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Effects of BNO 1016 on ciliary transport velocity and cell culture surface liquid height of sinonasal epithelial cultures

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Abstract

Background: BNO 1016 is an ethanolic extract of a mixture of five herbs that has been sold in different formulations for decades in the European market and more recently, in the United States market as an over-the-counter treatment for rhinosinusitis. Previous studies indicated activation of chloride secretion and increase in ciliary beat frequency by BNO 1016 but the functional consequences on mucociliary transport velocity and airway surface liquid homeostasis are unknown. This study intends to examine the effects of BNO 1016 on these properties in vitro.

Results: Human sinonasal epithelial cells were grown at an air-liquid interface, with addition of BNO 1016 basolaterally in each experiment. Polystyrene fluorescent microspheres were added to the apical surface of the culture, and distance traveled across the surface of the culture over a fixed time period was measured using live imaging. BNO 1016 concentrations of 50 μg/ml and 500 μg/ml were tested. Basolateral application of compound resulted in a non-dose-dependent increase in culture surface liquid height compared to controls at 30 min, and this effect persisted through the one-hour duration of the experiment ($p < 0.01$). Basolateral application of BNO 1016 also resulted in a non-dose-dependent increase in microsphere transport velocity at 45 and 60 min following compound application ($p < 0.01$).

Conclusions: Basolateral application of BNO 1016 at a concentration mimicking post-ingestion serum levels appears to elicit increases in cell culture surface liquid height and mucociliary clearance, as assessed by microsphere transport velocity. These properties can potentially be leveraged for therapeutic efficacy in diseases affecting mucus production and mucociliary transport.

Keywords: Sinusitis, Cilia, Therapeutics, Sinupret, BNO 1016

Background

Chronic rhinosinusitis (CRS) is a very common disease with debilitating symptoms, including rhinorrhea, nasal obstruction, facial pressure, headache, and hyposmia. The etiology of CRS is not entirely known, but is likely a combination of environmental exposures, microbiome perturbation, genetics, and dysfunctional mucociliary clearance (MCC) [1]. Effective MCC requires a coordinated movement of airway surface liquid (ASL) by cilia to maintain a healthy sinonasal tract. When this function is impaired, there is stasis of sinonasal secretions and significant local inflammation, which can be inciting or perpetuating factors in sinusitis [2, 3]. With associated increases in clearance of pathogens, particulate matter, and inflammatory milieu, drugs that improve MCC can serve as alternative therapies or as adjuncts with conventional treatments.

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BNO 1016 is an extract of five herbs: gentian root (Gentianae radix), primrose flowers (Primulae flos), elder flowers (Sambuci flos), common sorrel herb (Rumicis herba) and vervain herb (verbenae herba). BNO 1016 extract formulations have been extensively studied, with several randomized trials demonstrating therapeutic benefit in rhinosinusitis [4–6]. It is currently used for a variety of respiratory conditions to reduce acute and chronic sinonasal or lower airway symptoms, such as those observed in bronchitis [7]. These positive clinical studies are also backed by physiologic data showing mucolytic, secretomotoric, anti-inflammatory, and antibacterial effects of BNO 1016 as well as other preparations of this herb combination, including powdered non-extracted forms [8–11]. In mouse nasal epithelial cells, airway surface hydration occurs in response to addition of BNO 1016 through chloride ion secretion through the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) channel [8, 12]. This transepithelial ion movement causes an increase in mucus layer depth due to accompanying fluid shifts. Conversely, dysfunction of the CFTR channel in cystic fibrosis results in thick and difficult to clear secretions with an accompanying reduction in rate of MCC. For this reason, chloride ion secretagogues are increasingly used for treatment of diseases with inadequate MCC, including COPD, asthma, and CRS [13].

Work in human cells has demonstrated that BNO 1016 increases ciliary beat frequency and chloride efflux in respiratory epithelium [8, 9, 12]. Increased ASL hydration can be of therapeutic benefit in many circumstances [9], and even small increases in ciliary beating can have profound effects on MCC. However, functional consequences of BNO 1016 on mucociliary transport velocity and ASL homeostasis in human sinonasal epithelial cells are currently unknown, and previous studies have used dosages that are higher than concentrations reached in the serum following oral ingestion. The goal of the present study was to determine if prior murine and human findings translated into increased mucociliary transport, even at a lower dose, as assessed by "bead velocity" and cell culture surface liquid hydration through measuring cell culture surface liquid height in primary human upper respiratory epithelial cultures.

Methods

Reagents and experimental solutions

Phosphate-buffered saline (PBS) and Hank's Balanced Salt Solution (HBSS) were purchased from Sigma Aldrich. BNO 1016, in a formulation brand named as Sinupret® ethanolic dry extract was obtained from Bionorica. It consists of gentian root (Gentianae radix), primrose flowers (Primulae flos), elder flowers (Sambuci flos), common sorrel herb (Rumicis herba) and vervain herb (verbenae herba) in a 1:3:3:3:3 ratio. This dry extract was initially dissolved in 100% DMSO to obtain a concentration of 100 mg/ml. The resulting suspension was sonicated in an ultrasonic bath (HF 35 kHz) at room temperature for 30 min, and then vortexed for an additional 2 min. The suspension was centrifuged for 10 min at 3000 g at room temperature, and the supernatant was removed to be used for all BNO 1016 preparations at the concentrations used in experiments.

Human tissue acquisition

Tissue was obtained with informed consent and Institutional Review Board approval. Patients undergoing sinonasal surgery at the Department of Otorhinolaryngology, Division of Rhinology at the University of Pennsylvania and the Philadelphia Veterans Affairs Medical Center were recruited and tissue was taken from residual clinical material at surgery and processed immediately. Patients were excluded if they had antibiotic, corticosteroid, or antibiologic use within 1 month of surgery, or a history of systemic disease such as Wegener's or Cystic Fibrosis.

Air-liquid Interface (ALI) cultures

We have previously described the culture of human nasal epithelial cells at an ALI [14, 15]. Briefly, human sinonasal epithelial cells were enzymatically dissociated and grown with medium containing DMEM/Hem's F-12 and bronchial epithelial based medium (Clonetics), in addition to 100 U/ml penicillin and 100 μg/ml streptomycin for 7 days. Following this, cells were trypsinized and placed on porous polyester membranes in transwell cell culture inserts (Transwell-clear, 12-mm diameter, 0.4-μm pores; Corning). These inserts were coated with 100 μl of coating solution (BSA 0.1 mg/ml; Sigma-Aldrich), type 1 bovine collagen (30 μg/ml; BD), and fibronectin (10 μg/ml; BD) in LHC basal medium (Invitrogen). After 5 days, the apical compartment was cleared and the epithelium was allowed to differentiate using a medium of 1:1 DMEM (Invitrogen) and BEBM (Clonetics, Cambrex), with the Clonetics complements for hEGF (0.5 ng/ml), epinephrine (5 μg/ml), hydrocortisone (0.5 μg/ml), BPE (0.13 mg/ml), insulin (5 μg/ml), triiodothyronine (6.5 μg/ml), and transferrin (0.5 μg/ml), supplemented with 100 UI/ml penicillin, 100 g/ml streptomycin, 0.1 nM retinoic acid (Sigma-Aldrich), and 10% FBS (Sigma-Aldrich) in the basal compartment. For each experiment, three ALI cultures each obtained from three different human patients were utilized. Experiments were performed in paired fashion to control for inter-donor variability.

Bead transport velocity measurements

Mucociliary transport velocity was measured using in vitro fluorescent bead tracking using 2 μm polystyrene fluorescent microspheres (0.0025% by weight in 30 μl) that were added to the apical surface of the cultures after copious washing with PBS to remove mucus clumps as previously described [14]. To obtain optimal imaging of the beads, the filters containing the ALI cultures were cut out of the support and placed directly on a cover slip. Test solutions were added to the apical side but as the filters were cut out, the solution in essence is in contact with both basoateral and apical surface of the cells. Beads were imaged using an inverted Nikon TE2000E epifluorescence microscope (20 × 0.5 NA PlanFluor objective) equipped with a 12-bit QImaging camera and computer running ImageJ (W Rasband, NIH) and μManager. A bead streak had to have a visible beginning and ending (see results), within the field of view, to be included in the statistical analysis of the data. The distances beads traveled were calculated using ImageJ within-application measurements and distance was divided by time to calculate a transport velocity for each bead. Either an ND4 or ND8 filter was used for bead-tracking experiments, depending on the thickness of the individual epithelial culture and resulting brightness of the beads.

Cell culture surface liquid (ASL) depth measurements

ASL was labeled with 10 μl of Texas red dextran (10,000 MW; 2 mg/ml) in PBS followed by 24 h incubation at 37 °C in a humidified incubator to allow ALIs to absorb excess fluid and reach ASL homeostasis as previously described [16]. Upon removal from the incubator, cultures were immediately overlayed with 100 μL perfluorocarbon (PFC-77) to prevent ASL evaporation. The apical side of the cultures was otherwise unmodified; drug additions were made only basolaterally. Confocal images were then taken using a 60x (1.0 NA) water immersion objective with 0.33 μm step size.

Statistical analysis

All statistical analyses were perfomed using GraphPad Prism. All tests were two-tailed, and $p < 0.05$ was the curoff for statistical significance. Statistical analysis of the arithmetic means of the velocities or ASL height derived from each culture was performed using an ANOVA, followed by Dunnett's test comparing each treatment group within a test item concentration range to the vehicle treated samples.

Results

We evaluated BNO 1016 for effects on mucociliary transport velocity and cell culture surface liquid in sinonasal epithelium cell cultures. The extract was prepared at concentrations of 50 μg/ml and 500 μg/ml, and dissolved in 0.5% DMSO diluted with phosphate-buffered saline. These concentrations were specifically chosen by Bionorica SE to mimic serum concentrations of BNO 1016 following oral ingestion. Mucociliary transport velocity was assessed in proxy utilizing polystyrene fluorescent microspheres added to the surface of the cultured sinonasal epithelial cells. Sample results are shown in Fig. 1, demonstrating bead travel distance before and after application of extract. Bead distance travelled over a fixed 50 mSec time period allowed for calculation of changes in transport velocity, and between 20 and 25 bead measurements were made at each time point for each culture. Each experiment was repeated three times with unique ALI cultures. Application of both 50 μg/ml and 500 μg/ml BNO 1016 resulted in significant increases in mucocilary transport speed, expressed as a percentage change from baseline speed, at 30, 45, and 60 min following application ($p < 0.01$, $n = 3$ cultures per condition, Fig. 2). In the bead tracking paradigm, DMSO causes a persistent depression (≈20%) in bead velocity, which is noted at the earliest time point (15 min) post-application of BNO 1016.

Prior work had demonstrated that BNO 1016 increased chloride ion flux across the epithelial membrane [9], resulting in paracellular transport of water and increased hydration of the mucus barrier. To test this hypothesis, cell-culture surface liquid height changes were assessed using confocal microscopy of Texas red dextran added to the apical surface of the ALI prior to basolateral application of BNO 1016. Experiments were repeated 3 times with 3 unique ALI cultures and 5 cell-culture surface liquid height measurements per culture. With addition of both 50 μg/ml and 500 μg/ml BNO 1016 a time dependent significant increase in cell-culture surface liquid depth was observed between 30 and 60 min ($p < 0.01$, $n = 3$ cultures per condition, Fig. 3).

Discussion

Glucocorticoids and antibiotics are the mainstay of standard treatment for respiratory inflammatory/infectious diseases. However, patients and providers are increasingly exploring alternative and adjunctive therapies, with over one-quarter of patients using some type of herbal preparation for treatment of sinusitis [17]. BNO 1016, also known as Sinupret extract®, is an over the counter supplement that has been used for many years in respiratory ailments, including sinusitis and bronchitis. While there are several proposed and experimentally evaluated mechanisms of action, it is known that BNO 1016 increases ciliary beat frequency and chloride efflux in respiratory epithelium [9]. The objective of the current study was to advance these findings and determine if BNO 1016 accelerated mucociliary clearance and

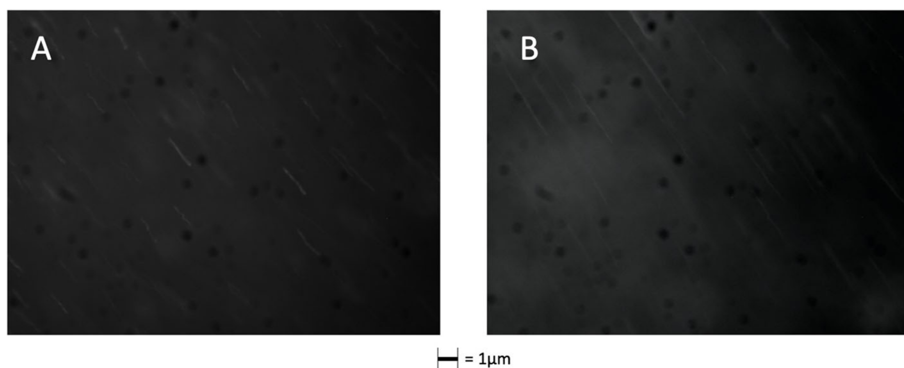

Fig. 1 In vitro fluorescent bead tracking as a proxy for mucociliary transport velocity. White streaks represent distance beads travelled over 50mSec at baseline (**a**) and 60 min following application of BNO 1016 (**b**)

increased mucus hydration in human tissues. Furthermore, we used a lower dosage of BNO 1016 than that used in previous experiments [12], to better mimic serum concentrations following oral ingestion of extract. Utilizing primary human sinonasal epithelial cultures, basolateral application of BNO 1016 significantly increased MCC and cell culture surface liquid height to comparable degrees at 50μg/ml and 500μg/ml in a time-dependent (≈30 min) manner. These properties may explain the clinical efficacy of BNO 1016 in respiratory diseases involving increased mucus production and decreased clearance, and can potentially be further leveraged for therapeutic efficacy.

Effective MCC is dependent on ciliary beating and appropriate ASL composition. In the present experiment, the increases in MCC were observed even in an environment with submerged apical cilia, demonstrating that this process is at least partially independent from changes in cell culture surface liquid hydration, and it is not solely chloride efflux that is responsible for increases in efficiency of ciliary movement. These independent mechanisms likely act synergistically to alleviate thick mucus overproduction and mucus stasis, common sequelae of respiratory infection and inflammation. Alleviation of these symptoms has marked impact on sinonasal quality of life [18]. In addition to these actions, BNO 1016 as well as non-extracted powder formulations of the herbal preparation have been shown to have antiviral activity in vitro and in vivo [10, 19] These properties are likely related to demonstrated efficacy of BNO 1016 as a treatment option in placebo-controlled studies for acute sinusitis and CRS [5, 20].

BNO 1016 is primarily administered orally and thus intended physiologic effects must function in a systemic fashion. While previous studies have examined the effects of apical application of BNO 1016, a basolateral application is more consistent with cellular exposure *in vivo*. Alternatively, topical therapies are better evaluated with apical addition of compound. In the present study, all ASL changes were observed with basolateral application of compound, while changes in mucociliary transport velocity were observed with application of

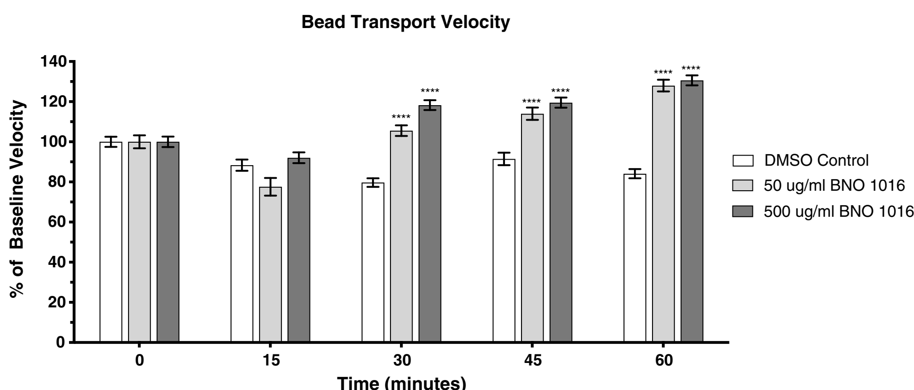

Fig. 2 Effects of BNO 1016 on bead transport velocity. Application of BNO 1016 resulted in significant ($p < 0.01$) acceleration of bead transport at 30, 45 and 60 min post application ($n = 3$ cultures per condition). Error bars on graph represent SEM

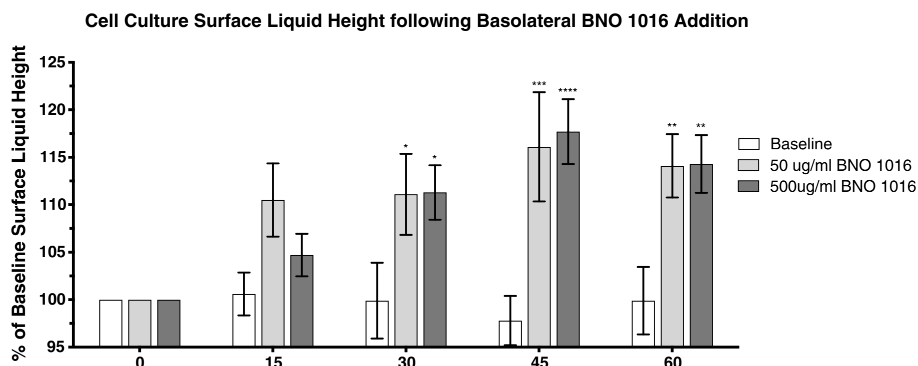

Fig. 3 Basolateral application of BNO 1016 significantly increases cell culture surface liquid height in a time dependent manner ($n = 3$ cultures per condition). Error bars on graph represent SEM. (*$p < 0.05$, **$p < 0.01$, **$p < 0.005$)

compound to the basolateral and apical sides of the ALI simultaneously, as the interface was fully submerged to fulfill the necessary experimental conditions. Another source of potential confounding is the effect of DMSO on mucociliary clearance, as control conditions with only DMSO showed a persistent depression of approximately 20% in bead velocity after exposure. This acts in a directly antagonistic fashion to BNO 1016, but it is unclear if BNO 1016 without DMSO would elicit mucociliary transport velocity increases even beyond those observed in our experimental paradigm. However, DMSO was chosen in this case and was necessary as the extraction solvent given the potential toxicity of ethanol to the cell cultures given the concentrations of extract utilized.

Conclusions

Overall, this study builds upon prior experimentation with mouse and human sinonasal epithelial cells, and demonstrates that BNO 1016 elicits increases of cell culture surface liquid height and mucociliary transport velocity, as assessed by fluorescent bead velocity, when applied basolaterally in vitro in a fashion comparable to systemic drug exposure. Sinonasal infection and inflammation is often associated with thickened mucus and mucus stasis, two symptoms that can cause severe reductions in quality of life of affected patients. The observed in vitro changes with BNO 1016 application might serve as a surrogate in accounting for health benefits of herbal medicinal products made from these herbal substances in clinical studies.

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Authors' contributions
ADW, IWM, VT, and NNP drafted the work, acquired data, interpreted data, have approved the submitted version, and are accountable for their contributions. CCL and ECK critically revised the work, interpreted data, have approved the submitted version, and are accountable for their contributions. DWK, JNP, NDA, and NAC made substantial contributions to the conception of the work, acquired data, substantively revised the work, have approved the submitted version, and are accountable for their contributions.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Tissue was obtained from patients undergoing sinonasal surgery at the Hospital of the University of Pennsylvania with informed consent and approval from the University of Pennsylvania Institutional Review Board.

Consent for publication
Not applicable.

Competing interests
The corresponding author (NAC) has a consultant relationship with Bionorica SE.

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