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The Bioflavonoid Compound, Sinupret, Stimulates Transepithelial Chloride Transport In Vitro and In Vivo

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Objectives/Hypothesis: Dehydration of airway surface liquid (ASL) disrupts normal mucociliary clearance in sinonasal epithelium leading to chronic rhinosinusitis. Abnormal chloride (Cl^-) transport is one mechanism that contributes to this disorder, as demonstrated by the disease cystic fibrosis. Identifying safe compounds that stimulate transepithelial Cl^- transport is critical to improving hydration of the ASL and promoting mucociliary transport. Sinupret (Bionorica, LLC, San Clemente, CA), a combination of naturally occurring bioflavonoids, is a widely used treatment for respiratory ailments in Europe. However, the effects of Sinupret on target respiratory epithelium have yet to be fully investigated. The present study evaluated the mechanisms underlying this bioflavonoid therapeutic on transepithelial Cl^- transport in respiratory epithelium.

Study Design: In vitro and in vivo investigation.

Methods: Well characterized murine nasal septal epithelial (MNSE) cultures, and murine nasal potential difference (NPD) techniques were used to evaluate the effects of Sinupret on Cl^- secretion.

Results: The change in Sinupret-stimulated current (ΔI_{SC} expressed as $\mu A/cm^2$) in MNSE, representing Cl^- secretion, was significantly increased when compared to controls (19.04 ± 1.67 $\mu A/cm^2$ vs. 1.8 ± 0.35 $\mu A/cm^2$, respectively; $P = .00005$). Transepithelial Cl^- transport measured in the murine NPD in vivo assay ($n = 42$) was also significantly enhanced when compared to controls (-0.8 mV vs. -0.9 mV; $P = .0004$). Importantly, Sinupret-stimulated Cl^- transport was substantially more robust in vivo than forskolin, a compound among the strongest known cystic fibrosis transmembrane conductance regulator activators (-3.8 mV vs. -1.65 mV; $P = .01$).

Conclusions: Sinupret strongly activates transepithelial Cl^- secretion through a mechanism known to hydrate the ASL of respiratory epithelium. This is one means by which the medication is likely to exert therapeutic benefit.

Key Words: Transepithelial ion transport, Sinupret, cystic fibrosis transmembrane conductance regulator, chronic sinusitis, chloride secretion, murine nasal culture, mucociliary clearance, nasal potential difference.

Level of Evidence: 1a.

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INTRODUCTION

Respiratory sinonasal epithelium is a highly regulated inert barrier that is primarily responsible for the mucociliary apparatus that eliminates inhaled pathogens from the nose and paranasal sinuses.[1] Mucociliary clearance is dependent, in part, on the biological properties of the airway surface liquid (ASL). The ASL is modified through vectorial transport of ions, such as chloride (Cl^-).[2] Dysfunctional Cl^- transport results in dehydration of the ASL and mucus stasis as demonstrated in the severe lower and upper respiratory disease, cystic

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Dr. Sorscher serves as a consultant for a Birmingham start up company (PNP Therapeutics, Inc.) but is not employed by the company and draws no salary from the company. His consulting includes a role as Director/Officer. He acts as a consultant with the approval of UAB and the UAB CIRB. He is compensated for consulting with stock. This company has no relationship to the current article. Dr. Sorscher and Dr. Woodworth are inventors on a patent submitted regarding the possible activity of chloride secretagogues for therapy of sinus disease (Provisional Patent Application Under 35 U.S.C. §111(b) and 37 C.F.R. §1.53(c) in the United States Patent and Trademark Office). Dr. Woodworth is a consultant for Gyrus ENT, ArthroCare ENT, and is on the GlaxcoSmithKline speaker's bureau.

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fibrosis. Dehydrated, inspissated mucus places patients at risk for bacterial infection and widespread chronic rhinosinusitis (CRS) refractory to medical management.[3] In practical terms, substances that increase vectorial ion transport are being sought that improve hydration of mucinous secretions and increase mucociliary transport.

Compounds that stimulate Cl^- secretion, specifically those that activate the cystic fibrosis transmembrane conductance regulator (CFTR), are an area of active investigation. Multiple flavonoids have been demonstrated to augment gating of the CFTR.[4] Although in vitro studies investigating flavonoid compounds have been promising, in vivo experiments have been limited, due to, for example, rapid metabolism of active Cl^- secretagogues.[5]

Sinupret (Bionorica, LLC, San Clemente, CA) is an herbal medicinal product that has been widely employed as a mucoactive agent for sinusitis or acute and chronic bronchitis in Germany and other European countries for many years. In conjunction with antibiotics, the medication has been shown to reduce the acute symptoms and signs of sinusitis.[6] Sinupret contains extracts of five herbs: elder (*Sambucus nigra, Caprifoliaceae)* flowers, primrose (*Primula veris, Primulaceae)* flowers with calyx, common sorrel (*Rumex acetosa, Polygonaceae*) herb, European vervain (*Verbena officinalis, Verbenaceae*) herb, and gentian (*Gentiana lutea, Gentianaceae*) root. The effects of this agent are believed to be related to its constituent, naturally occurring bioflavonoids. Perhaps based in part on the limited success of conventional therapy for CRS and the debilitating nature of the condition, herbal medicines are becoming increasingly popular and are frequently used by adults with rhinosinusitis.[7] Thirty-two percent of patients with CRS have used herbal therapy alone or as adjunctive treatment for their disease.[8] However, little is known about the cellular mechanisms by which Sinupret or related formulations may confer their well-established clinical benefit.

The purpose of the current study was to evaluate the effect of Sinupret on vectorial Cl^- transport in murine nasal septal epithelial (MNSE) cultures and murine nasal potential difference (NPD) measurements. One hypothesis underlying these experiments is that Sinupret increases vectorial Cl^- transport in these model systems in vitro and in vivo.

MATERIALS AND METHODS

University of Alabama at Birmingham Institutional Animal Care and Use Committee approval was obtained prior to initiation of the study.

Cell Culture

The culture technique of murine nasal septal epithelium differentiated at an air-liquid interface was used as described in our prior studies.[1,9–12] Tissue from genetically identical C57 mice was harvested and grown on Costar 6.5-mm-diameter permeable filter supports (Corning, Kennebunk, ME) submerged in culture medium. The media was removed from the surface of the monolayers on day 4 after reaching confluence, and cells were fed via the basal chamber. A total of 76 MNSE filters were studied when fully differentiated and exhibiting widespread ciliogenesis and transepithelial resistances $>500\ \Omega \cdot cm^2$. Differentiation and ciliogenesis occurred in all cultures within 10 to 14 days.

Electrophysiology

Solutions and chemicals. The bath solution contained (in mM) 120 NaCl, 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.8 K_2HPO_4, 1.2 $MGCL_2$, 1.2 $CaCl_2$, and 10 glucose. The pH of this solution is 7.3 to 7.4 when gassed with a mixture of 95% O_2-5% CO_2 at 37°C. Chemicals were obtained from Sigma (St. Louis, MO). Each chemical was prepared as a 1000× stock and used at 1× in the Ussing chamber. All experiments were performed with low Cl^- (6 mM) in the mucosal bath. Pharmacologic preparations were as follows: Sinupret (1 μg/mL, 100 μg/mL, 1000 μg/mL, 2.5 mg/mL), amiloride (100 μM), forskolin (2 μM), and INH-172 (10 μM). Sinupret tablets were dissolved in dimethylsulfoxide (DMSO) followed by removal of insoluble matter by filtration. The concentrations noted do not account for removal of insoluble particulates (i.e., concentrations above 2.5 mg/mL could not be obtained using this method).

Short circuit (I_{SC}) measurements. Transwell inserts (Costar; Corning Inc. Life Sciences, Lowell, MA) were mounted in a modified, vertical Ussing chamber to investigate pharmacologic manipulation of ion transport. Monolayers were continuously monitored under short-circuit conditions following fluid resistance compensation using automatic voltage clamps (VCC 600; Physiologic Instruments, San Diego, CA). Transwell filters were mounted in batch solutions warmed to 37°C, and each solution continuously gas lifted with a 95% O_2-5% CO_2 mixture. DMSO control solutions were tested for comparison. The I_{SC} was assessed at one current measurement per second. By convention, a positive deflection in I_{SC} was defined as the net movement of anions in the serosal to mucosal direction.

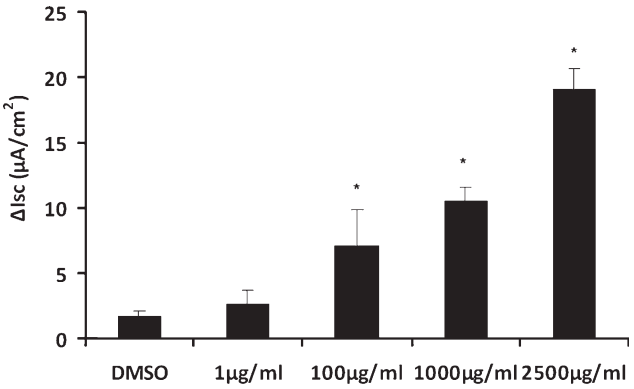
Nasal potential differences. A three-step protocol was used, as described previously.[13] First, nasal cavities of anesthetized mice (C57) were perfused with Ringer's solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and amiloride 100 μM (pH 7.3). Next, a low-chloride–containing solution was perfused (mM N-methyl-D-glucamine [NMDG], 6 mM Cl^-, pH 7.3). Cl^- channels were activated with Sinupret 2.5 mg/mL or forskolin 20 μM in the perfusate. DMSO vehicle in the perfusate served as a control. Because of the continuous presence of amiloride (50 μM) and the complete replacement of Na^+ with a membrane-impermeant cation (140 NMDG in the perfusion solution), hyperpolarization reflects Cl^- secretion rather than cation absorption. All traces were interpreted in a blinded fashion.

Statistical Analysis

Statistical analysis was performed using 2-tailed unpaired *t* test and analysis of variance where appropriate.

RESULTS

Change in Cl^- stimulated current (ΔI_{SC}) was significantly increased when MNSE cultures were exposed to Sinupret compared to vehicle treated (control) monolayers. This was demonstrated in dose-dependent fashion at 1 μg/mL, 100 μg/mL, and 1000 μg/mL, with maximal effects demonstrated at 2.5 mg/mL (ΔI_{SC}, 19.04 ± 1.67 $\mu A/cm^2$) when compared to DMSO (ΔI_{SC}, 1.8 ± 0.35 $\mu A/cm^2$; $P = .00005$) (Fig. 1). Although Sinupret stimulation of Cl^- secretion was very robust, the application of forskolin resulted in a further increase in I_{SC},



* P value < 0.05

Fig. 1. Sinupret stimulates transepithelial Cl^- secretion in a dose-dependent fashion. Maximal ΔI_{SC} was noted at 2.5 mg/mL, the highest concentration investigated.

indicating Sinupret does not maximally activate CFTR-dependent Cl^- transport (Fig. 2). To further confirm that Sinupret stimulated I_{SC} was mediated through CFTR, a separate set of experiments was performed to exclude the role of CFTR channels following Sinupret activation. There was significant inhibition following application of the specific CFTR channel blocker INH-172 (ΔI_{SC}, -16.1 ± 1.3 $\mu A/cm^2$) when compared to vehicle (DMSO) control (ΔI_{SC}, -2.4 ± 0.9 $\mu A/cm^2$; $P = .001$), indicating that CFTR-mediated pathways are stimulated by this formulation (Fig. 3).

Transepithelial Cl^- transport in vivo measured by the murine NPD assay was also significantly enhanced with Sinupret (n = 9; -3.8 ± 1.7 mV) when compared to controls (n = 20; -0.9 ± 1.7 mV, $P = .0004$) (Fig. 4). Importantly, Sinupret-stimulated Cl^- transport was significantly more robust in vivo than forskolin, a very efficient, and perhaps the most potent in vivo CFTR activator currently available (n = 13; -1.65 ± 1.8 mV, $P = .01$).

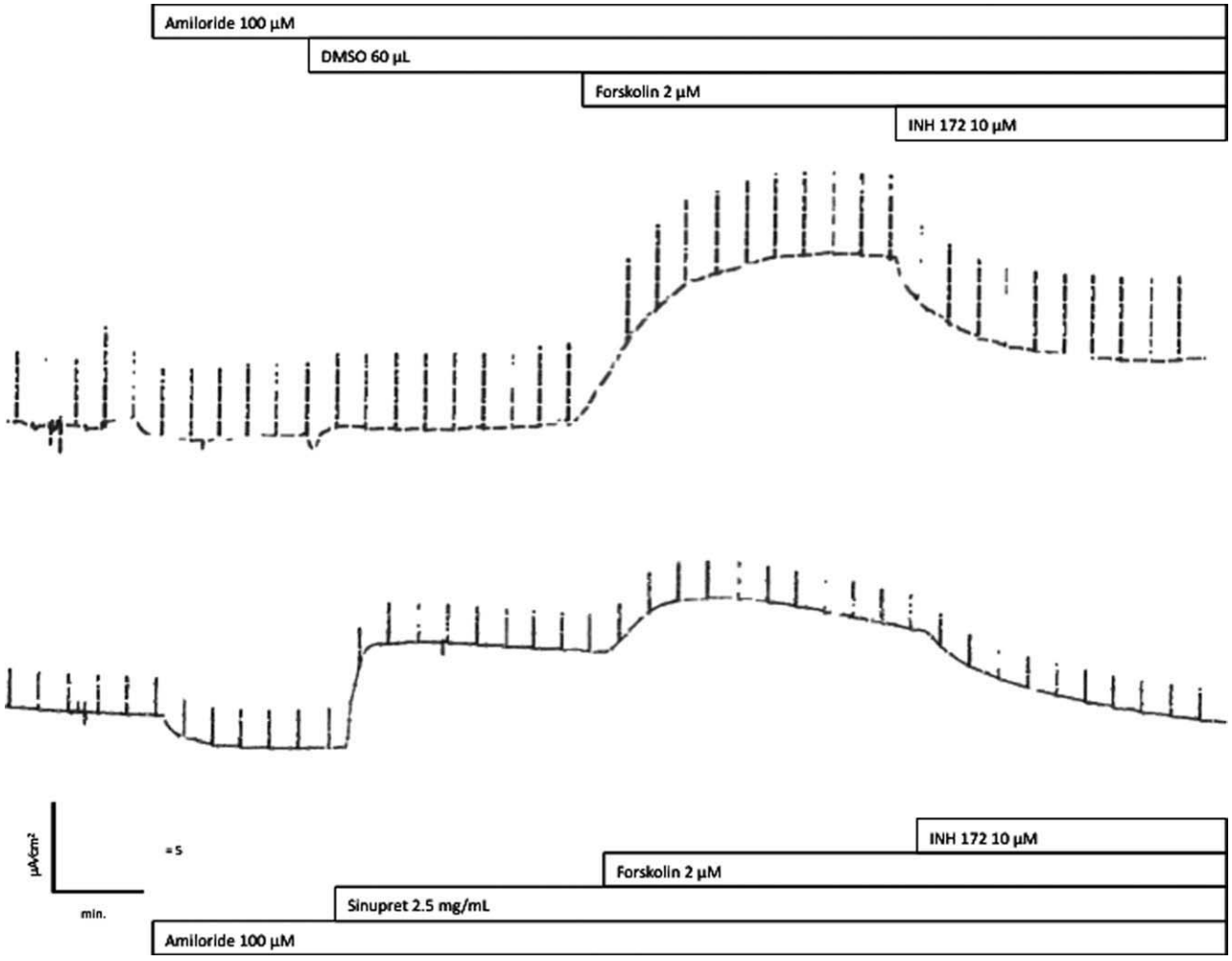


Fig. 2. Representative Ussing chamber analysis of wild type murine nasal septa epithelial (MNSE) cultures treated with Sinupret. Wild type MNSE cells grown on transwell permeable supports were mounted in Ussing chambers under short-circuit conditions and sequentially exposed to amiloride, Sinupret, forskolin, and INH-172. A positive deflection indicates the net movement of an anion from the serosal to the mucosal direction. Sinupret robustly stimulated Cl^- secretion. DMSO = dimethylsulfoxide.

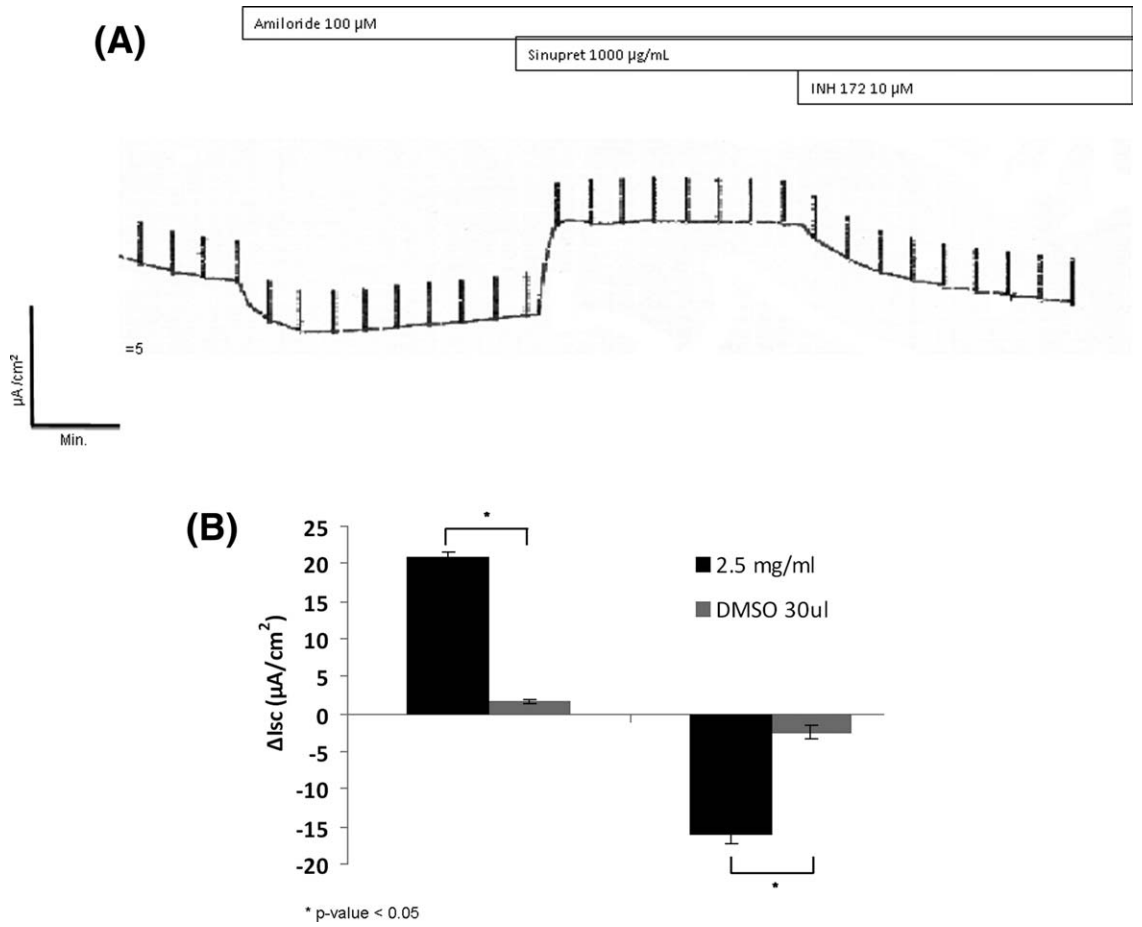


Fig. 3. Sinupret stimulates Cl^- secretion via cystic fibrosis transmembrane conductance regulator (CFTR)-mediated pathways. (A) A negative deflection of I_{SC} following the addition of the specific CFTR blocker INH-172 without forskolin indicates that stimulated Cl^- secretion has been inhibited via CFTR-dependent pathways. (B) There was significant inhibition following application of INH-172 (ΔI_{SC}, -16.1 ± 1.3 $\mu A/cm^2$) when compared to inhibition following dimethylsulfoxide (DMSO) controls (ΔI_{SC}, -2.4 ± 0.9 $\mu A/cm^2$; $P = .001$).

DISCUSSION

Sinupret has been sold in the European marketplace for more than 70 years and has a verified safety profile. Since 2003, the formulation has been available in the United States, primarily via mail order and professional sales. In 2008, Sinupret became available domestically in retail outlets, sold under the trade names Sinupret Plus/Sinupret Adult Strength and Sinupret Syrup for Kids. In Germany, this therapeutic was the most popular cough and cold remedy chosen for self medication in 2006 to 2008, and the tenth most commonly prescribed product, including all prescription medications, in 2003.[14]

Experimental studies have demonstrated that Sinupret and its individual components have mucolytic properties,[15] and prophylactic administration increases resistance to respiratory tract infection by intranasal application of Sendai virus (*Parainfluenza viridae*) in mice.[16] Sinupret also has demonstrated efficacy in several clinical studies of rhinosinusitis. A randomized, placebo-controlled trial involving 31 patients[17] with rhinosinusitis demonstrated significant improvement in radiologic outcomes and headache in patients receiving Sinupret alone. Other clinical trials[18] tested the effects of Sinupret as an adjunctive treatment to antibiotics and/or nasal decongestants for acute rhinosinusitis. All of these studies indicated a higher responder rate when Sinupret was included in the medical regimen, including two trials demonstrating statistical significance over placebo. Although clinical evidence indicates Sinupret could play an important role in the management of rhinosinusitis, there is minimal data regarding the cellular mechanism of action by which this widely used treatment may act.

CFTR is the major Cl^- channel in the apical membranes of respiratory epithelia and is crucial for modulating ASL via salt and water secretion and absorption. CFTR dysfunction results in the severe respiratory disease cystic fibrosis, and is also dysfunctional in a variety of other conditions, including cigarette smoke exposure.[1] Drug discovery efforts have centered on small molecules that target CFTR[19,20] to treat lower respiratory disease. Flavonoids are one such group of molecules with a demonstrable affect on CFTR activity, including CFTR-mediated Cl^- currents.[19,21,22]

In the present study, we evaluated the ability of Sinupret to activate CFTR dependent Cl^- transport in a murine primary cell culture model of sinonasal

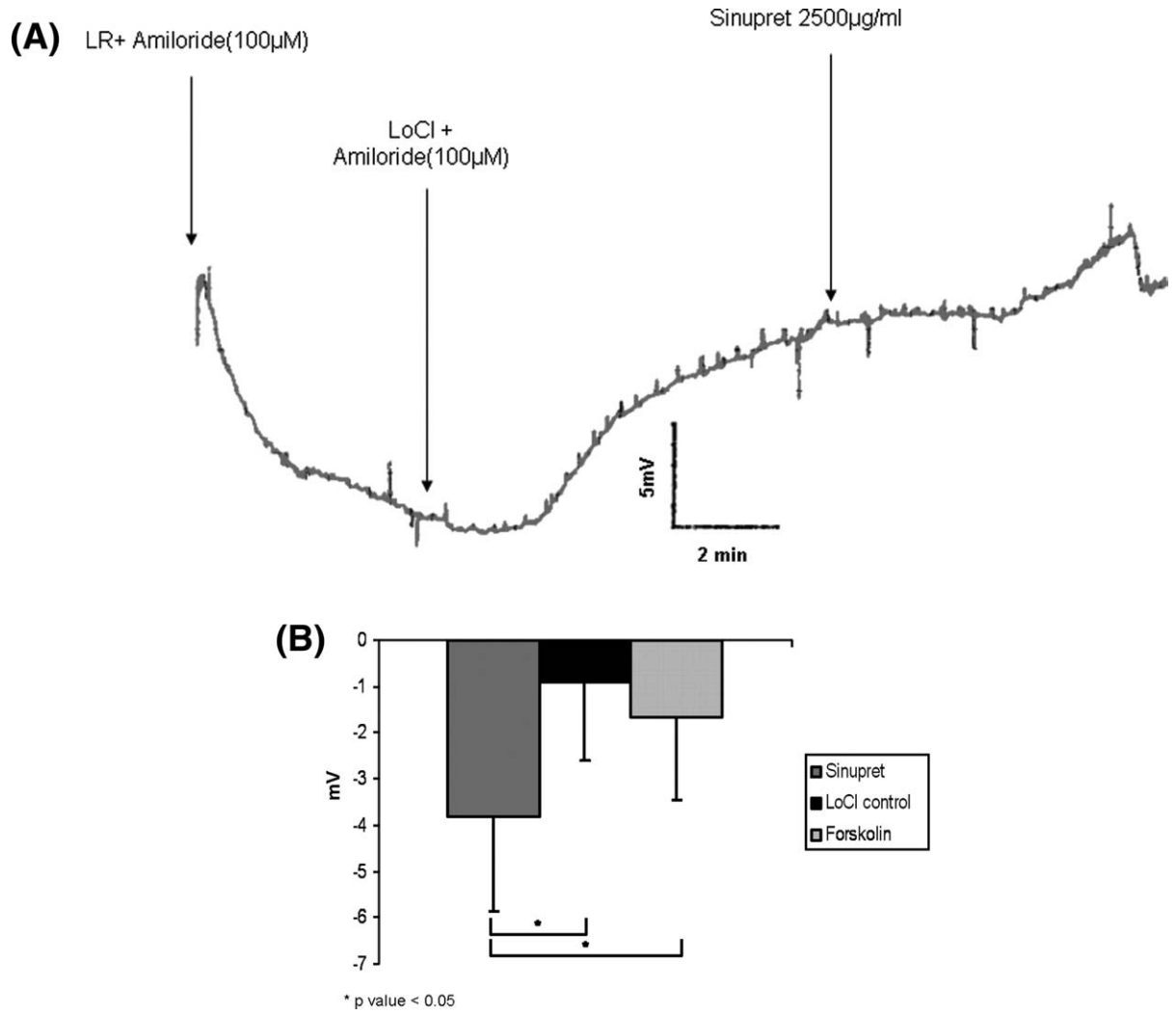


Fig. 4. Sinupret activates cystic fibrosis transmembrane conductance regulator (CFTR)-dependent ion transport across the murine nasal mucosa in vivo. Mice underwent a standardized nasal potential difference (NPD) protocol with the addition of 2.5 mg/mL Sinupret, 20 μM forskolin (or vehicle control), in the final perfusion solution. (A) Representative tracing from C57 mice stimulated with Sinupret in the final perfusate. (B) Summary data of studies described above. Sinupret perfusion resulted in a -3.8 mV mean NPD polarization that was significantly different than that seen in mice receiving vehicle alone, or exposed to forskolin. *$P < .05$.

epithelium and by in vivo murine NPD measurements. Our results indicate Sinupret robustly activates CFTR in upper airway epithelium. The formulation confers a dose-dependent increase in Cl^- secretion with maximal effects demonstrated at 2.5 mg/mL in MNSE cells (Fig. 1). Typically, flavonoids have characteristic stimulatory effects on CFTR at low concentrations and inhibitory effects at higher levels of administration.[21,23] However, we were not able to demonstrate an inhibitory concentration for the formulation in the current study, because Sinupret could not be concentrated beyond 2.5 mg/mL in DMSO.

Administration of Sinupret without forskolin indicates that preactivation with an adenylate cyclase activator is not necessary for function as a Cl^- secretagogue. Inhibition with the specific CFTR inhibitor INH-172 also demonstrated significant reduction in I_{SC} when compared to controls, establishing that Sinupret exerts its effects, at least in part, through interaction with CFTR dependent pathways. Whether this occurs through a protein kinase A-dependent mechanism or direct binding to the CFTR channel itself (as suggested previously for flavonoid activators of CFTR), is not known and will require further investigation.

Sinupret-stimulated Cl^- transport was significantly more robust in vivo than forskolin, which is an agent that is among the strongest known CFTR activators in murine nasal tissue. Because the NPD is measured following topical administration, our data suggest Sinupret could be a very effective stimulator of fluid/electrolyte secretion and mucociliary clearance (MCC). These studies thus provide new insight into the important and novel mechanisms underlying efficiency of a widely used therapy for sinusitis. Our data also provide new evidence that topical administration of CFTR activators—including potentiator agents that have recently received attention as cystic fibrosis therapeutics[24]—might also serve as local or regional treatments for individuals

afflicted with decreased MCC from infectious or inflammatory processes, including chronic and debilitating sinus disease.

CONCLUSION

Mucus viscosity is strongly influenced by epithelial Cl^- secretion. Increasing Cl^- secretion represents a means of improving mucus clearance in individuals affected by acute or chronic rhinosinusitis. Therapeutic agents with low toxicity and high efficacy are desired. Sinupret has a well-established safety profile in human studies. Together with our results demonstrating stimulation of CFTR-dependent anion transport in vitro as well as in vivo, this provides support for further studies investigating this agent in human protocols, particularly as a topical therapeutic.

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