Rational drug design for sore throat—An aspirin-based treatment that addresses bradykinin-induced inflammation

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Abstract

Often thought of as a minor health concern, sore throat or pharyngitis is an important public health issue. It is one of the most common symptoms of upper respiratory diseases including COVID-19 and is a leading cause of physician visits and antibiotic prescriptions. Despite being on the market for decades, few over the counter sore throat medications are proven to heal sore throat. In studying pharyngitis using organotypic human respiratory tissue stimulated with bradykinin, we saw an increase in prostaglandin E2 (PGE2) and interleukin-8 (IL-8) in response to bradykinin. Bradykinin is one of the first inflammatory signals for pharyngitis and it increases PGE2 in human subjects. If left unregulated, PGE2 may further increase inflammation via the COX pathway and via IL-8, a proinflammatory chemokine responsible for neutrophil infiltration and possibly thus, a cytokine storm. Acetyl salicylic acid (ASA), a non-specific COX inhibitor, was able to mitigate a bradykinin-induced increase in PGE2 in our studies. However, ASA was inflammatory above its small therapeutic window, greatly increasing levels of PGE2 and IL-8 above those seen with bradykinin stimulation alone. Similar to other systems, the respiratory epithelia maintains a delicate balance of pro-inflammatory and anti-inflammatory signals in order to keep the respiratory barrier intact. To our knowledge, this is the first study to try and elucidate the complex mechanisms involved in healing pharyngitis, an inflammatory condition of the respiratory epithelia. Biovanta™, a formula containing ASA mitigated bradykinin-induced inflammation more strongly than ASA alone in organotypic human respiratory tissues. Surprisingly, we found that many of the most common over the counter sore throat therapies exacerbate inflammation and IL-8 in organotypic human respiratory tissues, suggesting these common treatments may possibly increase the likelihood of further respiratory complications in people.

Competing interest statement: This study was funded entirely by Applied Biological Laboratories, a private company that owns the Biovanta™ product. Unless otherwise indicated all experiments were performed at Applied Biological Laboratories research facility located at the SUNY Downstate Biotechnology Incubator, a part of StartUP NY. All of the authors were employees of Applied Biological Laboratories at the time the experiments were performed.

Background

Prior to the COVID-19 pandemic, pharyngitis, or sore throat was among the leading causes of physician visits and antibiotic prescriptions in the United States. This is surprising since 85% of sore throat cases were not caused by bacterial infections, but by viruses, such as rhinovirus and influenza (1). Now, sore throat is considered a symptom of COVID-19 (2) and there is growing concern that antibiotic overtreatment of COVID-19 patients will lead to the emergence of antibiotic-resistant bacteria (3). Historically, one major reason for the over-prescription of antibiotics has been the high rate of inaccuracy with office-based tests for bacterial throat cultures. Even if patients decide against going to the doctor, over the counter medicines will not heal sore throat. Pharyngitis as the name implies, is characterized by pain and inflammation of the pharynx, but most available therapies focus only on numbing the pain. The biochemical pathways causing inflammation in the upper respiratory system are well characterized, however no available over the counter or prescription therapies seem to address inflammation when treating sore throat, and some may actually worsen it.

Most of the therapies currently on the market for common upper respiratory complaints, such as sore throat, were developed decades ago and are marketed under the FDA monograph system. Since these medications have come to market, scientific research techniques in the field have greatly advanced. Although there are still arguably very few reliable animal models to study upper respiratory disease, three-dimensional, organotypic tissue explants of upper respiratory mucosa are now available (4-6). These models more reliably mimic the function of living tissue than either animal models or cell culture monolayers and can greatly accelerate drug discovery (4, 6). One obstacle to wide-spread use of these tissue cultures is the need for human donors and the technical complexity involved in reliably generating reproducible histological patterns.

Several companies and research labs specialize in providing these organotypic respiratory tissues to the highest reproducible scientific and quality control standards and we have sourced the tissues for these studies from two of these companies, Epithelix Sarl (Geneva, Switzerland) and Mattek Corporation (Ashland MA) (5, 7). The tissue we use in our studies are generated either from nasal biopsy or trachea-bronchial tissue. The majority of the respiratory tree, from the nasal cavity to the bronchi, is lined by pseudostratified columnar ciliated epithelium and thus have similar histological and functional characteristics (8). Thus we are able to extrapolate our findings from these tissues to the pharynx.

The nasopharynx is the main site of viral replication during upper respiratory infection (URI) (9). Infections often start in the nose and are carried down to the nasopharynx. One of the first chemical signals generated by pharyngial cells that will ultimately result in a sore throat is bradykinin. From the nasopharynx, bradykinin will eventually stimulate nociceptive receptors in the oropharynx and throat. At least three prior human clinical studies have used bradykinin to induce sore throat in healthy subjects (10-12). According to these studies, bradykinin released from the nasopharynx causes inflammation and pain in the throat that we characterize as sore throat. Each of these studies cites *in vitro* research that outlines the biochemical pathways and

mechanism of action for the role of bradykinin. The role of bradykinin in allergy and airway hyperresponsiveness has also been elucidated in several, more recent studies.

Bradykinin has been shown to increase IL-8 production in a time and concentration dependent manner in airway smooth muscle cells and respiratory epithelial cells via COX enzymes and PGE2 (13, 14). We sought to determine whether bradykinin would also increase IL-8 in organotypic human respiratory tissue and if so, whether the same signaling pathway involving COX enzymes are involved. We decided to measure the effect of COX inhibition on prostaglandin E2 (PGE2) and interleukin 8 (IL-8) release using acetyl salicylic acid (ASA), a non-specific COX inhibitor and an NSAID.

Here, we show that bradykinin can indeed increase PGE2 and IL-8 production in two highly differentiated organotypic models of the human airway, one derived from nasal and one derived from tracheo bronchial tissues. One assay system we used in this study is MucilAir™. MucilAir™ is a pseudostratified and ready-to-use organotypic model of human airway epithelium, constituted with primary human epithelial cells freshly isolated from nasal polyp biopsies. When switched at the air-liquid interface, the progenitor cells undergo a progressive differentiation and polarization to a fully- ciliated epithelia. The mature MucilAir™ is composed of basal cells, ciliated cells and mucus cells. The proportion of these various cell types is preserved compared to what one observes *in vivo* (15). Another similar model used in our studies, which uses tissue derived from trachea-bronchial biopsies is the EpiAirway™ (AIR-200-PE6.5) human tissue model.

The pathway of IL-8 secretion in these tissues is similar to that reported in respiratory epithelial cells and in airway smooth muscle and it involves increased COX-2 and PGE2. We show that the bradykinin induced increase of PGE2 can be inhibited by acetyl salicylic acid (aspirin), a non-selective COX inhibitor, in these systems. Acetyl salicylic acid did not appreciably and consistently decrease levels of IL-8 protein, despite significantly decreasing PGE2 levels. This suggests, as Pang and Knox noted in 1998, that either other products of arachidonic acid may be involved in increasing IL-8 expression, or that it involves increased gene expression. Other studies have shown IL-8 to be stable over time in culture conditions (14).

Apart from the COX pathway, arachidonic acid is also responsible for the generation of leukotrienes via lipoxygenase. Inhibiting the COX pathway with COX inhibitors may result in the shunting of arachidonic acid to the lipoxygenase pathway to produce leukotrienes. One of the major lipoxygenase products, LTB4, has been shown to stimulate synthesis and release IL-8 (13). Thus, it is likely that lipoxygenase products also mediate bradykinin and arachidonic acid-induced IL-8 production in human respiratory epithelial cells. We found that in A549 cells and in the two *ex-vivo* models we studied, there was a small therapeutic window in which acetyl salicylic acid inhibited PGE2 and IL-8 release. At a ten-fold higher concentration than this therapeutic dose, IL-8 increased to levels two to five times higher than with bradykinin stimulation alone. IL-8 is known to be an inflammatory cytokine, and at high levels of protein expression, we observed negative cytopathic effect such as decrease in TEER and increase in LDH. According to our observations there is a delicate balance in the regulation of IL-8 production.

According to previous studies, bradykinin induces the release of neutrophil chemotactic activity in A549 cells and bronchial epithelial cells, however the factors responsible have not been identified. IL-8 may be a component of the neutrophil chemoattractant activity produced by airway epithelial cells (14). Bradykinin also induces the release of IL-8 in respiratory smooth muscle cells via the COX-prostaglandin pathway. Pain receptors impinge on respiratory smooth muscle where PGE2 and IL-8 are also involved in pain perception and signaling (16). The bradykinin-PGE2 system seems to be a very important homeostatic pathway. We started out investigating bradykinin's role and possible modes of inhibition in pharyngitis, but found, to our surprise, that high concentrations of aspirin (an NSAID) and many over the counter lozenges and syrups used to treat sore throat, lead to inflammation via this pathway. Given bradykinin's important role in the inflammatory response and pain signaling, it is important for its signaling pathways to be tightly regulated. However, our findings indicate that the many available treatments aimed at alleviating the pain and inflammation associated with pharyngitis may actually worsen it by either inducing osmotic stress or deregulating inflammatory checkpoints.

Methods

Cells. A-549 cells (ATCC CCL-185) were cultured in F-12K Medium (ATCC, Catalog No. 30-2004) supplemented with 10% heat-inactivated fetal bovine serum (Gibco 10082147), 100 U ml−1 penicillin and 100 U ml−1 streptomycin (Gibco, 15140122). Cells were seeded on 96 well cell culture plates at a density of 6400 cells/well. Before the experiment, when cells reached 90% confluency, media was replaced with serum free media. Human airway epithelia reconstituted in vitro were purchased from two different providers, EpiAirway™ tissues (AIR-100) from MatTek Corporation (Ashland, MA, USA) and MucilAir™ tissues from Epithelix Sàrl (Geneva, Switzerland). Specific cell culture media for each tissue were obtained from the same manufacturer. Basal media was replaced with fresh media every other day until the day of the experiment.

Reagents for treatment. Biovanta™ liquid and lozenge formulas are composed of the following ingredients lactoferrin, lysozyme, acetyl salicylic acid and menthol and can be purchased from leading pharmacies. Other over the counter treatments for sore throat and cold were obtained from a local pharmacy. Over the counter products in a lozenge presentation were prepared for use by dissolving the product in saliva buffer (17). Products in liquid form were used as-is. Three days before each study, the apical side of the EpiAirway™ and Mucilair™ tissue inserts was washed with 200 ul of media. Briefly, fresh-warmed media was gently added to the apical side of the insert to not disturb the tissues. The plates were incubated for 15 min at 37 C. After incubation, media from the apical side was gently pipetted up and down 3 times to remove the excess mucus formed on the tissue. The day of the experiment the inserts were transferred to new plates with fresh warmed media and then 10ul of each treatment was added to the cells or the apical surface of the human airway tissues, the plates were gently swirled and returned to 37C for 5 min. Every 24 hours, 10 ul of new treatment was applied.

Bradykinin Challenge. Cell cultures and tissues were challenged with different concentrations of bradykinin from TOCRIS, Minneapolis, MN. Bradykinin was diluted to the appropriate

concentration using saliva buffer (17). After tissues were treated, ten microliters of the bradykinin solution were added to the cells (A549 cells) or the apical surface of the human airway tissues. Plates were gently swirled and returned to 37C incubation. Samples from basal media were collected at different time points after challenge and stored at -80C until use. Bradykinin was not repeated after the initial application.

Measurement of Transepithelial Electrical Resistance (TEER). TEER was measured in the human airway epithelium inserts to determine the integrity of tight junctions with a Millicell ERS-2 volt-ohmmeter (Millipore Sigma, Burlington, MA, USA). Briefly, inserts were transferred to new plates with 600 ul of warmed media per well, then 200 ul of media were gently added to the side wall of the inserts apical side. Plates were incubated for 5 min at 37C and then the TEER measurements were collected. Resistance values (Ω) were converted to TEER (Ω.cm2) by using the following formula: TEER (Ω.cm2) = (resistance value (Ω) - 100(Ω)) x 0.33 (cm2), where 100 Ω is the resistance of the membrane and 0.33 cm2 is the total surface of the epithelium.

Cytotoxicity. Cytotoxicity was assessed via lactate dehydrogenase (LDH) concentrations measured in 100 ul of basolateral medium incubated with the reaction mixture of a cytotoxicity detection kit (Sigma-Aldrich, Roche, Saint Louis, MO, USA) following the manufacturer's instructions. To determine the percentage of cytotoxicity, the following equation was used (A = absorbance values): Cytotoxicity (%) = (A (exp value)-A (low control)/A (high control)-A (low control))*100. The high control value corresponds to a 10% Triton X-100 treatment applied to the culture for 24 h. A threshold limit of 5% of the toxicity index reflects the physiological cell turnover in human airway epithelium cultures.

Analysis of inflammatory response. To evaluate the inflammatory response, cell culture supernatant and basolateral media from tissues was collected at different time points after challenge. Cyclooxygenase-2-derived prostaglandin E2 (PGE2) release was measured with an enzyme-linked immunosorbent assay kits (ELISA; R&D systems, Minneapolis, MN) according to the manufacturer's instructions. Interleukin-8 (IL-8 or CXCL8) protein in the basolateral media was measured using a magnetic bead-based ELISA (Procartaplex, ThermoFisher Scientific, Waltham, MA) according to the instructions provided by the manufacturer and the plate were then read using a Luminex-based Bio-plex Multiplex system (Bio-Rad, Hercules, CA). Samples were diluted to 1:3 for PEG− 2 analysis and 1:20 for IL-8 measurements.

Histopathology. At the end of the experiment, inserts with the human airway epithelia cells were fixed with a formalin solution (neutral buffered, 10%) and kept at 4C until being sent to Histowiz (New York, NY, USA) for histology processing. Inserts were bisected to allow histological staining of paraffin-embedded sections. Paraffin-embedded tissues were cut into 5-µm sections, de-paraffinized, and the rehydrated sections were stained with hematoxylin–eosin. The images were collected from digitalized slides at a final magnification of 20X.

Statistical analysis. Data are presented as mean ± standard error of the mean. For statistical comparison of differences between groups, results were analyzed by an unpaired t test, Mann-Whitney, ANOVA or Kruskal-Wallis tests using the GraphPad Prism software (version 6.01, La Jolla, USA). A p-value ≤ 0.05 was considered significant.

Results

Bradykinin, one of the first chemokine signals for pharyngitis causes inflammation in A549 cells that can be blocked by acetyl salicylic acid.

Bradykinin increases PGE2 via the arachidonic acid-COX 2 pathway, and acetyl salicylic acid (ASA) is known to be a potent COX inhibitor (14). First, we sought to determine the effects of bradykinin on A549 cells. Various concentrations of bradykinin were applied to A549 cells to assess the downstream effects on PGE2 and IL-8 (Figures 1A and 1B). LDH was also measured to assess cytotoxicity (Figure 1C).

At least 10uM bradykinin (supplied by Tocris) was needed to stimulate a statistically significant increase in PGE2 compared to negative control, a 5.3-fold increase (Figure 1A). As a result of bradykinin stimulation, there was a trend towards increased IL-8 expression after 24 hours, although it was not statistically significant (Figure 1B).

Once we established the stimulatory effects of bradykinin, we asked whether it could be inhibited by acetyl salicylic acid (ASA). Prior to stimulating with 10uM bradykinin, we treated cells with various concentrations of ASA (Figure 1D). Treating the cells with 0.6 ug/ml did not affect the bradykinin-induced increase in PGE2 secretion, but 6 ug/ml and 60 ug/ml kept PGE2 and IL-8 at control levels (Figure 1D and data not shown). A high 600 ug/ml dose of ASA was inflammatory, leading to a significant increase of PGE2 and IL-8 over control levels (Figure 1D and data not shown).

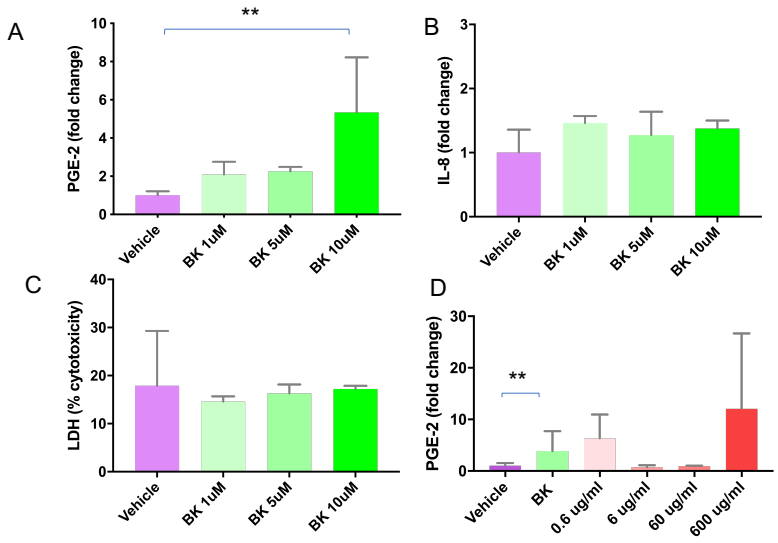

Figure 1 A549 cells were treated with culture media (vehicle) or the indicated concentrations of bradykinin (BK) in 96-well microtiter plates. PGE2 levels in media were measured at 4 hours post-inoculation (A). IL-8 (B) and LDH (C) levels were measured 24 hours post-inoculation. Cells were also treated with indicated concentrations of bradykinin after treatment with various concentrations of ASA and PGE2 was measured at 4 hours post-inoculation (D). Statistics were performed using GraphPad Prism software: Mann-Whitney test, p= 0.0048 (A), and p= 0.0152 (D). Statistics were performed on raw data without omissions, and not on fold-change values.

The bradykinin-induced inflammatory cascade can be replicated in an organotypic model of the human respiratory system.

MucilAir™ is composed of basal cells, ciliated cells and goblet cells that secrete mucus. The cells used to prepare these organotypic cultures are derived from nasal polyp biopsies. The proportion of the various cell types is preserved compared to what one observes *in vivo* (15) and the cytoarchitecture is virtually identical to that of the pharynx (8). We chose to use the organotypic tissue model because we hypothesized that they would be more predictive of human efficacy than animal models. These tissue models secrete most of the same cytokines that are secreted *in vivo*, and since the treatments we are designing are intended to be used topically, the metabolism and targeting of the ingredients should be virtually identical to what would be observed *in vivo* (6, 18).

In order to determine a dosage of bradykinin and the various ingredients we tested in A549 cells for use in the organotypic model, we approximated about a three order of magnitude increase in dosage would be necessary. A recent study (19) tested various doses of bromelain for inhibition of bradykinin in live animals and primary cell culture. The effective dose in cell culture was 20ug/ml and in live animals 100 mg/kg body weight. Assuming in animals an average of 95 ml blood per kg body weight, this translates to a dose of ~1mg/ml, or about a 5000-fold difference between primary cell culture and *in vivo*.

After testing several different concentrations (data not shown), we determined 25mg/ml to be the lowest effective dose of bradykinin that would reliably elicit a significant response of PGE2 in Mucilair™. This is approximately 2,500-fold greater than the 10 uM dose that was effective in A549 cells, which is in line with our expectations. Next, we investigated whether various concentrations of ASA could inhibit bradykinin-induced inflammation in Mucilair™. In vivo, ASA would be taken by mouth and then diluted by saliva before reaching the pharynx, where bradykinin is released physiologically. Therefore, after assuming a 1000-fold increase from A549 cells, we assumed a 10-30-fold dilution for salivary flow (for a formula dosed every 10-30min and a salivary flow rate of 1-3 ml/min), which would ultimately give us a 100-fold increase in concentration from A549 cells. Hence, we tested 0.6 mg/ml and 6mg/ml of ASA (100-fold and 1000-fold more concentrated than in A549 cells) with 24 mM of bradykinin in the MucilAir™ organotypic model.

We found that 0.6 mg/ml ASA inhibited the bradykinin-induced increase in PGE2, but it did not affect the bradykinin-induced increase in IL-8 levels (Figures 2A and 2B). As in A549 cells, the higher, 6mg/ml concentration of ASA led to inflammation and toxicity, manifested by a 5-fold increase in IL-8 levels (Figure 2B) and reduced TEER (Figure 2C). Possible reasons for the small therapeutic window of ASA will be discussed in the Discussion section.

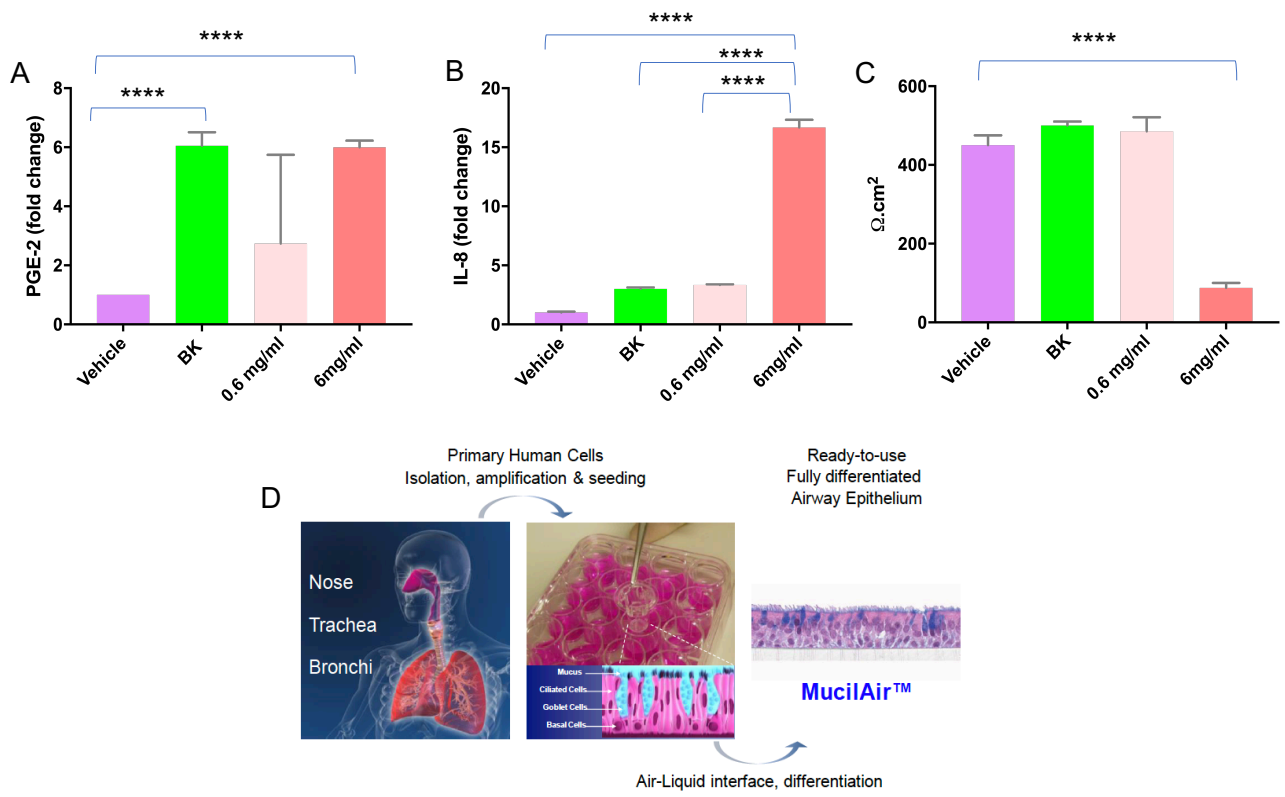

Figure 2 Mucilair™ respiratory tissues suspended in transwell inserts were treated with either saliva buffer (vehicle), 24 mM bradykinin, or 24 mM bradykinin following treatment with the indicated levels of acetyl salicylic acid (ASA). Levels of PGE2 in basal media were measured at 4 hours post-inoculation (A). IL-8 levels in basal media were measured at 24 hours post-inoculation, and transepithelial membrane resistance was measured at 24 hours post-inoculation (C). Statistics shown are results of unpaired t-test using GraphPad Prism software, p=0.0001 for all comparisons shown. Statistics were performed on raw data without omissions and not on fold-change values. Mucilair™ is obtained as described in the text and in D. The figures in D are reprinted with permission from Epithelix Sarl.

A Novel, ASA based formula is more effective than ASA alone at blocking bradykinin-induced PGE2 production in epithelial tissues.

Next, we sought to investigate the anti-inflammatory effects of a formula containing ASA, lysozyme, lactoferrin, aloe, glycerin, and menthol following bradykinin stimulation. Lysozyme and lactoferrin are potent anti-inflammatory molecules present in human nasal secretions. They are also used as preservatives and excipients to affect the rheological properties and viscosity of liquid formulations. Aloe, glycerin, and menthol are common excipients used in cough and cold products, are all natural, and were determined to not be toxic at the concentrations used (data not shown). We hypothesized that they could have beneficial anti-inflammatory effects and we determined optimal concentrations that would not elicit inflammation or cytotoxicity (data not shown).

We pretreated the tissues with either buffer, ASA alone, the formula containing ASA, or NS 398, prior to treating with bradykinin. As shown in figure 3A, ASA at a concentration of 0.6 mg/ml

decreased PGE2 expression, however the decrease was not statistically significant. The ASA-based formula and NS-398 decreased PGE2 levels almost three-fold which was highly significant (p=0.01). IL-8 levels were comparable to control levels after 24 hours in tissues treated with either ASA or the ASA-based formula and then treated with bradykinin. There was however a significant increase in IL-8 in tissues stimulated with bradykinin and then treated with NS-398 (p=0.01). ASA is a non-specific COX inhibitor, whereas NS-398 is a COX-2 specific inhibitor. This will be discussed in more detail in the Discussion Section.

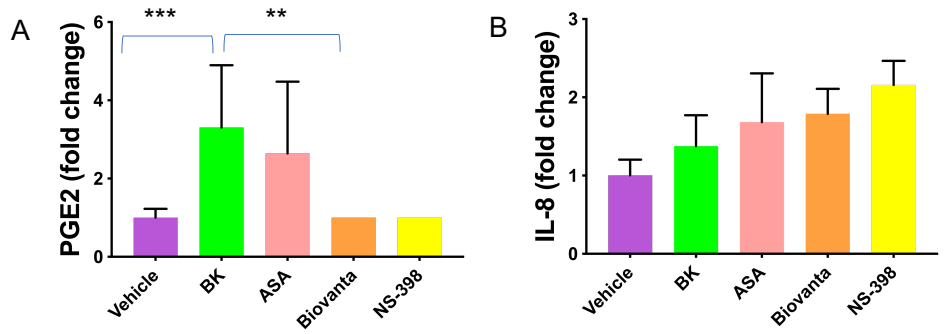

Figure 3 EpiAirway™ (AIR-200-PE6.5) respiratory tissues suspended in transwell inserts were treated with saliva buffer (vehicle), bradykinin (15 mM), or bradykinin following treatment with either ASA (0.6 mg/ml), Biovanta, or NS-398. PGE2 levels in basal media were measured at 4 hours post-inoculation, and Il-8 levels in basal media were measured 24 hours post-inoculation. Statistics shown are the results of Mann-Whitney tests using GraphPad Prism software. P< 0.0001 between negative control and bradykinin, and p=0.0091 between bradykinin and bradykinin + formula.

An ASA-based formula containing anti-inflammatory excipients decreases PGE2 in Mucilair™ without the cytotoxicity, or membrane damage caused by leading sore throat products.

After establishing the superiority of the ASA-based formula against ASA, we compared its efficacy in decreasing bradykinin-induced inflammation to leading sore throat products. Bradykinin at a concentration of 47 mM was used to induce inflammation after pretreating the tissues for five minutes with either buffer, NS-398 (100uM), Biovanta™ (liquid formula), Chloraseptic® Cherry Sore Throat Spray, Robitussin® Complete Formula (CF) Max Syrup, Zarbees™ Children's Cough Syrup + Mucus, or Halls Triple-Action Menthol-Lyptus Throat Drops. In tissues pre-treated with buffer and then bradykinin, prostaglandin E2 increased 1.54-fold after 4 hours and IL-8 increased 1.75-fold after 24 hours (Figure 4A and 4B). Both increases were statistically significant compared to negative control (p=0.02 and p=0.04, respectively). Note that at 48 hours, levels of IL-8 in tissues treated with sore throat products decrease, we believe this is due to cell death in the tissues because the corresponding values for TEER are very low and the corresponding values for LDH are high. Pretreatment with either NS-398 or the ASA-based formula kept PGE2 levels at control levels. At 24 hours post-stimulation, IL-8 levels in tissues pretreated with either NS-398 or the ASA-based formula were similar to tissues pretreated with buffer. However, at 48 hours post-stimulation, tissues pretreated with NS-398 showed a statistically significant increase in IL-8 above bradykinin-induced levels (p=0.01) (Figure 4C). This will be discussed in more detail in the Discussion section.

Of the leading sore throat products tested in this experiment, three were liquid formulations (either sprays or syrups) and one was a popular lozenge. None of the products prevented an increase in either PGE2 or IL-8. In fact, to our surprise, Choloroseptic® and Robitussin® pretreatment increased PGE2 levels compared to buffer pretreatment (Figure 4A). All of the products caused significant cytotoxicity as measured by increased IL-8 secretion, increased LDH secretion, and lowered TEER (Figure 4B-G).

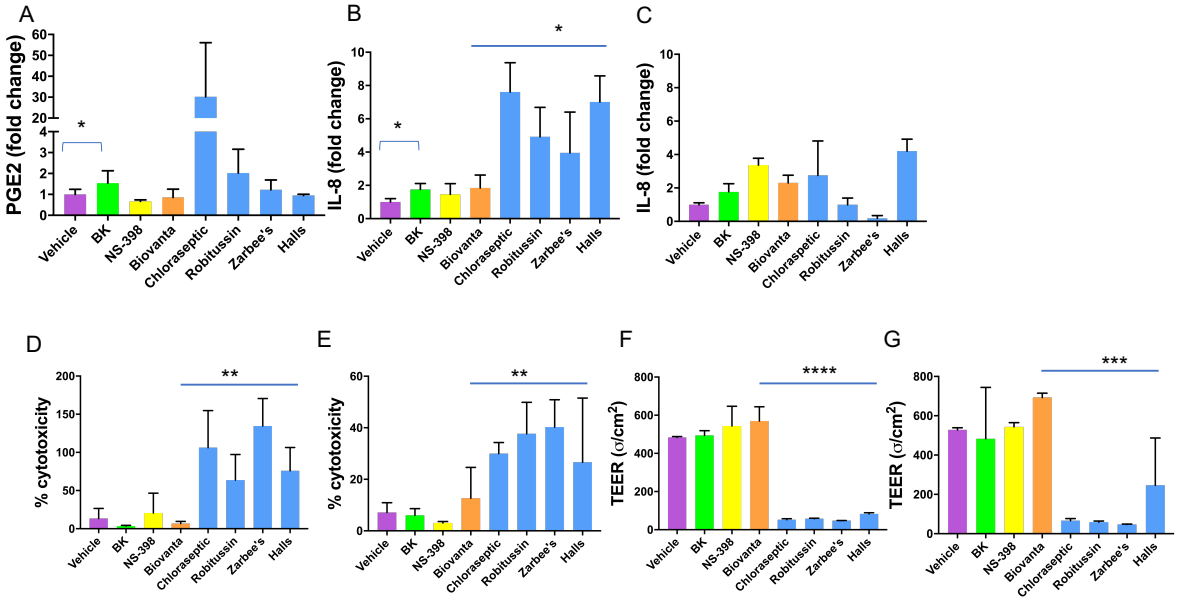

Figure 4 Mucilair™ respiratory tissues suspended in transwell inserts in 24 well plates were treated with saliva buffer (vehicle), bradykinin (BK) (47 mM) or BK following the indicated treatments. Levels of PGE2 in basal media were measured at 4 hours post-inoculation (A). IL-8 levels and LDH in basal media were measured at 24 hours (B and D) and 48 hours (C and E) post-inoculation, and transepithelial electrical resistance was measured at 24 hours (F) and 48 hours (G) post inoculation. Statistics shown are results of ANOVA and unpaired t-tests using GraphPad Prism software. Statistical analysis was performed on raw data without omissions and not on fold-change values.

We confirmed the inflammatory effects of leading over the counter sore throat products by testing them without bradykinin in the EpiAirway™ Model. We show that neither the ASA-based formula, nor a lozenge formulated with ASA and a low osmolarity sugar is inflammatory.

The following products were applied to the apical surface of EpiAirway™ to assess inflammatory effects: Ricola® Dual-Action Sore Throat and Cough Honey Lemon, Luden's® Wild Cherry Flavor Throat Flavor Dual Action, Cold-EEZE Natural Cherry Flavor Lozenge, Wedderspoon Organic Manuka Honey Drops, Zicam® Cherry Lozenge, Strepsils® Sore Throat and Cough Lozenges, and Vicks® Vapacool Severe Drops. These products were applied to tissues after being dissolved in saliva buffer. Briefly, and discussed in more detail below, the lozenges were weighed and dissolved in a predetermined w/v ratio of saliva buffer. Since lozenges are composed mainly of sugar, the volume was calculated as the minimum volume needed to dissolve the sugar in that lozenge as determined by its label. Biovanta™ liquid formula is available for purchase and is intended to be sprayed to the back of the throat in a total volume of 200ul. We considered the average salivary flow rate to be 2ml/min and that the liquid formula would be diluted 1:10 by

saliva in the mouth. Therefore, we diluted the liquid formula 1:10 before applying it to the tissues. After applying 10ul of the dissolved lozenge solution or Biovanta to the tissues, the tissues were placed at 37 degrees celsius for 5 minutes and then 10ul of saliva buffer was applied to the tissues. The tissues were not stimulated with bradykinin. As illustrated in figure 5, the products tested caused increases in inflammatory cytokines (PGE2, IL-8, or both), and LDH and also disrupted membrane integrity as illustrated by significantly lower TEER measurements.

In contrast, neither buffer, the ASA-based formula, nor a lozenge formulated with ASA and isomalt were inflammatory. In fact, levels of PGE2, IL-8, LDH, and TEER in response to the ASA-based formula and the lozenge were comparable to the buffer condition (Figure 5 A-D).

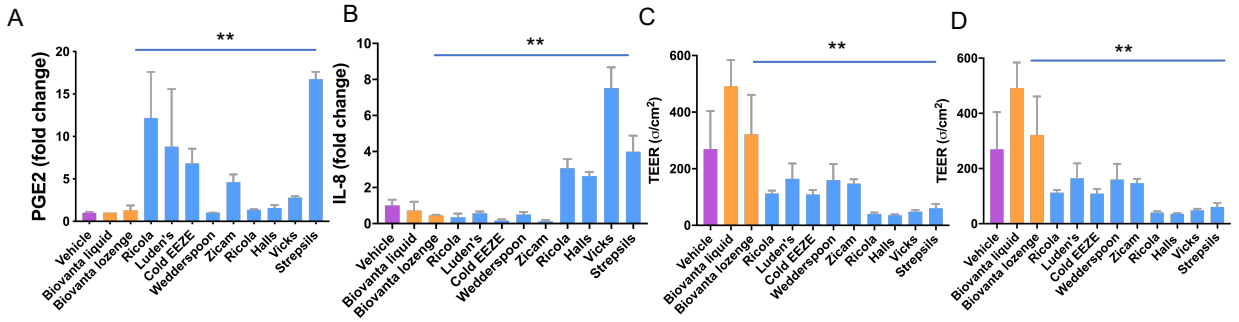

Figure 5 EpiAirway™ (AIR-200-PE6.5) respiratory tissues suspended in transwell inserts were treated with saliva buffer (vehicle), Biovanta liquid, Biovanta lozenges, or various leading brands of lozenges. Biovanta liquid and the lozenges were dissolved in saliva buffer before being applied to the apical surface of the respiratory tissues. Measurements of PGE2 at 4 hours (A), IL-8 at 24 hours (B), LDH at 24 hours, (C) and TEER at 24 hours (D) are shown. Statistics shown are results of Kruskal-Wallis tests performed using GraphPad Prism software, p=0.001 (A and B), p=0.003 (C), p=0.002 (D). Statistics were performed on raw data without omissions and not on fold-change values.

We show in a third-party blind placebo-controlled study that leading cold and sore throat remedies physically damage respiratory epithelia, but that a lozenge formulated with isomalt (a sugar with relatively low osmolality), ASA, and anti-inflammatory excipients does not.

Unlabeled Eppendorf tubes containing the various sore throat syrups and lozenges were sent to Mattek Corp (Ashland MA) for third party blind analysis. EpiAirway™ (AIR-200-PE6.5) were treated in triplicate with each of the syrups (undissolved) and each of the lozenges dissolved in buffer. Following a 5 min incubation, a 15 mM solution of bradykinin was added to the inserts. After incubating at 37 degrees for 48 hours, TEER measurements were taken.

Considering an average salivary flow rate of 2ml/min and an average recommended dosage for throat syrups of 20-30ml, we don't believe the syrups are intended to be dissolved in saliva but instead to bathe the oral pharynx. The lozenges were dissolved in buffer at a concentration determined by their weight and the type(s) of sugars they contain. For example, Halls® Relief Honey Lemon contains mainly glucose, according to its label. Glucose has a solubility of 91g/100ml of solution and a density of 1.02 g/ml. Each lozenge weighs 3.15g. It would therefore take about 1:0.75 w/v of lozenge to buffer (or saliva) to dissolve each lozenge. See table 1 for a list of the various products and the w/v ratio of buffer they were dissolved in.

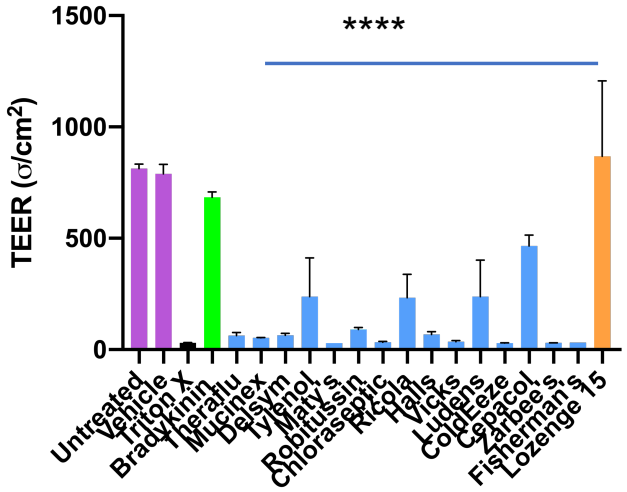

Figure 6 This study was performed in a double-blind format. EpiAirway™ (AIR-200-PE6.5) respiratory tissues suspended in transwell inserts were either left untreated, or treated with either saliva buffer (vehicle), triton-X, bradykinin, or various leading brands of lozenges. Following their respective treatments all inserts (except the untreated) were treated with 15mM bradykinin. The lozenges were dissolved in saliva buffer before being applied to the apical surface of the respiratory tissues. Statistics shown are results of a Kruskal-Wallis test using GraphPad Prism software, $p<0.0001$. Statistics were performed on raw data without omissions and not on fold-change values.

Table 1

Product	Form	Active Ingredient	Dosage	Predominant Sugar	w/v dissolution
Theraflu Express Max Nightime	Syrup	acetominiphen 650mg diphenhydramine HCl 25mg phenylephrine 10mg	30ml every 4 hours	n/a	n/a
Mucinex Fast Max. DM Max	Syrup	dextramethorphan HBr 20mg, guifenesin 400mg	20 ml: 12mg-- every 4 hours	n/a	n/a
Delsym	Syrup	dextramethorphan HBr 20mg, guifenesin 400mg	20 ml: 12mg-- no more than 6 doses in 24 hours	n/a	n/a
Tylenol Cold and Mucus Severe	Syrup	acetominophen 325mg, dextramethorphan HBr 10mg, guaifensein 200mg, phenylephrine HCl 5mg	30ml every 4 hours	n/a	n/a
Maty's cough syrup	Syrup	honey, apple cider vinegar, sea salt, clove, cinnamon, lemon balm, water, glycerin, lemon peel, marjoram, cayenne pepper	1-2 teaspoons as needed	n/a	n/a
Robitussin DM Max Cough and Chest Congestion	Syrup	dextramethorphan HBr 20mg, guifenesin 400mg	20ml every 4 hours	n/a	n/a
Chloraseptic max sore throat spray	Spray	phenol 1.5%, glycerin 33%	apply to affected area spit out after 15 seconds, use every 2 hours	n/a	n/a
Biovanta liquid	Spray	acetyl salicylic acid 6mg, menthol 5mg	apply one spray from each side to the back of the throat, repeat up to once every 30 min as needed	n/a	n/a
Ricola original	lozenge	menthol 4.8mg	dissolve 2 drops in the mouth, repeat every 2 hours	starch syrup, sugar	1:0.75
Halls Relief Honey Lemon	lozenge	menthol 7.5mg	dissolve 1 drop in the mouth every 2 hours	glucose syrup, sucralose, sucrose	1:0.75
Vicks Vapacool Severe	lozenge	menthol 20mg	allow one drop to dissolve in mouth, repeat as necessary	corn syrup	1:1
Ludens	lozenge	pectin 2.8mg	allow one drop to dissolve in mouth, repeat as necessary	corn syrup, sucrose	1:0.75

Coldeeze	lozenge	zincum gluconicum 2X (13.3mg zinc)	dissolve in mouth, repeat every 2-4 hours as needed	corn syrup, honey, glycine, sucrose	1:0.75
Cepacol Instamax	lozenge	benzocaine 15mg, menthol 20mg	1 lozenge every 2 hours	isomalt, maltitol, propylene glycol	1:1.4
Zarbees 96% honey cough soothers Natural Lemon Menthol	lozenge	english ivy leaf extract 20mg	dissolve one in mouth, repeat as needed	honey	1:0.6
Fisheman's Friend	lozenge	menthol 10mg	allow 1 lozenge to dissolve in mouth, repeat every 2 hours as needed	dextrin, licorice, sugar, tagacranth	1:1
Biovanta lozenge	lozenge	acetyl salicylic acid 6mg, menthol 5mg	allow 1 lozenge to dissolve in mouth, repeat up to every 30 minutes as needed	isomalt	1:1.4

Histological analysis confirms extensive tissue damage as measured by TEER.

Following the 48-hour experiment depicted in Fig 6, the organotypic respiratory tissues were fixed in 10% paraformaldehyde, embedded in parrafin, and sectioned into 5um thick sections taken from the center of each section after it was cut in half. The sections were then treated for hemotoxylin and eosin (H&E) staining to observe any histological changes. The average thickness of the untreated sections was 55-60um from the top of the cilia to the bottom of the basal cell layer. The groups treated with the most common over the counter sore throat products exhibited marked loss of cilia and pseudostratified columnar epithelial cells. In most cases, the columnar epithelial cells showed atrophy which decreased the thickness of the epithelia to about half of control values.

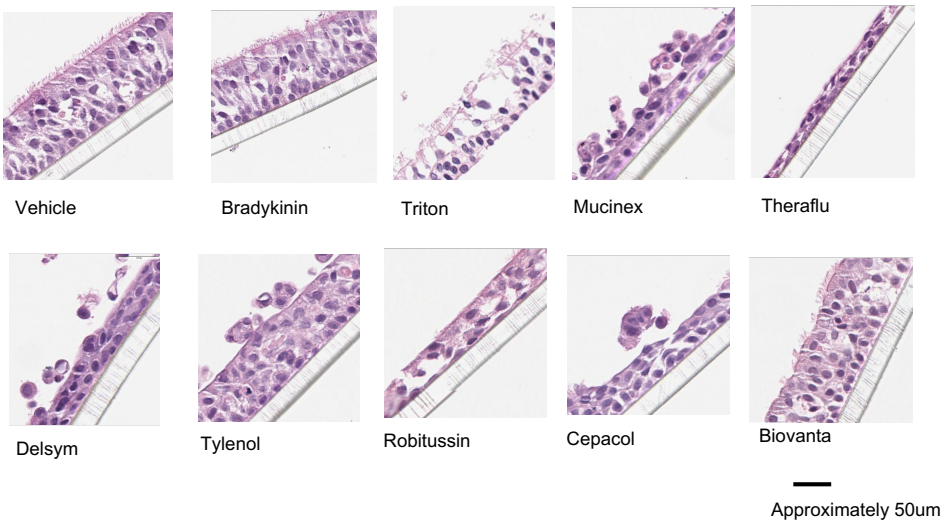

Figure 7 Histological analysis of the tissues analyzed in the double-blind study shown in figure 6. In all of the tissues, except those treated with saliva buffer (vehicle), bradykinin, or Biovanta, marked histological damage was seen in response to treatment. After fixation and embedding in parrafin, sections were cut into 5um thick section and stained with hemotoxylin and eosin.

Discussion

Despite the plethora of over the counter (OTC) products and supplements available to the US consumer, none have shown efficacy for sore throat in clinical studies. A Cochrane meta-analysis

found that non-steroidal anti-inflammatory drugs (NSAIDS) such as aspirin when taken orally are "somewhat effective" in relieving the discomfort caused by a cold (20). Considering the mechanism by which bradykinin induces inflammation through the COX 1, COX 2 pathway, it is surprising that systemically administered NSAIDs have not proven to be more effective. Perhaps, they need to be applied locally or at different dosages. Indeed, we found that there is a small therapeutic window at which aspirin decreases inflammation and above which it becomes inflammatory. At the therapeutic dose, we found that ASA and an ASA-based formula blocked the bradykinin-induced increase in PGE2. We expected to also see a decrease in IL-8 but this was not consistently observed. It is possible that the 24 and 48 hour time-points we used were too soon, or alternatively, it is possible that the bradykinin-induced increase in IL-8 is due to increased gene expression and therefore long-lived. At a dose 10-fold higher than the therapeutic dose, aspirin caused a 6-fold increase in PGE2 above baseline, a 15-fold increase in IL-8 above baseline, and a significant decrease in TEER, indicating membrane damage. Our results are consistent with a previous report of aspirin effectively countering bradykinin only at intermediate concentrations (21).

There are several reports suggesting that bradykinin plays an important role in the inflammation that follows the infection with SARS-CoV-2 (22, 23). Bradykinin levels can be elevated in SARS-CoV-2 patients due to the dysregulation of the angiotensin-converting enzyme levels (23). While it remains to be seen whether blocking bradykinin activity will be useful during SARS-CoV-2 infection, aspirin treatment was reported to be beneficial in SARS-CoV-2 patients (24), warranting a closer examination of the possible effects that other aspirin-based treatments could have on the prevention and treatment of SARS-CoV-2 infection.

A dose-dependent effect of aspirin has been detected in several different physiological systems (25). Aspirin is a non-selective COX-inhibitor, meaning that it inhibits both COX-1 (which is thought to be anti-inflammatory) as well as COX-2 (which is thought to be pro-inflammatory). To have an anti-inflammatory effect, a delicate balance must be reached in order to specifically inhibit COX-2 without excessively inhibiting COX-1. However, respiratory epithelial cells predominantly express COX-2, therefore this hypothesis may not hold true in this system (14). A more likely explanation for respiratory cells, given that we also see an increase in IL-8 when respiratory epithelia are treated with NS-398 (a COX-2 specific inhibitor), is that by inhibiting COX enzymes, arachidonic acid is shunted to producing leukotrienes.

Aspirin is already approved as an OTC for sore throat (internal analgesic), yet there are no throat sprays containing aspirin on the market. We have developed a formula containing aspirin and several other food-grade nutritional supplements which act synergistically to help the aspirin act locally and reduce inflammation and pain caused by bradykinin. Additionally, we found (to our surprise) that when measured against leading brands, this formula not only performed better but was much less inflammatory. We believe one reason for the inflammatory effects of the leading products is osmotic stress on the respiratory tissues. To our knowledge, the osmotic pressure of these products on respiratory epithelia has not been measured before. We observed significant decreases in TEER, indicating membrane perforation. We also observed liquid moving from the basolateral surface of the air-liquid interface to the apical surface (data not shown).

In addition, we observed increases in IL-8 in some cases. According to previous reports, higher osmolarities result in the secretion of proinflammatory cytokines (Interleukin-8, Interleukin-6, Interleukin-1β and Tumor Necrosis factor-α) (26). Prior research shows that a simple and primitive stress condition such as hyperosmolarity provides a complete but specific signal for IL-8 gene expression and synthesis in primary human monocytic cells (27).

IL-8 is a proinflammatory cytokine that is upregulated by different cellular stress stimuli in order to control the extent of neutrophil infiltration (28). Because of its importance, human cells are characterized by their marked capacity for varying the expression levels of IL-8 (28). The expression of IL-8 is regulated at both transcriptional and posttranscriptional levels (29), and the main MAPK pathways (p38, MEK1/2, and JNK) play a significant role in the release of IL-8 during the inflammatory process (16).

We have shown several instances of IL-8 increase by different possible inflammatory processes. One possible mechanism is the bradykinin-PGE2 pathway, another possible pathway is via leukotrienes when arachidonic acid is prevented from producing COX enzymes. Finally, it is possible that the increased IL-8 we observed following treatment with various sore throat products was due to osmotic pressure.

Epithelial cells are attached to their neighbors by cell-cell junctions, including tight junctions (found in the apical part of the lateral epithelial membrane), adherens junctions, gap junctions and desmosomes. These structures link with the cellular cytoskeleton via numerous adaptor proteins and form an impermeable mechanical barrier. They also maintain an apical to basal ionic gradient which facilitates directional secretion of many substances, enables communication between adjacent cells and regulate intercellular transport of soluble factors. Hyperosmotic solutions cause opening of tight junctions which increase the permeability of the paracellular pathway (30). Hyperosmotic solutions containing NaCl and mannitol cause changes in mucosal epithelia cell shape and thus open tight junctions (31).

Taken together, we believe the integrity and inflammatory state of the respiratory membrane epithelium is an important factor to consider in the prevention and treatment of respiratory disease. There are several inflammatory pathways known to be involved and clearly more to be discovered. From our observations, there seem to be several pathways involved in the control of IL-8 expression, including PGE2 and perhaps osmotic stress. Although we were not able to consistently decrease IL-8 levels, we managed to reduce PGE2 levels in respiratory epithelia, and to at least keep IL-8 levels in check. We were surprised to learn that many common OTC products on the market for cold symptoms such as sore throat, greatly exacerbate IL-8 levels. Ongoing studies will determine if this is due to osmotic stress or some other inflammatory signal. In either case, it is highly concerning given the key role IL-8 plays in exaggerating the immune response, thus increasing the likelihood of a “cytokine storm”.

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