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Article  (Published Version)

Danahay, H, Fox, R, Lilley, S, Charlton, H, Adley, K, Christie, L, Ansari, E, Ehre, C, Flen, A, Tuvim, M J, Dickey, B F, Williams, C, Beaudoin, S, Collingwood, S P and Gosling, M (2020) Potentiating TMEM16A does not stimulate airway mucus secretion or bronchial and pulmonary arterial smooth muscle contraction. FASEB BioAdvances, 2. pp. 464-477. ISSN 2573-9832

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Potentiating TMEM16A does not stimulate airway mucus secretion or bronchial and pulmonary arterial smooth muscle contraction

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Funding information
All work was funded by Enterprise Therapeutics Ltd.

Abstract
The calcium-activated chloride channel (CaCC) TMEM16A enables chloride secretion across several transporting epithelia, including in the airways. Additional roles for TMEM16A have been proposed, which include regulating mucus production and secretion and stimulating smooth muscle contraction. The aim of the present study was to test whether the pharmacological regulation of TMEM16A channel function, could affect any of these proposed biological roles in the airways. In vitro, neither a potent and selective TMEM16A potentiator (ETX001) nor the potent TMEM16A inhibitor (Ani9) influenced either baseline mucin release or goblet cell numbers in well-differentiated primary human bronchial epithelial (HBE) cells. In vivo, a TMEM16A potentiator was without effect on goblet cell emptying in an IL-13 stimulated goblet cell metaplasia model. Using freshly isolated human bronchi and pulmonary arteries, neither ETX001 or Ani9 had any effect on the contractile or relaxant responses of the tissues. In vivo, ETX001 also failed to influence either lung or cardiovascular function when delivered directly into the airways of telemetered rats. Together, these studies do not support a role for TMEM16A in the regulation of goblet cell numbers or baseline mucin release, or on the regulation of airway or pulmonary artery smooth muscle contraction.

Keywords
Anoctamin-1, bronchoconstriction, ETX001, mucin, vasoconstriction

Abbreviations: ALI, air-liquid interface; ATP, adenosine-5’-triphosphate; CaCC, calcium-activated chloride channel; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; CPA, cyclopiazonic acid; DMSO, dimethylsulfoxide; EC_{50}, concentration inducing 50% efficacy; EC_{20}, concentration inducing 20% efficacy; HBE, human bronchial epithelial; HPCD, 2-hydroxypropyl-beta-cyclodextrin; i.p., intraperitoneal; ISC, short-circuit current; NMP, N-Methyl-2-pyrrolidone; PMA, Phorbol 12-myristate 13-acetate; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; TEM, transmission electron microscopy; UTP, Uridine-5’-triphosphate; Vss, volume of distribution.

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1 | INTRODUCTION

The TMEM16A protein (anoctamin-1) is now recognized as a calcium-activated chloride channel (CaCC) and is the native CaCC in a variety of cell types including secretory epithelia, smooth muscle, and neurons. The long-recognized functions of the CaCC in these cell types range from the regulation of ion transport and fluid homeostasis to the control of contractile responses in excitable tissues. With the identification of TMEM16A as the CaCC, genetic models and pharmacological modifiers have begun to introduce an expanding list of proposed biological roles for this protein.

With respect to the function in the airways, Caputo et al demonstrated that TMEM16A was the CaCC responsible for vectorial chloride transport across the bronchial epithelium and that both expression and function were upregulated by the Th2 cytokines IL-4 and IL-13. Enhancing TMEM16A-mediated chloride secretion thereby represents a therapeutic approach to hydrating the hyperconcentrated mucus in the airways of patients with a variety of diseases associated with mucus obstruction including cystic fibrosis (CF). In support of this concept, a novel low molecular weight potentiator of TMEM16A, ETX001, has been demonstrated to promote airway mucosal hydration and to promote mucus clearance in vivo.

Of further relevance to lung physiology, it has been proposed that TMEM16A can positively regulate both mucin secretion from goblet cells and the IL-13-dependent formation of goblet cells although a recent report challenges some of these observations. Additionally, there are several reports indicating that TMEM16A positively regulates the contractile responses of murine, guinea pig, and human airway smooth muscle in vitro and murine airways in vivo. TMEM16A blockers, including niclosamide and benzbromarone, have been demonstrated to relax airway smooth muscle, while a reported TMEM16A activator, E₄₅, induces contractile responses. To this end it has been suggested that blocking TMEM16A therapeutically may provide a greater overall benefit in the lungs of patients, by simultaneously bronchodilating the airways and reducing the burden of mucus, vs the benefit of enhancing TMEM16A function and improving mucosal hydration and mucus clearance. Similarly, enhancing the activity of TMEM16A with agents such as ETX001 could potentially increase mucus secretion and goblet cell numbers and cause bronchoconstriction which may represent safety issues in the clinic.

The aim of the present study was, therefore, to test the hypothesis that enhancing the activity of TMEM16A using potentiators of channel function, such as ETX001, would induce bronchoconstriction, enhance baseline airway mucus secretion, increase goblet cell numbers, and alter pulmonary vascular tone. Using a variety of model systems, including primary human bronchial epithelial (HBE) cells, in vivo airway mechanics, and freshly isolated human bronchi and human pulmonary arteries, we demonstrate that ETX001 and related TMEM16A potentiators do not induce changes in airway smooth muscle function, do not influence either baseline mucus secretion or the formation of goblet cells, and do not influence human pulmonary artery smooth muscle contraction. The observed lack of activity of the potent and selective TMEM16A inhibitor Ani9 in these same airway models further challenges the conclusions of the published studies and a role for TMEM16A in these processes.

2 | METHODS

Unless otherwise stated, all chemicals were purchased from Sigma (Poole, Dorset, UK; St Louis, MO, USA) and cell culture/immunohistochemistry reagents from Thermo Fisher Scientific (Inchinnan, Renfrew, UK; Carlsbad, CA, USA).

2.1 | Cell culture

HBE were provided by Dr Scott Randell from both the University of North Carolina Chapel Hill collection and the Cystic Fibrosis Foundation Therapeutics repository. Cells were cultured at air-liquid interface (ALI) as previously described. In brief, following an expansion step on plastic, HBE were seeded onto either Snapwell or Transwell inserts in submerged culture followed by 2 weeks at ALI in either defined ALI media or DMEM:F12 media supplemented with Ultraser G (2% v/v; Pall, Cergy, France). At all stages of culture, cells were maintained at 37°C in 5% CO₂ in an air incubator.

2.2 | Ion transport

HBE that had been cultured on Snapwell inserts for 2 weeks at ALI in Ultraser G-based media, were treated with IL-13 (Peprotech, London, UK) for 48-96 hours (10 ng/mL) prior to use in functional studies, to upregulate TMEM16A expression. In some studies, HBE were also treated with either ETX001 (1 µmol/L) or vehicle (0.01% dimethylsulfoxide [DMSO]) for 96 hours while in culture, with media and treatments refreshed at 48 hours.

HBE were mounted in Ussing chambers in symmetrical Ringers solutions and voltage clamped to 0 mV as previously described. Following the addition of amiloride to inhibit the ENaC-mediated short-circuit current (ISC), the sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA)-pump inhibitor cyclosporin acid (CPA) or uridine-5’-triphosphate (UTP) were added to the cells to elevate intracellular calcium ([Ca²⁺]ᵢ) levels and to enable the efficacy of ETX001 to be
evaluated. In the CPA-format, an concentration inducing 20% efficacy (EC\textsubscript{20}) concentration of the SERCA-pump inhibitor was added to the cells to induce a stable, TMEM16A-mediated current response, upon which a concentration-response to ETX001 could be generated, as previously described.\textsuperscript{8} In the UTP format, increasing concentrations of UTP were added cumulatively and the area-under-the-curve was quantified to determine the magnitude of the anion secretory response.

2.3 | QPatch automated electrophysiology

Cell lines stably expressing full-length variants of either mouse (NM\_178642.5) or rat (NM\_01107564.1) were used (Scottish Biomedical, UK). Cells were cultured in Ham's F12 medium supplemented with 10% of fetal bovine serum, 2 mmol/L of L-glutamine, 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 250 µg/mL of hygromycin B. Cells were maintained at 37°C in 5% CO\textsubscript{2} in air incubator and grown to 70%-80% confluence prior to passaging or use in electrophysiology experiments. Whole-cell patch clamp recordings were made as previously described\textsuperscript{8} using a QPatch automated electrophysiology platform. In brief, currents were quantified using a combined step (−70 mV to +70 mV) and voltage ramp (−90 mV to +90 mV) protocol in experiments using anion selective recording solutions to isolate the TMEM16A current. The extracellular recording solution consisted of (mmol/L) 130 NMDG-Cl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 HEPES with the pH adjusted to 7.3 with HCl and the osmolarity to 320-330 mOsm with sucrose. The intracellular recording solution consisted of (mmol/L) 130 NMDG-Cl, 1 MgCl\textsubscript{2}, HEPES 10, EGTA 10, BAPTA 10, Mg-ATP 2 with CaCl\textsubscript{2} added to provide a free [Ca\textsuperscript{2+}]\textsubscript{i} experimentally derived to be the EC\textsubscript{20} for TMEM16A channel activation. For the CHO-mTMEM16A (murine) and rTMEM16A (rat) this was 260 and 415 mmol/L, respectively. The effect of test compounds on TMEM16A currents was quantified as the percentage change in current relative to the starting baseline current. The compound effect was analyzed by calculating the percentage change in current relative to the starting baseline current. The compound effect was always measured as the maximum current change within each concentration incubation. Concentration-response curves were generated using these values and fitted using a Hill plot within the QPatch software to determine the concentration inducing 50% efficacy (EC\textsubscript{50}) value.

2.4 | Baseline mucus secretion

In vitro: CF-HBE from 2 donor codes, both homozygous for F508delCFTR, were cultured in defined ALI media\textsuperscript{20} and used for these studies. Half of the inserts were treated with IL-13 (10 ng/mL) for 48 hours to increase the expression of TMEM16A and MUC5AC. After washing the apical surface to remove any accumulated mucus, cells were treated with vehicle (0.1% DMSO) or ETX001 (1 µmol/L) for 24 hours. After treatment, cell washings (0.5 mmol/L tris (2-carboxyethyl) phosphate in saline; 150 µL/insert; 30 minutes) were collected from ≥12 inserts per donor code. Phorbol 12-myristate 13-acetate (PMA; 300 nmol/L) was used as a positive control to confirm the capacity to increase goblet cell exocytosis. Washings were separated by electrophoresis and probed with antibodies directed against MUC5AC (mouse monoclonal 45M1) and MUC5B (rabbit polyclonal UNC414, provided by Ehre laboratory). Signal intensity was analyzed using the LiCor Odyssey software and was normalized to an untreated control group. To assess any effects of treatment on mucin granule numbers in goblet cells, unwashed inserts (n = 3) were fixed (osmium perfluorocarbon) and were processed for transmitted electron microscopy (TEM) as previously described.\textsuperscript{22} Briefly, inserts were carefully fixed in 2.5% of glutaraldehyde/ 2% paraformaldehyde in 0.1 mol/L of sodium cacodylate buffer at 37°C. Fixed samples were embedded, polymerized, serially sectioned via a TEM grid and ultrathin sections (~80 nm) were stained prior to imaging. The entire length of cell inserts was imaged using a JEOL 1230 transmission electron microscope at 8000x magnification. The average number of granules per field of view were counted across the entire length of each insert. A one-way ANOVA was used to test for differences between treatment groups.

In vivo pharmacokinetics and mucus secretion assay: To dose ETX001 directly into mouse airways would require the compound to be formulated as a suspension. To avoid any potential artifact induced by particulate instillation into the lungs in this model, a metabolically stable analogue of ETX001, ETX004, was dosed to mice by intraperitoneal injection to achieve systemic exposures that were in excess of those required to fully engage the target. To select these doses, ETX004 was first dissolved in 5% N-Methyl-2-pyrrolidone (NMP)/95% hydroxypropyl-beta-cyclodextrin (HPCD) (20%) and dosed by intra-peritoneal injection to male, C57bl6 mice (26-32 g; 0.003 to 3.0 mg/kg [10 mL/kg]). At regular intervals after dosing, blood was sampled from the tail vein (25 µL) and diluted 1:1 with water. A parallel group of mice were dosed with ETX004 by intravenous injection to enable the volume of distribution (V\textsubscript{ss}) of ETX004 to be calculated. Diluted blood was stored at −20°C prior to bioanalysis. ETX004 concentrations in blood were determined using standard LCMS-MS methods. Having established the PK profile of ETX004, airway goblet cell metaplasia was induced in C57bl6 mice by intrapharyngeal IL-13 instillation (4 × 2 µg in 40 µL dosed on days 1, 3, 5 & 13) as previously described.\textsuperscript{23} On day 15, mice were administered either vehicle or ETX004 (0.3 and 3.0 mg/kg i.p.). At 2.5 hours after
dosing, mice were exposed to an aerosol of either normal saline or adenosine-5′-triphosphate (ATP) (100 mmol/L in saline) for 20 minutes. At 10 minutes after completion of the aerosol dosing, animals were euthanized and lungs fixed, processed, and stained with PAFS as previously described.\textsuperscript{24} Images were acquired using an upright microscope (Olympus BX 60) with a ×40 NA 0.75 objective lens, and intracellular mucin was measured around all the circumferential section of the axial bronchus using ImagePro (Media Cybernetics).\textsuperscript{25} Data are presented as the epithelial mucin volume density, signifying the measured volume of mucin overlying a unit area of epithelial basal lamina, derived as described previously.\textsuperscript{24} Images were analyzed by investigators blinded to treatment.

\subsection*{2.5 | Goblet cell formation}

Following 14 days of differentiation at ALI in Ultroser-G-based media, HBE were treated with either vehicle (0.1% DMSO), Ani9 (10 µmol/L), ETX001 (1 µmol/L), or the combination of ETX001 and Ani9. These study groups were replicated both in the presence and absence of IL-13 (10 ng/mL) treatment. All treatments were refreshed at 48 hours and maintained to 96 hours. At 96 hours, wells were fixed in 4% of formaldehyde and were stained with antibodies to MUC5AC (45M1) and acetylated α-tubulin (6-11B-1) as previously described\textsuperscript{26} with secondary antibodies to enable fluorescence detection of both proteins (Alexa Fluor). The MUC5AC\textsuperscript{+} stained area was visualized using a Zeiss Axiovert epifluorescence microscope with a motorized stage that was used to image the same nine regions of interest on each insert. Image J was used to quantify the MUC5AC\textsuperscript{+} and stained area per insert which was normalized to the vehicle control group. The process was repeated for the acetylated α-tubulin\textsuperscript{+} stained area. A one-way ANOVA with post hoc Sidak test was used to enable statistically significant differences between specific groups to be identified.

\subsection*{2.6 | Wire myography}

Non-transplantable fresh lungs from three organ donors were used for these studies. Donors with a history of asthma, chronic obstructive pulmonary disease (COPD), emphysema, lung cancer, CF, pulmonary fibrosis, pulmonary hypertension, or pneumonia were excluded. Airways and pulmonary arteries were dissected free from the surrounding parenchyma and used for wire myography studies as previously described.\textsuperscript{17} In brief, bronchi and arteries were cut into 2 mm rings and mounted under isometric conditions in 5 mL myographs containing a physiological salt solution, aerated with 95% O\textsubscript{2}/5% CO\textsubscript{2} at 37°C. Airway preparations also had indomethacin (5 µmol/L) included in the salt solution. Following stabilization, tissue viability was assessed by challenging airways with carbachol (10 µmol/L) and isoprenaline (10 µmol/L) to confirm a bronchoconstrictor and dilation response, respectively. Airways that failed to respond were excluded. The viability of human pulmonary arteries was similarly confirmed using a high K\textsuperscript{+} (62.5 mmol/L) challenge to induce vasoconstriction. In addition, U44619 (100 nmol/L) and acetylcholine (10 µmol/L) were used to test endothelial integrity with constrictor and relaxation responses, respectively. Following the viability assessment, all preparations were washed, and tension returned to starting levels. U46619 (100 nmol/L) was added to pulmonary arteries selected for relaxation study. Test items were added to the preparations in increasing concentrations from 0.1 nmol/L to 10 µmol/L. At the completion of treatment, theophylline (2.2 mmol/L) was added to the airway preparations to fully relax the tissue. A two-way ANOVA was used to test for significant differences between the test and vehicle treated groups.

\subsection*{2.7 | Lung and cardiovascular function in vivo}

Male Sprague-Dawley rats (500-625g) had previously been implanted with a HD-S10 telemetry transmitter (Data Sciences International), with the pressure catheter positioned in the ascending aorta in order to measure blood pressure. A total of 10 animals were allocated to study, from which 8 were chosen, based on blood pressure signal and values. At weekly intervals, each animal received administration of either vehicle (0.05% Tween-80 in normal saline) or ETX001 (0.1-2.5 mg/kg) by intratracheal instillation while under isoflurane-induced anesthesia.

Respiratory parameters were measured using whole body, bias flow plethysmography equipment designed by Buxco Research Systems (Data Sciences International). Respiratory parameters (respiratory rate, tidal volume and minute volume) were derived from the changes in pressure associated with the warming and humidification of the air breathed in by the animal. This was monitored by specific probes located within the plethysmograph chambers. Bias flow (room air) was set at approximately 2.5 L/min. On one occasion prior to the first day of dosing, the animals were habituated to the plethysmographs for approximately 2 hours. On each test session, the animals were placed into plethysmograph chambers for pre-dose recording of respiratory parameters (respiratory rate, tidal volume, minute volume) for approximately 1 hour. Animals were then removed from the plethysmograph chambers and returned to their home cages. The animals were then dosed and returned to the plethysmograph chambers and
continuously recorded for approximately 4 hours post-dose. Respiratory rate, tidal volume, and minute volume were reported every 30 minutes up to 4 hours post-dose. Time 0 was the mean value of the last 20 minutes of data recorded during the 1 hour pre-dose period. All post-dose timepoints were the mean of 10 minutes recordings around each timepoint (eg, 10 minutes before and 10 minutes after) with the exception of the 4 hours timepoint, which was the mean value of the last 20 minutes of data recorded.

Cardiovascular parameters (systolic, diastolic, and mean blood pressure and heart rate derived from blood pressure) and core body temperature were recorded from the implanted transmitter using a dedicated Dataquest OpenART data capture system linked with a Ponemah data analysis system (Data Sciences International).

For the respiratory and cardiovascular data (including core body temperature) each post-dose timepoint and parameter was analyzed separately using analysis of covariance (ANCOVA). Factors in the model included day of measurement and treatment. Pre-dose data were included in the model as a covariate. ETX001 treatment was compared to vehicle treatment using Williams’ test.

2.8 | Study approval

The in-life experimental procedures undertaken in this study were subject to either the provisions of the United Kingdom Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 (the Act) or in accordance with the protocol approved by the Institutional Animal Care and Use Committees of The University of Texas MD Anderson Cancer Center (00001214-RN01). The number of animals used was the minimum that is consistent with scientific integrity and regulatory acceptability, consideration having been given to the welfare of individual animals in terms of the number and extent of procedures to be carried out on each animal.

3 | RESULTS

3.1 | Epithelial ion transport

As previously described, ETX001 (1 µmol/L) potentiated the anion secretory response of the endogenously expressed, native TMEM16A in CF-HBE to the P2Y2 receptor agonist,
UTP (Figure 1). ETX001 treatment increased the sensitivity of the epithelium to UTP and enhanced the maximum efficacy (Figure 1A,B). The activity of ETX001 was independent of the duration of treatment, with both a 5 minutes (apical) and 96 hours (basolateral) exposure potentiating TMEM16A function to a similar degree (Figure 1B). The amiloride-insensitive ISC was significantly enhanced following the 96 hours treatment with ETX001 (5.0 ± 0.4 µA/cm² in control vs 9.7 ± 0.4 µA/cm² in the 96 hours ETX001 group; P = .002, n = 6 inserts per group) and was sensitive to inhibition by Ani9 (data not shown). To date, ETX001 has demonstrated similar potency and efficacy in each of the 11 donor codes of CF-HBE tested (reference 8 and present study). The present studies were performed using three donors of CF-HBE, including a code that was homozygous for F508del CFTR.
Following treatment of CF-HBE with CPA (2 μmol/L), ETX001 induced a concentration-dependent increase in the ISC. The potency and efficacy of ETX001 was independent of the sidedness of compound addition (Figure 1C). Similarly, the efficacy of the TMEM16A blocker Ani9, was independent of apical or basolateral addition. Figure 1D-F illustrates the attenuation of a UTP-stimulated anion-secretory response by Ani9 when administered to either the apical or basolateral side of the CF-HBE for 5 minutes before apical stimulation with the P2Y2-receptor agonist. These data validated the treatment of CF-HBE with either ETX001 or Ani9 by addition to the basolateral side of the epithelium in subsequent assays.

**FIGURE 3** ETX004 does not stimulate airway goblet cell exocytosis in vivo. A sample raw data trace (A) illustrating the potentiation of recombinant murine TMEM16A channels by ETX004, as assessed by automated patch electrophysiology together with the associated concentration-response data (B; mean data ± SEM; n = 6). The voltage protocol used to generate the recording is shown above and dotted line indicates the zero-current level. Following a single intraperitoneal dose to mice, ETX004 was detected in blood in a dose-dependent manner to achieve concentrations in excess of the EC50 value for ETX004 on murine TMEM16A (38 nmol/L; dotted line) at the 0.3 and 3.0 mg/kg doses (C). Mean data ± SEM are shown (n = 3 mice per dose group). On a background of an IL-13 induced goblet cell metaplasia in mice, ETX004 (0.3 and 3.0 mg/kg i.p.) was without effect on the emptying of airway goblet cells (D). The positive control, inhaled ATP, induced a significant emptying of goblet cells (D,E). A sample histology image (E) illustrating the presence of PAFS stained material in airway goblet cells (white arrowheads) in vehicle treated mice compared with the reduced staining after aerosol exposure to ATP to induce exocytosis. Mean data ± SEM (n = 10 mice per group). **P = .001 using a Holm-Sidak’s multiple comparison test.
3.2 | Baseline mucus secretion in vitro

Treatment of CF-HBE with ETX001 for 24 hours failed to increase the secretion of either MUC5AC or MUC5B (Figure 2B,C). This was supported by TEM imaging and quantification of mucin granules in the goblet cells which likewise showed no evidence of an enhanced secretory response in the presence of the TMEM16A potentiator (Figure 2D,E). In contrast, treatment with PMA (300 nmol/L) induced a significant increase in both the secretion of the mucins MUC5AC and MUC5B and also a reduction in the numbers of mucin granules in the secretory cells, consistent with the competence of the epithelium to undergo stimulated exocytosis of the goblet cells when suitably activated. In parallel experiments, cells were treated with IL-13 for 48 hours to upregulate TMEM16A expression and function as well as increasing MUC5AC protein expression (Figure 2A) to maximize the opportunity to observe a TMEM16A-dependent regulation of goblet cell function. IL-13 treatment significantly increased MUC5AC protein expression and reduced MUC5B expression but did not influence the lack of effect of ETX001 on mucin secretion or goblet cell exocytosis (Figure 2B,C). These studies were performed in two donors of CF-HBE, both homozygous for F508delCFTR.

3.3 | Mucus secretion in vivo

To avoid the potential of a bolus topical dose of a particulate suspension to influence goblet cell secretion, a metabolically stable analogue of ETX001, ETX004, was administered by i.p. injection to achieve sustained blood levels of a TMEM16A potentiator. In vitro, ETX004 potentiated murine TMEM16A activity to a maximum 210% efficacy and with an EC50 value of 38 nmol/L (n = 6) (Figure 3A,B). A solution formulation of ETX004 at doses of 0.3 and 3.0 mg/kg achieved maximum blood exposures of 131 and 939 nmol/L, respectively (Figure 3C), approximately 3-25-fold over the EC50 for ETX004 on murine TMEM16A. These exposures were reached by 30 minutes after dosing. With the Vss calculated to be 3.5 L/kg from the iv dosed group (Figure S1), ETX004 was not plasma restricted and levels of ETX004 greater than plasma will be available in tissue, including well-perfused lung tissue. ETX004, at these levels of exposure, did not stimulate any measurable emptying of goblet cell mucin content (Figure 3D,E) in contrast to the positive control ATP, a potent secretagogue, that induced a 45% reduction in goblet cell mucin content (P = .001).

3.4 | Goblet cell formation in vitro

HBE cultured at ALI for 14-21 days (in Ultroser G-based ALI media) formed a well-differentiated epithelium, evidenced by the presence of both acetylated α-tubulin positive ciliated cells and MUC5AC positive goblet cells (Figure 4). Treatment of these cells with IL-13 for 96 hours significantly increased the MUC5AC positive stained area, consistent with an increase in goblet cell numbers. There was no significant effect of IL-13 on the numbers of ciliated cells (Figure S2). The parallel treatment of these cells with ETX001 failed to increase the MUC5AC positive stained area (Figure 4B-D). Similarly, co-treatment with both ETX001 and IL-13 failed to influence the MUC5AC positive stained area beyond the effect of IL-13 alone (Figure 4B-D). Treatment of cells with the TMEM16A blocker Ani9 was also without effect on the MUC5AC positive stained area in either the normal or IL-13 treated HBE (Figure 4B-D). The ciliated cell population was unaffected by any of the treatments based on the acetylated α-tubulin stained area (Figure S2).

3.5 | Smooth muscle contraction ex vivo

Freshly isolated human airways under an imposed tone showed a concentration-dependent relaxation to the positive control isoprenaline (Figure 5A). ETX001 failed to induce any change in airway tone, either relaxation or constriction. In contrast, the reported TMEM16A blocker niclosamide (10 µmol/L) did significantly relax the tissues, although Ani9, a more potent and structurally diverse TMEM16A blocker did not have any effect. All airways studied had previously responded to carbachol challenge with a bronchoconstrictor response (data not shown).

Freshly isolated human pulmonary arteries showed no response to ETX001 when studied in either the relaxation or constriction modes, although positive controls showed the predicted efficacy (Figure 5B,C). Niclosamide (10 µmol/L) induced a vasoconstrictor response in both assay formats, an effect that was not evident with Ani9 treatment.

3.6 | Respiratory and cardiovascular function in vivo

ETX001 potentiated rat TMEM16A to a maximum 337% efficacy and with an EC50 value of 88 nmol/L (Figure 6A,B). When dosed by intratracheal administration to telemetered rats, there were no ETX001-induced effects on either respiratory (Figure 6C-E) or cardiovascular function (Figure 7). At the highest dose studied (2.5 mg/kg) there was a significant reduction in minute volume (Figure 6C), but only at a single, isolated timepoint (1.5 hours) with no evidence of time-dependent response as there were no trends at the timepoints either side (1.0 and 2.0 hours). Respiratory rate and tidal volume were not different from the values in the vehicle control group at any time during the study. There was a small (4%)
decrease in diastolic blood pressure measured at a single time-point (0.5 hours) in the 2.5 mg/kg treated group (Figure 7B). Again, there was no evidence of a time-dependent response.

4 | DISCUSSION

Since the identification of TMEM16A as the gene encoding the native CaCC, a number of physiological roles have been validated and expanded upon using a combination of genetic models and pharmacological modulators. Of relevance to the lung, TMEM16A loss-of-function studies have reported effects on goblet cell number and function\(^9\)-\(^{13}\) as well as relaxation of airway smooth muscle\(^{10,15-17}\) culminating in the recent proposal that inhibiting TMEM16A function would be of benefit in patients with respiratory diseases associated with mucus obstruction.\(^{18}\) Using the TMEM16A potentiator compounds ETX001 and ETX004, we have tested the hypothesis that positive modulation of the airway CaCC will enhance baseline mucin release, increase goblet cell numbers and cause airway and pulmonary arterial smooth muscle constriction. Despite illustrating the clear in vitro efficacy of ETX001 (Figure 1 for human data; Figure 6A,B for rat data) and ETX004 (Figure 3A,B for murine data) as potentiators of TMEM16A, we have not observed any effects on goblet cell number or function on or airway or pulmonary arterial smooth muscle contractility.

The functional upregulation of the airway CaCC by IL-13 in primary HBE, and under conditions that also induced a goblet cell metaplasia, was originally considered to be a coordinated and complimentary physiological response.\(^{27,28}\) An increase in mucin production would require an increased fluid secretory capacity of the airway to maintain adequate hydration of the mucus gel. TMEM16A is abundantly expressed in goblet cells together with NKCC1,\(^{29}\) the chloride transporter required to support vectorial chloride secretion across the airway epithelium. Having an apically located CaCC (TMEM16A) together with NKCC1 in the basolateral membrane supports a central role for a TMEM16A-dependent anion-secretory response mediated through the goblet cell. Recently, however, additional roles for TMEM16A in goblet cell biology have been proposed.

A potential role of TMEM16A as being a key driver of the IL-13 induced goblet cell metaplasia has been suggested by several groups.\(^{11-13}\) These studies have used nonselective blockers of TMEM16A such as niflumic acid, benzbro- marone, and T16Ainh-A01 to demonstrate a reduction in MUC5AC expressing goblet cell numbers in various airway epithelial cell models. However, the lack of effect of the potent TMEM16A blocker Ani9 in the present study is in direct contrast to these published studies and does not support a role for TMEM16A in the formation of goblet cells under IL-13 stimulation. The lack of efficacy of Ani9 on goblet cell formation cannot be due to a lack of access to target, as Figure 1D,E clearly demonstrates that Ani9 effectively blocks TMEM16A function in this cell system. The lack of effect of the TMEM16A potentiator ETX001 on goblet cell numbers similarly cannot be due to lack of access to target as data in Figure 1A,B illustrates efficacy of the compound in this cellular system and thereby further challenges the proposed role for TMEM16A channel function as a driver of differentiation toward the mucin producing phenotype. Consistent

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**FIGURE 4** Neither potentiation nor inhibition of TMEM16A affects goblet cell numbers in primary HBE cells. MUC5AC positive goblet cell numbers were quantified in three independent codes of non-CF HBE following treatments with IL-13, Ani9 (10 µmol/L), or ETX001 (1 µmol/L). Sample immune-stained images (green = MUC5AC+ cells) reflecting nine images per individual insert (A). Panels (B-D) illustrate mean MUC5AC+ stained area ± SEM (n ≥ 4 inserts per group) normalized to the untreated control for each of the three donor codes. A one-way ANOVA with post hoc Sidak test was used to test for significant differences between groups (*P < .001 compared with the untreated control group).
**Figure 5** Neither potentiation nor inhibition of TMEM16A affects contractile or relaxant responses of human bronchi or human pulmonary preparations ex vivo. Effects of ETX001, Ani9, and niclosamide on isolated human airways (A) and pulmonary artery preparations in relaxant (B) or constrictor (C) format using wire myography. Mean data ± SEM are shown (n = 3-5 replicates per group from individual donated lungs). A two-way ANOVA with post hoc Dunnett’s test was used to test for differences between test items and vehicle (*P < .01, **P < .0001).

**Figure 6** ETX001 has no effect on lung function in rats in vivo. A sample raw data trace (A) illustrating the potentiation of recombinant rat TMEM16A channels by ETX001, as assessed by automated patch electrophysiology together with the associated concentration-response data (B; mean data ± SEM; n = 41). The voltage protocol used to generate the recording is shown above and dotted line indicates the zero-current level. The effects of ETX001 (0.1-2.5 mg/kg) or vehicle (0.05% Tween-80 in normal saline) on respiratory rate (C), tidal volume (D), and minute volume (E) when dosed by intratracheal instillation to male SD-rats. Mean data ± SEM (n = 8 animals per group).
with our observations, a recent publication has also suggested that the upregulation of MUC5AC and TMEM16A are independent of one another and that there is no role for TMEM16A in goblet cell formation. One potential explanation for the disconnect between the Simões report and our present study with the literature may relate to the specificity of the TMEM16A blockers widely used in the earlier studies to establish the phenotype. For example, niflumic acid is a nonselective chloride channel blocker, inhibiting cyclooxygenase and several potassium channels. T16Ainh-A01 and benzbromarone likewise have additional pharmacological and toxicological activities, that may have influenced the outcome of the published studies.

Attenuated, goblet cell-derived, baseline mucin secretion secondary to the loss of expression of Tmem16a in mice has been described by Benedetto and colleagues. When Tmem16a gene expression was specifically silenced in Foxj1+ multiciliated cells, the authors described an attenuated paracrine signaling mechanism to the goblet cell which inhibited the baseline release of mucins. Using well-differentiated HBE cultures in the present study, both multiciliated and goblet cells are represented, and therefore, presumably any putative paracrine signaling pathway. In this system, potentiation of TMEM16A with ETX001 for 24 hours failed to influence the baseline secretion of mucus irrespective of pretreatment with IL-13 to increase the expression of both TMEM16A and MUC5AC. The reported TMEM16A activator Eact has also been demonstrated to induce airway goblet cell emptying of stored mucus. Although originally reported to not directly influence [Ca2+]i, Eact has recently been demonstrated to induce a nonspecific elevation of [Ca2+]i, which then drives an indirect activation of TMEM16A. It therefore seems likely that any Eact induced emptying of airway goblet cells is due to a TMEM16A-independent elevation of [Ca2+]i, a well-documented mechanism for exocytosis. Importantly, ETX001 was demonstrated to have no direct effect on [Ca2+]i, and also did not influence a P2Y2 receptor driven elevation of [Ca2+]i. These data would appear to challenge the direct linkage between the positive regulation of TMEM16A function and baseline mucus release in human airway epithelial cells. It cannot, however, be excluded that potentiating TMEM16A function may regulate mucin secretion at higher levels of [Ca2+]i than are achieved using these “resting conditions” in vitro. These observations were, therefore, expanded into an in vivo model of goblet cell-derived mucin secretion where [Ca2+]i and mucin release would be dynamically regulated.
by a range of endogenous signaling pathways and mediators. In vivo, extended systemic exposure to a potent TMEM16A potentiator ETX004 also failed to demonstrate any evidence of goblet cell emptying. In view of the moderate to high Vss of ETX004 (3.5 L/kg) which would result in a preference for compound to partition into tissue rather than remain compartmentalized in blood, in addition to the sustained blood levels of ETX004 that were in excess (up to 25×) of the on target EC50, it is unlikely that the lack of effect of ETX004 is due to a failure to access the target in well-perfused lung tissue. These data further support the lack of a role for TMEM16A in the regulation of baseline mucin release from goblet cells. It cannot, however, be excluded that potentiation of TMEM16A may regulate stimulated mucin release at higher [Ca2+]i, than have been achieved in the present in vivo model.

The role of the CaCC in smooth muscle contractility has been well documented to contribute toward depolarization of the plasma membrane and subsequent Ca2+ influx. Huang and colleagues10 implicated a role for TMEM16A in the regulation of murine airway smooth muscle function and more recently it has been demonstrated that nonselective TMEM16A blockers including benz bromarone, T16Ainh-A01, and niclosamide relax airway smooth muscle preparations.15-17 The reported TMEM16A activator Eact, has also been shown to induce airway smooth muscle contraction and bronchospasm in vitro and in vivo.15,16 The present study has confirmed the broncho-relaxant effect of niclosamide in isolated human bronchi. However, the lack of effect of both Ani9 and ETX001 in this system challenges whether niclosamide is working through a TMEM16A-dependent mechanism. Furthermore, when dosed directly into the airways of rats in vivo where [Ca2+]i, in airway smooth muscle will be dynamically regulated by a range of mediators and signaling pathways, ETX001 failed to show any robust effects on lung function parameters. The doses of ETX001 used in this study (0.5-2.5 mg/kg) were in excess of the doses administered to conscious sheep (35-100 µg/kg) to observe efficacy in a model of mucociliary clearance.8 Given that ETX001 shows good species cross-reactivity in rat and in vivo, it seems unlikely that the lack of effect of ETX001 would be due to inadequate exposure of the airway smooth muscle to compound. A potential explanation for the inconsistency between the present data with ETX001 and Ani9 and published studies may again relate to the lack of selectivity of the TMEM16A blockers and activator, Eact, that have been widely used in the literature. Of particular note, niclosamide is a mitochondrial toxin within the same concentration range required to block TMEM16A.41 As discussed above, Eact, is not a direct TMEM16A activator but rather induces an elevation of [Ca2+]i, that would induce smooth muscle contraction, irrespective of any putative effect on TMEM16A.

Roles for TMEM16A in the regulation of vascular smooth muscle have been similarly proposed although the function is controversial.42 To this end, we have been unable to observe any effects of either Ani9 or ETX001 in isolated human pulmonary artery preparations. Niclosamide induced a contractile effect in both assay formats evaluated, although a role for TMEM16A inhibition cannot be ascribed for the reasons relating to selectivity outlined above. ETX001 also failed to influence cardiovascular function in vivo following local delivery into the airways at doses up to and including the highest dose studied (2.5 mg/kg). However, it should be noted that ETX001 has been designed as an inhaled drug with high plasma clearance and short plasma half-life that will limit systemic exposure.

The data presented in the current study are, therefore, inconsistent with the hypothesis that the positive modulation of TMEM16A will necessarily enhance baseline mucin secretion, stimulate goblet cell metaplasia, induce bronchoconstriction or cause pulmonary artery constriction. Furthermore, inhibition of TMEM16A with the potent and selective channel blocker Ani9, did not relax human bronchial smooth muscle, influence goblet cell function or alter pulmonary artery tone, phenotypes previously observed with nonselective blockers using similar assay formats. Together, these data challenge the potential therapeutic utility of TMEM16A blockers for the treatment of respiratory diseases18 and support the evaluation of TMEM16A potentiators as an approach to hydrate mucus in diseases such as CF.5-7

CONFLICT OF INTEREST
All work has been funded by Enterprise Therapeutics Ltd. HD, SPC, and MG are full-time employees of Enterprise Therapeutics Ltd.

AUTHOR CONTRIBUTIONS
HD, LC, EA, CE, MJT, BFD, CW, SB, SPC, and MG designed the research; HD, RF, SL, HC, KA, LC, CE, AF, MJT, SB, and MG performed the research. HD, RF, SL, HC, KA, LC, EA, CE, AF, MJT, CW, SB, and MG analyzed the data. HD and MG wrote the paper.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Danahay H, Fox R, Lilley S, et al. Potentiating TMEM16A does not stimulate airway mucus secretion or bronchial and pulmonary arterial smooth muscle contraction. FASEB BioAdvances. 2020;2:464–477. https://doi.org/10.1096/fba.2020-00035