAME) (11 ± 7 ; $n = 5$ cultures from three patients; $p < 0.01$) but not D-N^C-nitroarginine methyl ester (91 ± 13 ; $n = 4$ cultures from three patients; $p =$ not significant [n.s.]), U73122 (90 ± 14 ; $n = 3$ cultures from three patients; $p =$ n.s.), or U73343 (88 ± 17 ; $n = 4$ cultures from three patients; $p =$ n.s.). (C) DAF-FM fluorescence increases in response to another anthocyanidin, cyanidin (90 μM ; 112 ± 17 ; $n = 5$ cultures from three patients), were likewise inhibited by L-NAME (8 ± 1 ; $n = 3$ cultures from three patients; $p < 0.01$) but not U73122 (93 ± 9 ; $n = 3$ cultures from three patients; $p =$ n.s.). (D) The anthocyanin keracyanin similarly caused an increase in RNS production at 3 μM (58 ± 10 ; $n = 5$ cultures from four patients) and 30 μM (107 ± 8 ; $n = 7$ cultures from four patients). Response to 30 μM was inhibited by L-NAME (8 ± 6 ; $n = 3$ cultures from three patients; $p < 0.01$) but not U73122 (104 ± 12 ; $n = 7$ cultures from four patients; $p =$ n.s.). For all bar graphs, $**p < 0.01$ compared with control (first bar) by one-way analysis of variance with the Dunnett posttest.

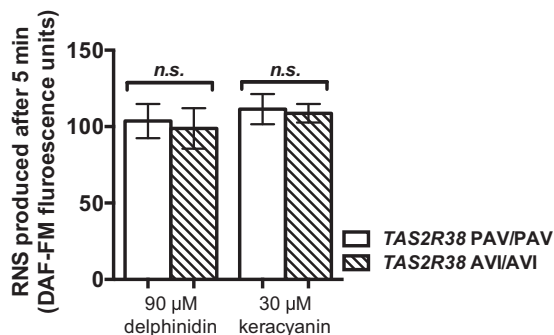


Figure 3. T2R38 is not involved in anthocyanin- and/or anthocyanidin-stimulated nitric oxide (NO) production. 4-Amino-5-methylamino-2',7'-difluorescein (DAF-FM) measured NO production in human sinonasal epithelial cells in response to delphinidin or keracyanin stimulation did not differ between patient cultures homozygous for the functional "taster" TAS2R38 haplotype (PAV) or the nonfunctional "nontaster" haplotype (AVI). DAF-FM fluorescence increases were 104 \pm 11 units (90 μ M delphinidin, PAV/PAV; n = 6 cultures from three patients), 99 \pm 13 units (90 μ M delphinidin, AVI/AVI; n = 6 cultures from three patients), 111 \pm 10 units (30 μ M keracyanin; PAV/PAV; n = 6 cultures from three patients), and 109 \pm 6 units (30 μ M keracyanin; AVI/AVI; n = 6 cultures from three patients). Significance determined by one-way analysis of variance with the Bonferroni posttest comparing bracket-indicated conditions.

nm. Variation was minimized by normalizing values to LB only (control) conditions grown on the same plate.

P. aeruginosa and *S. aureus* Planktonic Growth Assay

Briefly, 100 μ L of anthocyanidin solution was added to at least three wells of a 96-well plate, followed by 100 μ L of an overnight culture diluted to the optical density of a 0.5 McFarland standard in LB, and subsequent incubation for 2 hours at 37°C, with periodic shaking at 30-minute intervals. Before spotting on the LB agar plates, dilutions were made at 1:10, 1:100, 1:1000, and 1:10,000 with LB. Colony-forming units (CFUs) were manually counted after overnight incubation.

Plate-based Assays for *P. aeruginosa* Motility

Plates were prepared as described elsewhere,^{53,54} with some slight modifications, by using M9 media, 0.5% casamino acids, 0.01% MgSO₄, 0.2% glucose, and 0.4% (swimming) or 0.8% (swarming) agar. Agar solutions were allowed to cool to the touch while stirring to avoid any heat deactivation of the flavonoid compounds, added after autoclaving. The plates were inoculated with 5 μ L of an overnight *P. aeruginosa* culture grown at 37°C in LB diluted with LB to an optical density of 0.5 and allowed to dry on the bench. Once dry, the plates were incubated at 37°C for 16–20 hours and subsequently imaged.

Data Analysis and Statistics

Data were analyzed in SAVA and/or Fiji.⁵⁵ Statistical analyses were performed in Excel (Microsoft Corporation, Redmond, WA) and/or Prism (GraphPad Software, Inc., La Jolla, CA) with p < 0.05 considered statistically significant. For multiple comparisons with one-way analysis of variance, the Bonferroni posttest was used when preselected pairwise comparisons were performed, and the Dunnett posttest was used when values were compared with a control value. All data are presented as mean \pm standard error of the mean (SEM).

RESULTS

Anthocyanidins Trigger Sinonasal Epithelial Cells to Produce NO

NO generation can be activated by receptor-driven changes in intracellular calcium concentrations that stimulate NO synthase activation.^{19,42,56} Cellular NO production was imaged in real time by using DAF-FM, which reacts with NO-derived reactive nitrogen species to form a fluorescent benzotriazole.¹⁹ Treating the apical side of ALIs with delphinidin, a representative anthocyanidin found in Cabernet Sauvignon grapes,^{57,58} elicited DAF-FM fluorescence increases over ~5 minutes, which signaled production of NO (Fig. 2 A). This was confirmed by using the NO synthase inhibitor L-N^G-nitroarginine methyl ester (L-NAME) and inactive stereoisomer D-N^G-nitroarginine methyl ester. L-NAME, but not D-N^G-nitroarginine methyl ester, blocked DAF-FM fluorescence increases in response to delphinidin (Fig. 2 B), which demonstrated that this indeed reflected NO production. However, the DAF-FM fluorescence responses were not blocked by phospholipase C inhibitor U73122 (Fig. 2 B). The inactive analog of U73122, U73343, also showed no effect (Fig. 2 B). The structurally similar cyanidin activated similar NO responses that were inhibited by L-NAME but not U73122 (Fig. 2 C).

Anthocyanidins are often found as glycosylated anthocyanins (Fig. 1). Keracyanin is the anthocyanin derivative of cyanidin, found in black currants and raspberries.⁵⁹ To determine if anthocyanins have similar effects, we tested keracyanin on sinonasal ALIs. Keracyanin similarly stimulated NO production that was inhibited by L-NAME but not by U73122 (Fig. 2 D). In addition, delphinidin- and keracyanin-induced DAF-FM fluorescence responses were not dependent on the TAS2R38 gene, which encodes T2R38.^{60,61} There was no significant difference between cultures homozygous for the functional TAS2R38 PAV "taster" allele and those homozygous for the nonfunctional AVI "nontaster" allele (Fig. 3), which confirmed the lack of involvement of this taste receptor in these responses.

Anthocyanidins and Anthocyanins Do Not Impact Ciliary Beat Frequency

To determine if anthocyanin- and/or anthocyanidin-stimulated NO production results in increased ciliary beating, we imaged CBF

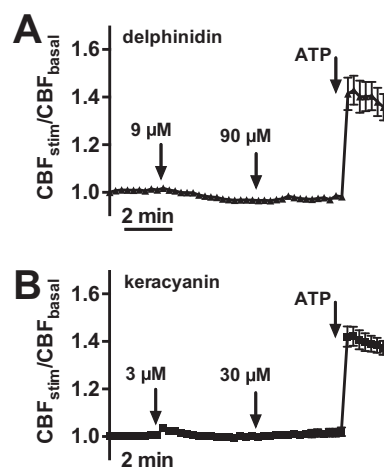


Figure 4. Delphinidin and keracyanin did not stimulate significant increases in ciliary beat frequency (CBF). Mean traces of normalized CBF are shown for delphinidin (A) (n = 6 cultures from three patients) and keracyanin (B) (n = 6 cultures from three patients). Responses to ATP, added at the end, confirmed the responsiveness and viability of cultures. ATP = adenosine 5'-triphosphate.

in vitro by using high-speed digital imaging. Changes in CBF were reported as a ratio of stimulated basal frequencies. No significant differences were observed when ALLs were stimulated with delphinidin (Fig. 4 A) or keracyanin (Fig. 4 B). Application of adenosine 5'-triphosphate (ATP), a purinergic agonist and a positive control to assess responsiveness, induced a robust 40–50% CBF increase.

Anthocyanidins and Anthocyanins Have Minimal Effects on *P. aeruginosa* and *S. aureus* Growth

We sought to determine if anthocyanidins or anthocyanins affect biofilm or planktonic growth of *P. aeruginosa* or *S. aureus*, common sinonasal pathogens sensitive to sinonasal NO production.¹⁴ We tested two wild-type *P. aeruginosa* strains, ATCC 27853 and PAO1. Although biofilm mass appeared to be slightly reduced in the pres-

ence of both keracyanin and delphinidin, the results were not statistically significant (Fig. 5 A), which indicated that these compounds had minimal or no antibiofilm effects. We also tested these compounds on *P. aeruginosa* planktonic growth. No significant reduction of CFUs was observed with either delphinidin or keracyanin (Fig. 5 B). As a control, the potent gram-negative antibiotic colistin sulfate reduced CFUs by ~1000-fold (Fig. 5 B). Similarly, we tested biofilm and planktonic growth in methicillin-resistant *S. aureus* strain M2. There was no significant difference in biofilm mass in the presence of cyanidin or delphinidin, although there seemed to be a slight reduction in the latter. A small but statistically significant reduction was observed in the presence of keracyanin (Fig. 5 C). There was no significant difference in the percentage of *S. aureus* CFUs that remained in the presence of cyanidin, delphinidin, or keracyanin relative to an LB-only control (Fig. 5 D).

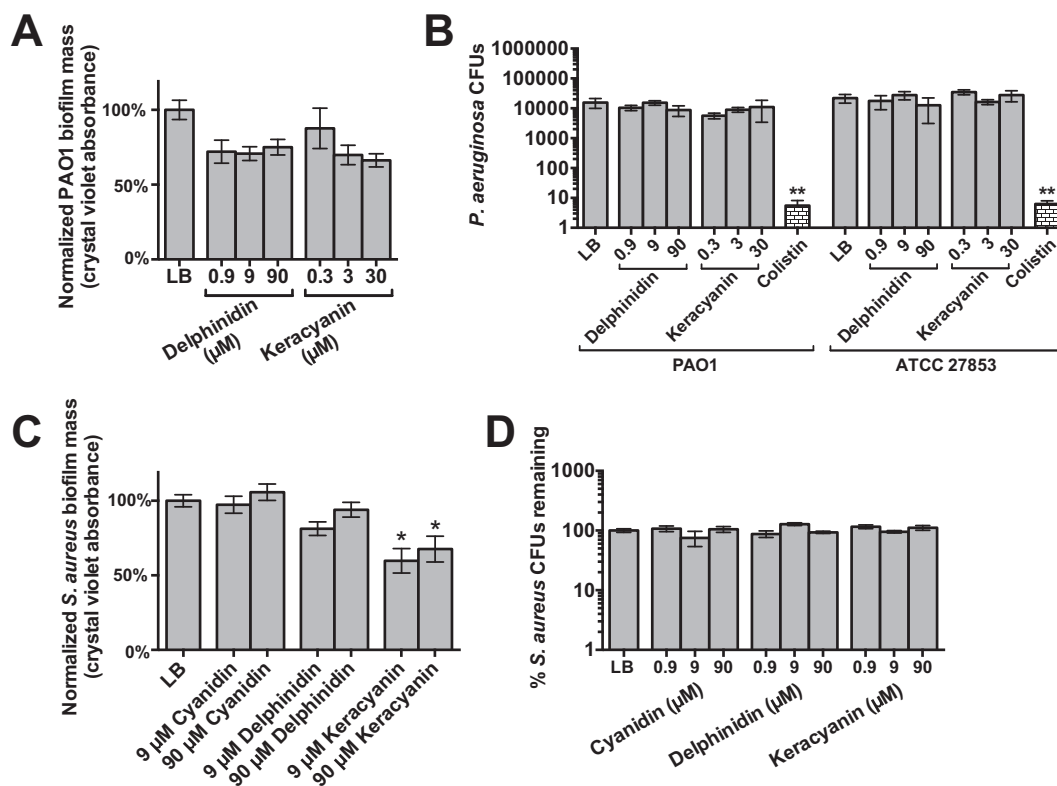


Figure 5. Anthocyanidins had minimal effects on *Pseudomonas aeruginosa* and *Staphylococcus aureus* planktonic and biofilm growth. (A) Bar graph showing *P. aeruginosa* normalized biofilm mass (PAO1) grown in the presence of LB-only (control) or LB that contained delphinidin or keracyanin at indicated concentrations. Although biofilm mass seemed to be slightly reduced, the values were not significant when compared with LB-only by one-way analysis of variance (ANOVA) with the Dunnett posttest. Each biofilm mass measurement represents the average of five experiments, with each experiment being the average of at least eight wells grown in a single 96-well plate. Normalized biofilm masses were 100 ± 6 (LB-only), 72 ± 7 (0.9 μM delphinidin), 71 ± 5 (9 μM delphinidin), 75 ± 5 (90 μM delphinidin), 88 ± 14 (0.3 μM keracyanin), 70 ± 6 (3 μM keracyanin), and 66 ± 4 (30 μM keracyanin). (B) Bar graph, showing planktonic growth, measured by a colony-forming unit (CFU) spotting and counting assay of two *P. aeruginosa* wild-type strains, PAO1 and ATCC 27853. CFUs were not significantly reduced in the presence of delphinidin or keracyanin at indicated concentrations (ANOVA with the Dunnett posttest). The potent gram-negative antibiotic colistin sulfate was included as a control; ***p* < 0.01. CFUs recovered for PAO1 were 15,571 ± 2119 (LB), 10,417 ± 1031 (0.9 μM delphinidin), 15,333 ± 1312 (9 μM delphinidin), 8,722 ± 1370 (90 μM delphinidin), 5,667 ± 623 (0.3 μM keracyanin), 9,000 ± 816 (3 μM keracyanin), 11,000 ± 3391 (30 μM keracyanin), and 5 ± 3 (10 μg/mL colistin). CFUs recovered for ATCC 27853 were 22,000 ± 3536 (LB), 17,667 ± 4327 (0.9 μM delphinidin), 27,667 ± 4249 (9 μM delphinidin), 12,667 ± 4784 (90 μM delphinidin), 35,000 ± 3342 (0.3 μM keracyanin), 16,333 ± 1546 (3 μM keracyanin), 27,667 ± 5573 (30 μM keracyanin), and 6 ± 2 (10 μg/mL colistin). (C) Bar graph, showing *S. aureus* normalized biofilm mass grown in the presence of LB-only (control) or LB that contained cyanidin, delphinidin, or keracyanin at indicated concentrations. Normalized *S. aureus* biofilm masses were 100 ± 4% (LB), 97 ± 6% (9 μM cyanidin), 106 ± 5% (90 μM cyanidin), 81 ± 5% (9 μM delphinidin), 94 ± 5% (90 μM delphinidin), 59.7 ± 8% (9 μM keracyanin), and 68 ± 9% (90 μM keracyanin). (D) Normalized *S. aureus* CFUs remaining were 100 ± 7% (LB), 108 ± 11% (0.9 μM cyanidin), 75 ± 22% (9 μM cyanidin), 105 ± 12% (90 μM cyanidin), 88 ± 11% (0.9 μM delphinidin), 128 ± 6% (9 μM delphinidin), 93 ± 4% (90 μM delphinidin), 116 ± 8% (0.9 μM keracyanin), 95 ± 4% (9 μM keracyanin), 111 ± 10% (90 μM keracyanin); **p* < 0.05 by ANOVA with Dunnett post-test. LB = lysogeny broth.

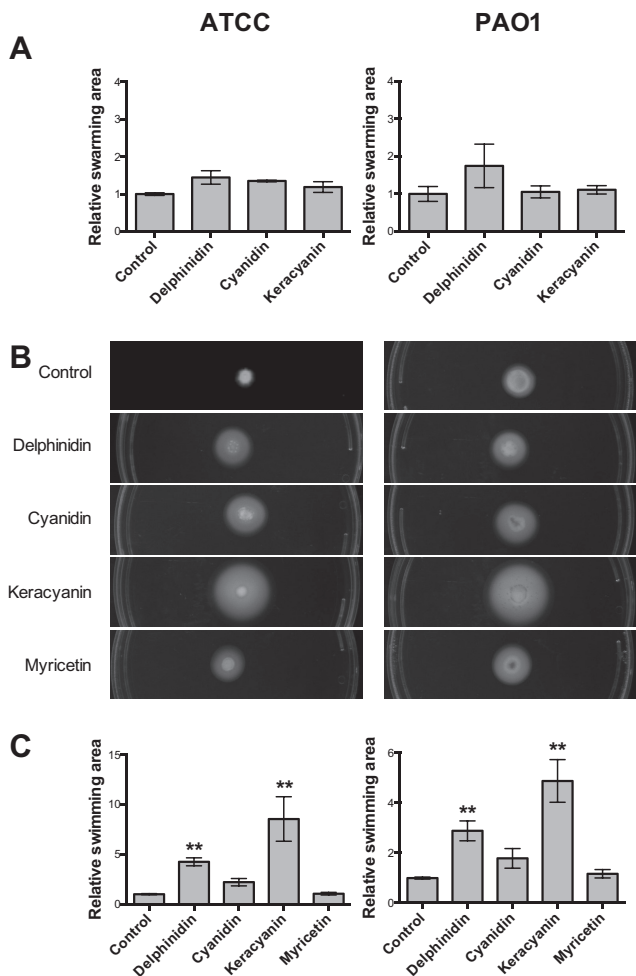


Figure 6. Effects of flavonoid compounds on swarming and swimming motility of two *Pseudomonas aeruginosa* strains. (A) Summary of bacterial swarming results for each condition. For both strains, ATCC 27853 (left) and PAO1 (right), delphinidin, cyanidin, and keracyanin did not have a statistically significant effect on relative swarming area. Relative bacterial swarming area for ATCC 27853 was 1.00 ± 0.03 (control), 1.45 ± 0.18 (delphinidin), 1.35 ± 0.02 (cyanidin), and 1.19 ± 0.14 (keracyanin). Relative bacterial swarming area for PAO1 was 1.00 ± 0.20 (control), 1.75 ± 0.58 (delphinidin), 1.06 ± 0.16 (cyanidin), and 1.11 ± 0.11 (keracyanin); $n = 3-4$ experiments each, 4–6 plates per experiment. (B) Representative images of bacterial swimming results for each condition. (C) Summary of bacterial swimming results. For both strains, the anthocyanidin delphinidin and the anthocyanin keracyanin demonstrated statistically significant increases in the relative bacterial swimming area (** $p < 0.01$). Cyanidin and myricetin did not produce statistically significant differences in the relative swimming area. The relative bacterial swimming area for ATCC 27853 was 1.00 ± 0.06 (control), 4.23 ± 0.41 (delphinidin), 2.21 ± 0.37 (cyanidin), 8.55 ± 2.23 (keracyanin), and 1.06 ± 0.13 (myricetin). The relative bacterial swimming area for PAO1 was 1.00 ± 0.04 (control), 2.89 ± 0.40 (delphinidin), 1.79 ± 0.39 (cyanidin), 4.88 ± 0.85 (keracyanin), and 1.17 ± 0.17 (myricetin).

Delphinidin, Cyanidin, and Keracyanin Do Not Inhibit Bacterial Swarming Motility

Swarming motility, evidenced by a characteristic uneven radiance of colonies from a spot on semisolid high-agar plates that mimics gelatinous viscous surfaces, e.g., a mucosal membrane,⁵⁴ plays a pivotal role in bacteria spreading and the onset of biofilm formation.⁵⁴

Swarming requires intact flagellar function and the production of rhamnolipids,^{54,62} linked to airway infection,^{63,64} as well as 3-hydroxyalkanoic acids.⁵⁴ However, a plate-based swarming assay⁵⁴ revealed that neither delphinidin, cyanidin, nor keracyanin caused reductions in bacterial swarming (Fig. 6A).

Delphinidin and Keracyanin Promote Bacterial Swimming Mobility

Swimming is a method of *Pseudomonas* motility that occurs near surfaces,^{65,66} is involved in chemotaxis,⁶⁷ requires twitching⁶⁸ and flagellar motility, and is implicated in pathogenesis^{69,70} and biofilm formation.^{71,72} Swimming is evidenced by an even colony spread, characteristic of uncooperative motion, on lower-agar plates.⁵³ Delphinidin and keracyanin caused a statistically significant increase in swimming of both *P. aeruginosa* strains ($p < 0.01$) (Fig. 6, B and C). However, this was not statistically significant with cyanidin and the non-anthocyanidin flavonoid myricetin, which indicated that variation exists in terms of flavonoid stimulation of bacterial swimming.

DISCUSSION

Our results indicated that anthocyanidins stimulated sinonasal NO production, previously shown to have antibacterial effects,^{19,73,74} but not through bitter taste receptor stimulation. Although cyanidin can activate both T2R39 (effective concentration [EC], $32 \mu\text{M}$; EC_{50} , $187 \mu\text{M}^{24}$) and T2R14 (EC, $250 \mu\text{M}^{24}$), the lower EC observed in this study ($90 \mu\text{M}$) combined with the lack of inhibition by U73122 indicated that these effects of anthocyanidins are not mediated through T2Rs. Moreover, cells from patients who were homozygous for both functional (PAV) T2R38 and nonfunctional (AVI) T2R38 responded equally to delphinidin and keracyanin, which indicated that T2R38, a major T2R isoform in sinonasal cilia, was not involved. Phospholipase C β 2 is an essential downstream component of taste signal transduction,^{12,13,15,16,19,20} and the lack of an effect of U73122 here indicated that the anthocyanidin-stimulated production of NO observed is likely not due to a taste receptor response. We previously demonstrated that U73122 inhibits NO downstream of the T2R38 taste receptor expressed in sinonasal cilia.^{19,42} The mechanism of this NO production remains unknown. We recently reported on a secreted *S. aureus* compound that likewise stimulated acute taste receptor-independent NO production.⁴⁷ It remains to be determined if anthocyanidins activate this same pathway.

Mucociliary clearance, driven by ciliary beating, removes foreign debris, including bacteria, from the respiratory tract.⁷ NO can elevate CBF through activation of protein kinase G. It remains unclear why anthocyanidin-driven NO increases do not increase CBF. Possibilities include off-target effects, including changes in intracellular pH or protein kinase C activity⁷⁵ to “cancel out” stimulatory effects of protein kinase G, or, perhaps the NO response is somehow uncoupled from downstream protein kinase G signaling. The lack of CBF increases, lack of inhibition of biofilm or planktonic growth or swarming, and promotion of bacterial swimming indicated that anthocyanidins may have less therapeutic benefit than compounds that stimulate both NO and CBF together (e.g., agonists that activate sinonasal T2R isoforms¹⁹). Analysis of these data also indicated that anthocyanidins alone had minimal antibacterial effects against *Pseudomonas* and *Staphylococcus* biofilm or planktonic growth. Although a statistically significant reduction in *S. aureus* biofilm growth was seen with keracyanin, it is unclear whether this small decrease ($<1 \log$) *in vitro* would result in clinically significant biofilm reduction *in vivo*. Cranberry proanthocyanidins, another flavonoid class, were previously shown to have antibiofilm properties⁷⁶ and block *P. aeruginosa* swarming,⁷⁷ which indicates that they may be more useful as therapeutics than anthocyanidins.

Analysis of these results indicates that *in vitro* screening of compounds under investigation for topical application in CRS, including

naturally occurring compounds, *e.g.*, anthocyanidins, against both human sinonasal cells *in vitro* and sinonasal bacteria can be useful for identification and prioritization of which compounds may have the most therapeutic benefit. Although a lack of effect *in vitro* (*e.g.*, as with the lack of effects on ciliary beating observed in this study) does not necessarily mean a lack of benefit *in vivo*, the *in vitro* testing of two of the most common natural CRS therapeutics, Sinupret (Bionorica SE, Neumarkt in der Oberpfalz, Germany)^{78,79} and GeloMyrtol (G. Pohl-Boskamp GmbH & Co. KG, Hohenlockstedt, Germany),⁸⁰ demonstrated significant *in vitro* enhancement of components of sinonasal innate immunity. *In vitro* screening may also be useful to identify compounds with off-target effects that may increase bacterial virulence.

CONCLUSION

Although analysis of our data indicates that some anthocyanin or anthocyanidin compounds activate a potentially beneficial NO response from sinonasal cells, the same compounds can stimulate activation of a possibly pathogenicity-enhancing response in *P. aeruginosa*. Further study is required to determine (1) whether this bacterial response would offset the potential beneficial responses of anthocyanins and/or anthocyanidins *in vivo* and (2) which specific anthocyanidin isoforms might stimulate the most efficacious host NO production without modulating bacterial physiology. However, therapeutic effects of anthocyanins and/or anthocyanidins nonetheless may be reduced by their lack of a net effect on cilia. Analysis of these data indicated that *in vitro* screening of compounds against multiple parameters of epithelial and bacterial physiology may allow prioritization of classes of compounds for *in vivo* testing by identifying which compound isoforms activate the most beneficial responses.

ACKNOWLEDGMENTS

The authors thank Danielle R. Reed and the Monell Chemical Senses Center Genotyping Core, supported by National Institutes of Health grant P30DC011735.

REFERENCES

- Settipane RA, Peters AT, and Chandra R. Chapter 4: Chronic rhinosinusitis. *Am J Rhinol Allergy* 27(suppl. 1):S11–S15, 2013.
- Gudis D, Zhao KQ, and Cohen NA. Acquired cilia dysfunction in chronic rhinosinusitis. *Am J Rhinol Allergy* 26:1–6, 2012.
- Fokkens WJ, Lund VJ, Mullol J, et al. European Position Paper on Rhinosinusitis and Nasal Polyps 2012. *Rhinol Suppl* (23):3 p preceding table of contents, 1–298, 2012.
- Bhattacharyya N. Incremental health care utilization and expenditures for chronic rhinosinusitis in the United States. *Ann Otol Rhinol Laryngol* 120:423–427, 2011.
- Bhattacharyya N, Grebner J, and Martinson NG. Recurrent acute rhinosinusitis: Epidemiology and health care cost burden. *Otolaryngol Head Neck Surg* 146:307–312, 2012.
- Smith KA, Orlandi RR, and Rudmik L. Cost of adult chronic rhinosinusitis: A systematic review. *Laryngoscope* 125:1547–1556, 2015.
- Antunes MB, Gudis DA, and Cohen NA. Epithelium, cilia, and mucus: Their importance in chronic rhinosinusitis. *Immunol Allergy Clin North Am* 29:631–643, 2009.
- Al-Mutairi D, and Kilty SJ. Bacterial biofilms and the pathophysiology of chronic rhinosinusitis. *Curr Opin Allergy Clin Immunol* 11: 18–23, 2011.
- Toledano A, Rodriguez G, Martin AM, et al. Quality of life in patients with smell loss due to upper respiratory tract infections. *Am J Otolaryngol* 32:504–510, 2011.
- Manes RP, and Batra PS. Bacteriology and antibiotic resistance in chronic rhinosinusitis. *Facial Plast Surg Clin North Am* 20:87–91, 2012.
- Kennedy JL, and Borish L. Chronic rhinosinusitis and antibiotics: The good, the bad, and the ugly. *Am J Rhinol Allergy* 27:467–472, 2013.
- Lee RJ, and Cohen NA. Bitter and sweet taste receptors in the respiratory epithelium in health and disease. *J Mol Med* 92:1235–1244, 2014.

- Lee RJ, and Cohen NA. Taste receptors in innate immunity. *Cell Mol Life Sci* 72:217–236, 2015.
- Lee RJ, and Cohen NA. Role of the bitter taste receptor T2R38 in upper respiratory infection and chronic rhinosinusitis. *Curr Opin Allergy Clin Immunol* 15:14–20, 2015.
- Lee RJ, and Cohen NA. Sinonasal solitary chemosensory cells “taste” the upper respiratory environment to regulate innate immunity. *Am J Rhinol Allergy* 28:366–373, 2014.
- Lee RJ, and Cohen NA. The emerging role of the bitter taste receptor T2R38 in upper respiratory infection and chronic rhinosinusitis. *Am J Rhinol Allergy* 27:283–286, 2013.
- Adappa ND, Howland TJ, Palmer JN, et al. Genetics of the taste receptor T2R38 correlates with chronic rhinosinusitis necessitating surgical intervention. *Int Forum Allergy Rhinol* 3:184–187, 2013.
- Adappa ND, Zhang Z, Palmer JN, et al. The bitter taste receptor T2R38 is an independent risk factor for chronic rhinosinusitis requiring sinus surgery. *Int Forum Allergy Rhinol* 4:3–7, 2014.
- Lee RJ, Xiong G, Kofonow JM, et al. T2R38 taste receptor polymorphisms underlie susceptibility to upper respiratory infection. *J Clin Invest* 122:4145–4159, 2012.
- Lee RJ, Kofonow JM, Rosen PL, et al. Bitter and sweet taste receptors regulate human upper respiratory innate immunity. *J Clin Invest* 124:1393–1405, 2014.
- Mfuna Endam L, Filali-Mouhim A, Boisvert P, et al. Genetic variations in taste receptors are associated with chronic rhinosinusitis: A replication study. *Int Forum Allergy Rhinol* 4:200–206, 2014.
- Adappa ND, Farquhar D, Palmer JN, et al. TAS2R38 genotype predicts surgical outcome in nonpolypoid chronic rhinosinusitis. *Int Forum Allergy Rhinol* 6:25–33, 2016.
- Ksonzekova P, Mariychuk R, Eliasova A, et al. In vitro study of biological activities of anthocyanin-rich berry extracts on porcine intestinal epithelial cells. *J Sci Food Agric* 96:1093–1100, 2016.
- Roland WS, van Buren L, Gruppen H, et al. Bitter taste receptor activation by flavonoids and isoflavonoids: Modeled structural requirements for activation of hTAS2R14 and hTAS2R39. *J Agric Food Chem* 61:10454–10466, 2013.
- Narukawa M, Noga C, Ueno Y, et al. Evaluation of the bitterness of green tea catechins by a cell-based assay with the human bitter taste receptor hTAS2R39. *Biochem Biophys Res Commun* 405:620–625, 2011.
- Edwards M, Czank C, Woodward GM, et al. Phenolic metabolites of anthocyanins modulate mechanisms of endothelial function. *J Agric Food Chem* 63:2423–2431, 2015.
- Jeong JH, Yoo HS, Lee SH, et al. Nasal and exhaled nitric oxide in chronic rhinosinusitis with polyps. *Am J Rhinol Allergy* 28:e11–e16, 2014.
- Phillips PS, Sacks R, Marcells GN, et al. Nasal nitric oxide and sinonasal disease: A systematic review of published evidence. *Otolaryngol Head Neck Surg* 144:159–169, 2011.
- Pala CU, Zorba NN, and Ozcan G. Microbial inactivation and physicochemical properties of ultrasound processed pomegranate juice. *J Food Prot* 78:531–539, 2015.
- Leitao DP, Polizello AC, Ito IY, and Spadaro AC. Antibacterial screening of anthocyanin and proanthocyanin fractions from cranberry juice. *J Med Food* 8:36–40, 2005.
- Junqueira-Goncalves MP, Yanez L, Morales C, et al. Isolation and characterization of phenolic compounds and anthocyanins from Murta (*Ugni molinae* Turcz.) fruits. Assessment of antioxidant and antibacterial activity. *Molecules* 20:5698–5713, 2015.
- Krauze-Baranowska M, Majdan M, Halasa R, et al. The antimicrobial activity of fruits from some cultivar varieties of *Rubus idaeus* and *Rubus occidentalis*. *Food Funct* 5:2536–2541, 2014.
- Viskeliš P, Rubinskiene M, Jasutiene I, et al. Anthocyanins, antioxidative, and antimicrobial properties of American cranberry (*Vaccinium macrocarpon* Ait.) and their press cakes. *J Food Sci* 74:C157–C161, 2009.
- Ombra MN, Fratianni F, Granese T, et al. In vitro antioxidant, antimicrobial and anti-proliferative activities of purple potato extracts (*Solanum tuberosum* cv Vitelotte noire) following simulated gastrointestinal digestion. *Nat Prod Res* 29:1087–1091, 2015.
- Lacombe A, Wu VC, Tyler S, et al. Antimicrobial action of the American cranberry constituents; Phenolics, anthocyanins, and organic acids, against *E. coli* O157:H7. *Int J Food Microbiol* 139:102–107, 2010.

36. Kim JM, Kim KM, Park EH, et al. Anthocyanins from black soybean inhibit *Helicobacter pylori*-induced inflammation in human gastric epithelial AGS cells. *Microbiol Immunol* 57:366–373, 2013.
37. Faria A, Fernandes I, Norberto S, et al. Interplay between anthocyanins and gut microbiota. *J Agric Food Chem* 62:6898–6902, 2014.
38. Cisowska A, Wojnicz D, and Hendrich AB. Anthocyanins as antimicrobial agents of natural plant origin. *Nat Prod Commun* 6:149–156, 2011.
39. Aura AM, Martin-Lopez P, O'Leary KA, et al. In vitro metabolism of anthocyanins by human gut microflora. *Eur J Nutr* 44:133–142, 2005.
40. Xia EQ, Deng GF, Guo YJ, and LI HB. Biological activities of polyphenols from grapes. *Int J Mol Sci* 11:622–646, 2010.
41. Chang JH, Song KJ, Kim HJ, et al. Dietary polyphenols affect MUC5AC expression and ciliary movement in respiratory cells and nasal mucosa. *Am J Rhinol Allergy* 24:e59–e62, 2010.
42. Lee RJ, Chen B, Redding KM, et al. Mouse nasal epithelial innate immune responses to *Pseudomonas aeruginosa* quorum-sensing molecules require taste signaling components. *Innate Immun* 20:606–617, 2014.
43. Zhao KQ, Cowan AT, Lee RJ, et al. Molecular modulation of airway epithelial ciliary response to sneezing. *FASEB J* 26:3178–3187, 2012.
44. Lee RJ, Chen B, Doghramji L, et al. Vasoactive intestinal peptide regulates sinonasal mucociliary clearance and synergizes with histamine in stimulating sinonasal fluid secretion. *FASEB J* 27:5094–5103, 2013.
45. Abad A, Fernandez-Molina JV, Bikandi J, et al. What makes *Aspergillus fumigatus* a successful pathogen? Genes and molecules involved in invasive aspergillosis. *Rev Iberoam Micol* 27:155–182, 2010.
46. Lai Y, Chen B, Shi J, et al. Inflammation-mediated upregulation of centrosomal protein 110, a negative modulator of ciliogenesis, in patients with chronic rhinosinusitis. *J Allergy Clin Immunol* 128:1207–1215.e1, 2011.
47. Carey RM, Workman AD, Chen B, et al. *Staphylococcus aureus* triggers nitric oxide production in human upper airway epithelium. *Int Forum Allergy Rhinol* 5:808–813, 2015.
48. Zhao KQ, Goldstein N, Yang H, et al. Inherent differences in nasal and tracheal ciliary function in response to *Pseudomonas aeruginosa* challenge. *Am J Rhinol Allergy* 25:209–213, 2011.
49. O'Toole GA. Microtiter dish biofilm formation assay. *J Vis Exp* (47): pii: 2437, 2011.
50. Antunes MB, Chi JJ, Liu Z, et al. Molecular basis of tobacco-induced bacterial biofilms: An in vitro study. *Otolaryngol Head Neck Surg* 147:876–884, 2012.
51. Goldstein-Daruech N, Cope EK, Zhao KQ, et al. Tobacco smoke mediated induction of sinonasal microbial biofilms. *PLoS One* 6:e15700, 2011.
52. Harro JM, Daugherty S, Bruno VM, et al. Draft genome sequence of the methicillin-resistant *Staphylococcus aureus* isolate MRSA-M2. *Genome Announc* (1): pii: e00037–12, 2013.
53. Ha DG, Kuchma SL, and O'Toole GA. Plate-based assay for swimming motility in *Pseudomonas aeruginosa*. *Methods Mol Biol* 1149:59–65, 2014.
54. Ha DG, Kuchma SL, and O'Toole GA. Plate-based assay for swarming motility in *Pseudomonas aeruginosa*. *Methods Mol Biol* 1149:67–72, 2014.
55. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: An open-source platform for biological-image analysis. *Nat Methods* 9:676–682, 2012.
56. Forstermann U, and Sessa WC. Nitric oxide synthases: Regulation and function. *Eur Heart J* 33:829–837, 837a–837d, 2012.
57. Nyman NA, and Kumpulainen JT. Determination of anthocyanidins in berries and red wine by high-performance liquid chromatography. *J Agric Food Chem* 49:4183–4187, 2001.
58. Ryan JM, and Revilla E. Anthocyanin composition of Cabernet Sauvignon and Tempranillo grapes at different stages of ripening. *J Agric Food Chem* 51:3372–3378, 2003.
59. Tulio AZ Jr, Reese RN, Wyzgoski FJ, et al. Cyanidin 3-rutinoside and cyanidin 3-xylosylrutinoside as primary phenolic antioxidants in black raspberry. *J Agric Food Chem* 56:1880–1888, 2008.
60. Lipchock SV, Mennella JA, Spielman AI, and Reed DR. Human bitter perception correlates with bitter receptor messenger RNA expression in taste cells. *Am J Clin Nutr* 98:1136–1143, 2013.
61. Bufe B, Breslin PA, Kuhn C, et al. The molecular basis of individual differences in phenylthiocarbamide and propylthiouracil bitterness perception. *Curr Biol* 15:322–327, 2005.
62. Caiazza NC, Shanks RM, and O'Toole GA. Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa*. *J Bacteriol* 187:7351–7361, 2005.
63. Read RC, Roberts P, Munro N, et al. Effect of *Pseudomonas aeruginosa* rhamnolipids on mucociliary transport and ciliary beating. *J Appl Physiol* 72:2271–2277, 1992.
64. Zulianello L, Canard C, Kohler T, et al. Rhamnolipids are virulence factors that promote early infiltration of primary human airway epithelia by *Pseudomonas aeruginosa*. *Infect Immun* 74:3134–3147, 2006.
65. Conrad JC, Gibiansky ML, Jin F, et al. Flagella and pili-mediated near-surface single-cell motility mechanisms in *P. aeruginosa*. *Biophys J* 100:1608–1616, 2011.
66. Keller JB. Effect of viscosity on swimming velocity of bacteria. *Proc Natl Acad Sci U S A* 71:3253–3254, 1974.
67. Sampedro I, Parales RE, Krell T, and Hill JE. *Pseudomonas* chemotaxis. *FEMS Microbiol Rev* 39:17–46, 2015.
68. Mattick JS. Type IV pili and twitching motility. *Annu Rev Microbiol* 56:289–314, 2002.
69. Shi W, and Sun H. Type IV pilus-dependent motility and its possible role in bacterial pathogenesis. *Infect Immun* 70:1–4, 2002.
70. Kazmierczak BI, Schniederberend M, and Jain R. Cross-regulation of *Pseudomonas* motility systems: The intimate relationship between flagella, pili and virulence. *Curr Opin Microbiol* 28:78–82, 2015.
71. Li Y, Xia H, Bai F, et al. Identification of a new gene PA5017 involved in flagella-mediated motility, chemotaxis and biofilm formation in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* 272:188–195, 2007.
72. Adams H, Horrevoets WM, Adema SM, et al. Inhibition of biofilm formation by Camelid single-domain antibodies against the flagellum of *Pseudomonas aeruginosa*. *J Biotechnol* 186:66–73, 2014.
73. Marcinkiewicz J. Nitric oxide and antimicrobial activity of reactive oxygen intermediates. *Immunopharmacology* 37:35–41, 1997.
74. Fang FC. Perspectives series: Host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. *J Clin Invest* 99:2818–2825, 1997.
75. Salathe M. Regulation of mammalian ciliary beating. *Annu Rev Physiol* 69:401–422, 2007.
76. Ulrey RK, Barksdale SM, Zhou W, and van Hoek ML. Cranberry proanthocyanidins have anti-biofilm properties against *Pseudomonas aeruginosa*. *BMC Complement Altern Med* 14:499, 2014.
77. O'May C, and Tufenkji N. The swarming motility of *Pseudomonas aeruginosa* is blocked by cranberry proanthocyanidins and other tannin-containing materials. *Appl Environ Microbiol* 77:3061–3067, 2011.
78. Virgin F, Zhang S, Schuster D, et al. The bioflavonoid compound, sinupret, stimulates transepithelial chloride transport in vitro and in vivo. *Laryngoscope* 120:1051–1056, 2010.
79. Zhang S, Skinner D, Hicks SB, et al. Sinupret activates CFTR and TMEM16A-dependent transepithelial chloride transport and improves indicators of mucociliary clearance. *PLoS One* 9:e104090, 2014.
80. Lai Y, Dilidaer D, Chen B, et al. In vitro studies of a distillate of rectified essential oils on sinonasal components of mucociliary clearance. *Am J Rhinol Allergy* 28:244–248, 2014. □