

# TMEM16A Potentiation: A Novel Therapeutic Approach for the Treatment of Cystic Fibrosis

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## Abstract

**Rationale:** Enhancing non-CFTR (cystic fibrosis transmembrane conductance regulator)-mediated anion secretion is an attractive therapeutic approach for the treatment of cystic fibrosis (CF) and other mucoobstructive diseases.

**Objectives:** To determine the effects of TMEM16A potentiation on epithelial fluid secretion and mucociliary clearance.

**Methods:** The effects of a novel low-molecular-weight TMEM16A potentiator (ETX001) were evaluated in human cell and animal models of airway epithelial function and mucus transport.

**Measurements and Main Results:** Potentiating the activity of TMEM16A with ETX001 increased the Ca<sup>2+</sup>-activated Cl<sup>-</sup> channel activity and anion secretion in human bronchial epithelial (HBE) cells from patients with CF without impacting calcium signaling.

ETX001 rapidly increased fluid secretion and airway surface liquid height in CF-HBE cells under both static conditions and conditions designed to mimic the shear stress associated with tidal breathing. In ovine models of mucus clearance (tracheal mucus velocity and mucociliary clearance), inhaled ETX001 was able to accelerate clearance both when CFTR function was reduced by administration of a pharmacological blocker and when CFTR was fully functional.

**Conclusions:** Enhancing the activity of TMEM16A increases epithelial fluid secretion and enhances mucus clearance independent of CFTR function. TMEM16A potentiation is a novel approach for the treatment of patients with CF and non-CF mucoobstructive diseases.

**Keywords:** mucus clearance; mucus hydration; anoctamin-1; calcium-activated chloride channel

Cystic fibrosis (CF) is a recessive genetic multiorgan disease that is primarily associated with pulmonary, gastrointestinal, and reproductive tract dysfunction (1). CF is estimated to affect 75,000 patients globally and is caused by loss of function mutations in the anion channel, CFTR (cystic fibrosis

transmembrane conductance regulator). Impaired CFTR function results in defective fluid and solute transport across epithelia with consequences for the hydration status and pH of the mucosal environment (2, 3). In the lungs, a major phenotype associated with the loss of CFTR function is a

hyperconcentrated mucus gel that is poorly removed from the airways by mucociliary and cough clearance mechanisms (4–6), leading to chronic bacterial infection and a progressive loss of lung function. CFTR modulators, which can partially rescue the function of mutated CFTR, have

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## At a Glance Commentary

### Scientific Knowledge on the

**Subject:** Although increasing anion conductance via CFTR (cystic fibrosis transmembrane conductance regulator) modulation is a clinically validated approach for treating cystic fibrosis (CF), it is only suited to patients with specific mutations.

### What This Study Adds to the Field:

This report describes a novel positive modulator of the calcium-activated chloride channel, TMEM16A, which enhances anion and fluid secretion in epithelial cells from patients with CF and accelerates mucociliary clearance *in vivo*. This approach offers the potential to treat all patients with CF as both a standalone therapy and in combination with CFTR modulators.

demonstrated improved clinical outcomes in those patients genetically suited to these therapies and have received recent U.S. Food and Drug Administration approval (7). However, those patients who do not share mutations in CFTR that are susceptible to these drugs will require alternative therapeutics that are mutation independent.

CFTR-independent approaches to treat the ion transport defect in CF include targeting the epithelial sodium channel (ENaC) with blockers to attenuate fluid reabsorption from the airways and increasing the activity of the  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channel (CaCC) to promote fluid secretion (8). Before understanding the molecular identity of the airway epithelial CaCC, denufosal, an inhaled P2Y2-receptor agonist, was developed as a treatment for CF lung disease (9, 10). *In vitro*, the denufosal-mediated increase in intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) activated the CaCC and attenuated ENaC function in cultured airway epithelial cells, resulting in a transient increase in airway fluid volume (10–12). The subsequent lack of clinical benefit of denufosal has been ascribed to the short pharmacodynamic activity of a compound that depleted intracellular  $\text{Ca}^{2+}$  stores and desensitized the receptor. This would render the epithelium refractory to further stimulation by either residual denufosal or the endogenous P2Y2-receptor ligands, ATP

and uridine-5'-triphosphate (UTP) (12, 13), potentially slowing mucus transport in the longer term. Furthermore, a global elevation of cellular  $\text{Ca}^{2+}$  in the airway epithelium by denufosal also stimulated an acute goblet cell exocytosis (9) that may have negatively impacted changes in lung function subsequent to the increased volume of mucus in the airways. The therapeutic utility of upregulating the CaCC pathway thereby remained an unanswered question.

In 2008, TMEM16A (anoctamin-1) was identified as the gene encoding the airway CaCC (14–16). Like CFTR, TMEM16A conducts both  $\text{Cl}^-$  and  $\text{HCO}_3^-$  across the airway epithelium and is expressed in both the surface epithelium and submucosal glands. In contrast to CFTR, TMEM16A is also abundantly expressed in goblet cells and becomes upregulated under inflammatory conditions (14, 17). Using high-throughput screens of compound libraries together with medicinal chemistry optimization, we have identified novel, selective “potentiators” of TMEM16A. We define a TMEM16A potentiator as a compound that enhances the activity of the channel to an endogenous stimulus, rather than directly activating the channel through an elevation of  $[\text{Ca}^{2+}]_i$ , as was the case with denufosal. Herein, we report that the potentiation of TMEM16A channel function using ETX001 enhances airway epithelial anion and fluid secretion in CF bronchial epithelial cells and promotes mucus clearance in *in vivo* models of mucociliary clearance (MCC) in sheep. These data support the concept of positive modulation of TMEM16A as a target for respiratory diseases and that compounds such as ETX001 represent therapeutic candidates for clinical development. Some of the results of these studies have been previously reported in the form of abstracts (18–20).

## Methods

For full details of methods used, data analysis, and the use of statistical tests, please refer to the online supplement.

### Cell Culture

Human bronchial epithelial (HBE) cells were provided by Dr. Scott Randell (University of North Carolina at Chapel Hill) from both the University of North Carolina at Chapel

Hill and the Cystic Fibrosis Foundation Therapeutics cell collections. A total of eight individual donor-derived cell codes were used that included both F508del homozygous mutations, as well as F508del heterozygous cells in which the second allele was of minimal function (*see* online supplement for full details). Cells were cultured at air–liquid interface (ALI), as previously described (21). In some studies, HBE cells were treated with IL-13 (10 ng/ml; 48 h) to increase the TMEM16A assay window (22). FRT-hTMEM16A cells (Fischer rat thyroid cells stably expressing human TMEM16Aabc) were provided by Dr. Luis Galletta (Genoa, Italy) and were cultured, as previously described (14). Chinese hamster ovary cells stably expressing full-length sheep TMEM16A (CHO-sTMEM16A) were obtained from SB Drug Discovery (Glasgow, Scotland).

### Whole-Cell Patch-Clamp Assay

Whole-cell voltage-clamp recordings from FRT-hTMEM16A or CHO-sTMEM16A were made using the QPatch planar patch-clamp system, as described previously (23). TMEM16A currents were assessed using chloride-selective solutions with calculated free  $[\text{Ca}^{2+}]_i$  buffered at 260 nM, a value measured to give 20% of full current activation. Current rectification ratios were calculated by dividing the magnitude of the outward current at +90 mV by the magnitude of the inward current at –90 mV.

### Short-Circuit Current Measurements

HBE cells mounted in Ussing chambers in symmetrical Ringer's solutions were voltage clamped, as previously described (21). Following the addition of amiloride to inhibit the ENaC-mediated short-circuit current (ISC), the sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase pump inhibitor cyclopiazonic acid (CPA) or UTP was added to the cells to elevate  $[\text{Ca}^{2+}]_i$  levels to enable the efficacy of ETX001 to be evaluated.

### Intracellular $\text{Ca}^{2+}$ Measurements

CF-HBE cells, cultured at ALI for 14 to 21 days and pretreated with IL-13 (10 ng/ml; 48 h), were loaded with the  $\text{Ca}^{2+}$ -sensitive fluorescent reporter dye Calcium 6 (Molecular Devices) for 120 minutes at 37°C in Hanks' balanced salt solution buffered with 20 mM N-2-hydroxyethylpiperazine-N'-ethane

sulfonic acid (pH 7.4). Calcium 6 was used because its  $K_d$  for calcium (320 nM) effectively covers the reported physiological range of  $[Ca^{2+}]_i$  mobilization by purinoceptors in these cells (0.1–1  $\mu$ M; 24, 25). The effects of UTP and ETX001 were then tested for direct effects on  $[Ca^{2+}]_i$  by addition to the apical surface of the epithelia before reading in a PHERAstar plate reader (BMG Lab Tech). Cumulative concentration responses to UTP were also established in the presence or absence of ETX001 to test for the potential for the compound to enhance a UTP-stimulated increase in  $[Ca^{2+}]_i$ .

### Airway Surface Liquid Height Assay

HBE cells were washed to remove accumulated mucus and the airway surface liquid (ASL) was stained with Texas-Red dextran, as previously described (23). XZ-confocal microscopy (SP5; Leica) was used to monitor changes in ASL height both before and after the imposition of shear stress (0.5 dyne/cm<sup>2</sup>) at 14 cycles per minute to mimic tidal breathing (26). CF-HBE cells used for these studies were not pretreated with IL-13.

### In Vivo Studies

All animal studies were approved by the Institutional Animal Care and Use Committee of the Mount Sinai Medical Center (Miami, FL). Tracheal mucus velocity (TMV) was measured in conscious sheep that had been administered aerosolized CFTR<sub>Inh172</sub>, a CFTR blocker, to slow the rate of TMV, as previously described (27). Test compounds were administered to sheep by aerosolization into the lungs and effects on TMV were measured. Alternatively, whole-lung MCC was assessed in conscious sheep following inhalation of technetium-99 (<sup>99m</sup>Tc)-labeled sulfur colloid particles (21). These animals had not been pretreated with CFTR<sub>Inh172</sub>. Test compounds were again administered by aerosolization but at 4 hours before assessing MCC.

## Results

### ETX001 Potentiates Recombinant TMEM16A Currents

To identify positive modulators of TMEM16A, parallel fluorescence and electrophysiological screening campaigns were used to interrogate a large (>150 K

compounds) low-molecular-weight chemical library. After hit triage and confirmation, a low potency primary hit was identified that weakly enhanced TMEM16A currents produced by 260 nM  $[Ca^{2+}]_i$  in the whole-cell patch-clamp assay at a concentration of 30  $\mu$ M (Figures 1A and 1B). Subsequent rounds of medicinal chemistry optimization improved potency and efficacy and delivered ETX001. ETX001 robustly potentiated TMEM16A currents in both the inward and outward directions, preserving the characteristic biophysical properties of the channel of outward rectification and time-dependent activation/inactivation (14–16). However, the outward rectification was significantly reduced by ETX001, consistent with a more pronounced effect on the inward (outward chloride ion flow) than the outward currents (inward chloride ion flow). The rectification ratio of  $20.3 \pm 4.5$  in the absence of compound was reduced to  $3.7 \pm 0.2$  by 3.33  $\mu$ M ETX001 ( $n = 10$ ;  $P < 0.002$ ). The effects of ETX001 were concentration dependent with a half-maximal effective concentration (EC<sub>50</sub>) of  $116 \pm 16$  nM up to a maximum magnitude of  $345 \pm 36\%$  ( $n = 14$ ) (Figures 1C–1E). When  $[Ca^{2+}]_i$  was reduced to 0 nM, below the threshold for TMEM16A activity, ETX001 was without effect, consistent with its effect being channel potentiation rather than activation (see Figure E1 in the online supplement). Using the identical FRT-hTMEM16A cell line cultured as a monolayer on Snapwell inserts, ETX001 also potentiated UTP-stimulated current changes in ion transport experiments (see Figure E2).

### Potentiation of Calcium-activated Anion Secretory Currents in Primary HBE Cells from Patients with CF by ETX001

The effects of ETX001 on the anion secretory currents in fully differentiated HBE cells from patients with CF grown at ALI and pretreated with IL-13 for 48 hours was assessed in two formats of the ISC assay. They first used a concentration required to give 20% of the maximal effect (EC<sub>20</sub>) of the sarco/endoplasmic reticulum  $Ca^{2+}$ -ATPase pump inhibitor CPA to elevate  $[Ca^{2+}]_i$ , providing a stable baseline to assess the cumulative concentration response to ETX001. In this format, ETX001 effectively enhanced the anion secretory currents, an effect that was

fully inhibited by the selective TMEM16A blocker, Ani9 (Figure 2A). The EC<sub>50</sub> for ETX001 was  $177 \pm 23$  nM with a  $262 \pm 22\%$  potentiation of baseline current ( $n = 52$  inserts from eight different CF donor codes). Potency and efficacy values from the primary cell assay were found to correlate well with the recombinant system used in the QPatch assay for the ETX series of TMEM16A potentiators (Figure 2C).

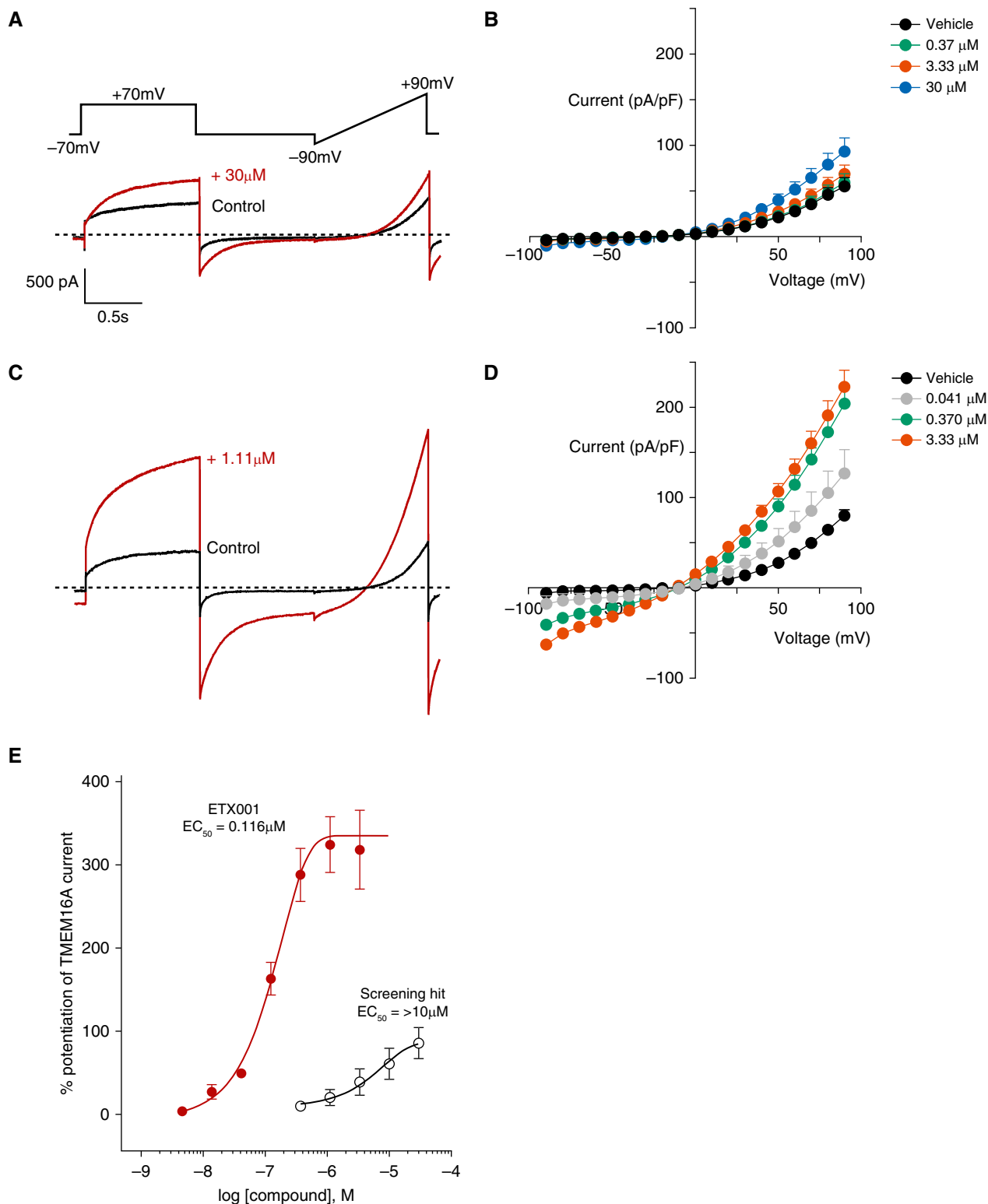
The second assay format assessed the effect of ETX001 on anion currents elicited in HBE cells using a physiologically relevant stimulus, the P2Y2-receptor agonist UTP. As shown in Figure 2D, ETX001 (1  $\mu$ M) significantly potentiated the magnitude of the anion current response to increasing concentrations of UTP, enhancing both the peak current and the duration of the current response as evidenced by the residual current level at the end of each UTP response. Integrating the area under the curve for each addition, the potentiating effect of ETX001 could be quantified (Figures 2E and E4). ETX001 also potentiated the UTP-stimulated anion current in non-IL-13 treated CF-HBE cells (Figures 2F and 2G).

The ion transport effect of ETX001 in HBE cells was selective and limited to the calcium-activated chloride conductance. Pretreatment of HBE cells with ETX001 revealed no effects on either the amiloride-sensitive ISC response (ENaC-dependent) or the forskolin-stimulated anion secretion (CFTR-dependent) (see Figure E5).

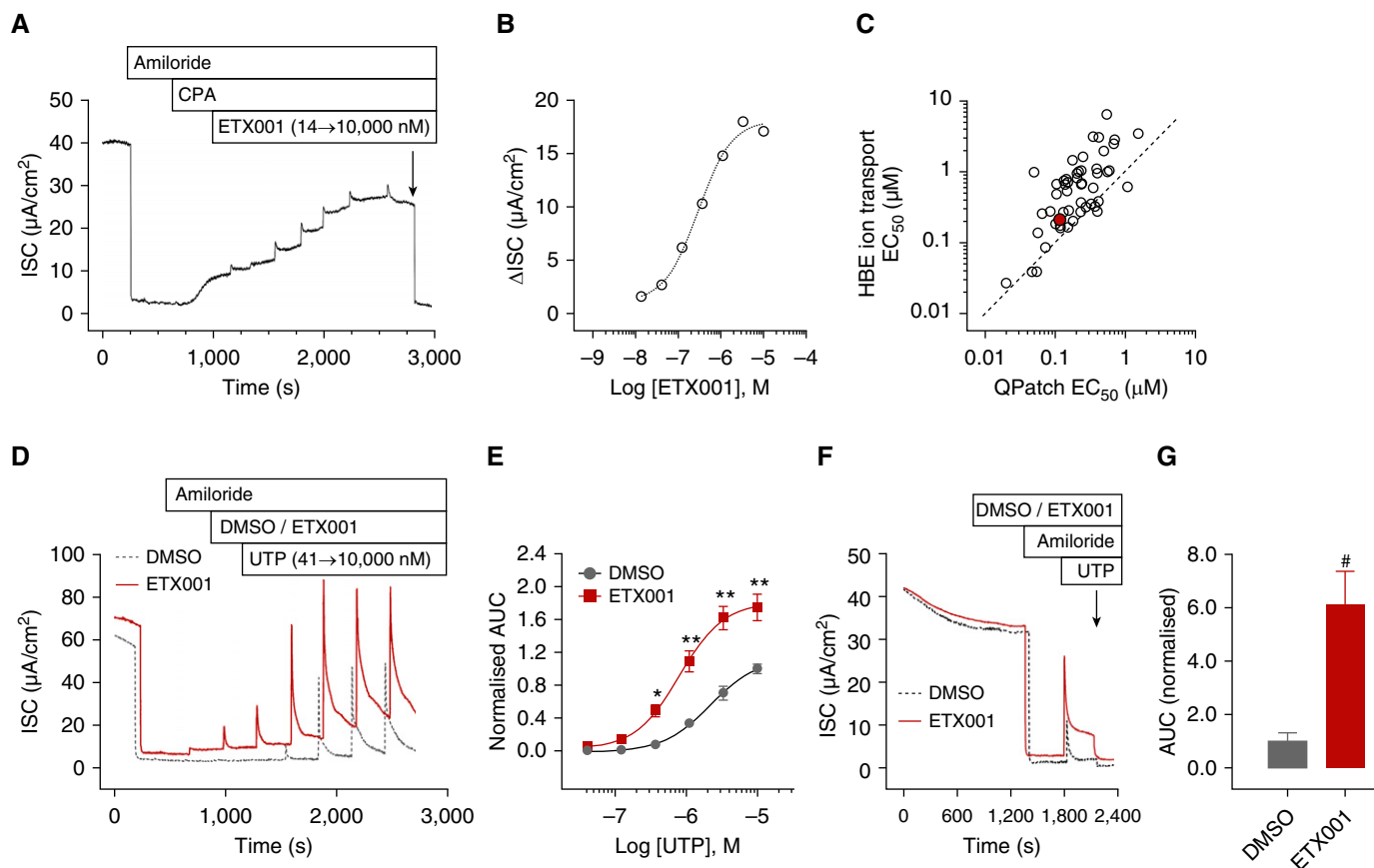
To build confidence in a direct effect of ETX001 on TMEM16A function, we evaluated whether there was any impact of ETX001 on  $[Ca^{2+}]_i$  in CF-HBE cells. It has been suggested that TMEM16A activity can regulate the levels of  $[Ca^{2+}]_i$  in epithelial cells (28) and activation of TMEM16A can elevate  $[Ca^{2+}]_i$ . ETX001 was without effect on the baseline levels of  $[Ca^{2+}]_i$  *per se* (Figure 3B), or the magnitude and sensitivity of the response to the calcium-mobilizing P2Y2-receptor agonist UTP (Figures 3B and 3C).

### ETX001 Increases Fluid Secretion and ASL Height in CF-HBE Cells

To establish whether potentiation of TMEM16A-mediated anion currents could ultimately enhance airway fluid secretion, a model of shear stress-induced mucosal hydration was used (26). In this system,



**Figure 1.** ETX001 is a potent and efficacious potentiator of human recombinant TMEM16A (anoctamin-1). (A and C) Whole-cell patch-clamp electrophysiological recordings from Fischer rat thyroid cells stably expressing hTMEM16A in the absence (black lines) and presence (red lines) of primary screening hit (A) and the optimized compound ETX001 (C) at indicated concentrations. Dashed lines indicate the zero-current level. (B and D) Current-voltage relationships for vehicle and indicated concentrations of compounds are shown. (E) Concentration-response curves for the primary screening hit and ETX001. Mean data ( $\pm$ SEM) (screening hit  $n=5$ ; ETX001  $n=14$ ) are shown.  $\text{EC}_{50}$  = half-maximal effective concentration.



**Figure 2.** ETX001 potentiates the calcium-activated anion secretory current in primary cystic fibrosis (CF)-human bronchial epithelial (HBE) cells. (A) Sample raw data trace illustrating the experimental protocol for assessing the effects of TMEM16A (anoctamin-1) potentiator compounds. Increasing concentrations of ETX001 were added to CF-HBE cells following stimulation of the short-circuit current (ISC) with a submaximal ( $EC_{20}$ ) concentration of cyclopiazonic acid (CPA). Ani9 ( $10\ \mu\text{M}$ ; downward arrow) was added at the completion of each experiment to confirm the sensitivity of the ISC response to the TMEM16A blocker. (B) Sample concentration-response data derived from the raw data in A. (C) Correlation of data from the CPA ISC assay to the QPatch whole-cell patch-clamp assay. ETX001 is shown as a solid red circle. (D) Sample raw data trace illustrating the effects of ETX001 ( $1\ \mu\text{M}$ ) on the UTP-stimulated anion current response. After inhibition of the ENaC current with amiloride ( $10\ \mu\text{M}$ ), inserts were treated with either DMSO (gray dashed trace) or  $1\ \mu\text{M}$  ETX001 (red line) before the cumulative addition of increasing concentrations of UTP. (E) Mean data illustrating the potentiation of the UTP concentration response by ETX001. Data are expressed as the mean of 6 to 8 inserts of CF-HBE cells (donor: KK036H). See online supplement for additional donor-derived CF-HBE cell data. All CF-HBE cells in A to E were IL-13 treated ( $10\ \text{ng/ml}$ ) for 48 hours before ion transport assay to optimize the TMEM16A assay window. (F and G) CF-HBE cells that had not been treated with IL-13 also showed a UTP-stimulated ( $10\ \mu\text{M}$ ) anion secretory response that was potentiated by ETX001. \* $P < 0.005$  and \*\* $P < 0.001$  using a two-way ANOVA with Siadak's multiple comparison test; # $P < 0.05$  using two-tailed Student's *t* test. AUC = area under the curve;  $EC_{50}$  = half-maximal effective concentration; UTP = uridine-5'-triphosphate.

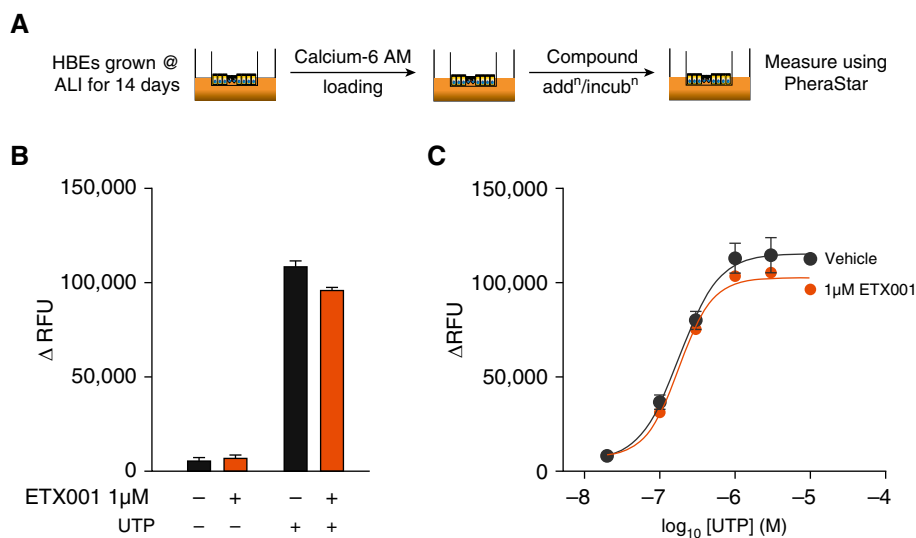
CF-HBE cells were exposed to conditions designed to mimic the shear stress associated with tidal breathing. These forces stimulate the endogenous release of ATP and adenosine, which regulate both  $[\text{Ca}^{2+}]_i$  and  $[\text{cAMP}]_i$ , respectively, thereby controlling TMEM16A and CFTR function (29, 30) (Figure 4A). Fluid secretion could then be quantified by measuring the change in height of the ASL using confocal microscopy. ETX001 ( $1\ \mu\text{M}$ ) was able to significantly increase fluid secretion and ASL height in CF-HBE cells from multiple donors. Following exposure to ETX001 for 60 minutes under static conditions (no

shear stress), ASL height increased compared with the vehicle control (Figures 4B and 4C). The subsequent imposition of cyclical shear stress induced an increase in ASL height in the vehicle-treated CF-HBE cells that was enhanced in the ETX001-treated epithelia (Figures 4B and 4C).

#### Potentiation of TMEM16A Enhances MCC *In Vivo*

*In vitro*, ETX001 potentiated recombinant sheep TMEM16A with an  $EC_{50}$  of  $127 \pm 21\ \text{nM}$  and a magnitude of  $554 \pm 104\%$  ( $n = 7$ ; see Figure E6). We also demonstrated the

expression of TMEM16A in primary cultures of ovine tracheal epithelial cells and the presence of a robust UTP-stimulated anion secretory current in these cells (see Figure E6). The ability of inhaled ETX001 to affect the rate and magnitude of mucus clearance *in vivo* was assessed using two sheep models. Dosing sheep with inhaled CFTR<sub>Inh172</sub> slowed the rate of TMV over a 4-hour period after administration (Figure 5A). TMV remained attenuated for the duration of the study. Dosing with inhaled ETX001 after previously slowing TMV with CFTR<sub>Inh172</sub> restored the rate of TMV to 90% to



**Figure 3.** ETX001 does not affect calcium handling in differentiated cystic fibrosis–human bronchial epithelial (HBE) cells. (A) A cartoon illustrating the experimental protocol for assessing effects of compounds on  $[Ca^{2+}]_i$  in air–liquid interface cultures of HBE cells. (B and C) ETX001 (1  $\mu$ M) had no direct effect on  $[Ca^{2+}]_i$  levels (B) and also had no effect on the UTP-stimulated increase in  $[Ca^{2+}]_i$  (C). Mean data  $\pm$  SD ( $n=6$  inserts per group) are shown from a single donor. add<sup>n</sup>/incub<sup>n</sup> = addition/incubation; ALI = air–liquid interface; AM = acetoxymethyl ester; RFU = relative fluorescence units; UTP = uridine-5'-triphosphate.

95% of the healthy baseline rate (Figure 5A). The second model assessed whole-lung MCC rates by measuring the clearance of aerosolized <sup>99m</sup>Tc sulfur colloid in naive sheep (i.e., with fully functional CFTR). Clearance was assessed 4 hours after inhaled dosing of either vehicle or ETX001. As shown in Figure 5B, ETX001 gave a robust, durable, and dose-dependent enhancement of MCC in this model.

## Discussion

In this study we present data that validates the concept that potentiating the activity of the  $Ca^{2+}$ -activated chloride conductance TMEM16A in airway epithelia can enhance MCC, thus offering a novel therapeutic approach for the treatment of patients with CF and other diseases characterized by mucus obstruction. Importantly, because TMEM16A expression and function is independent of the disease-causing mutations in CFTR, this therapeutic approach is predicted to be suitable for all patients with CF (8).

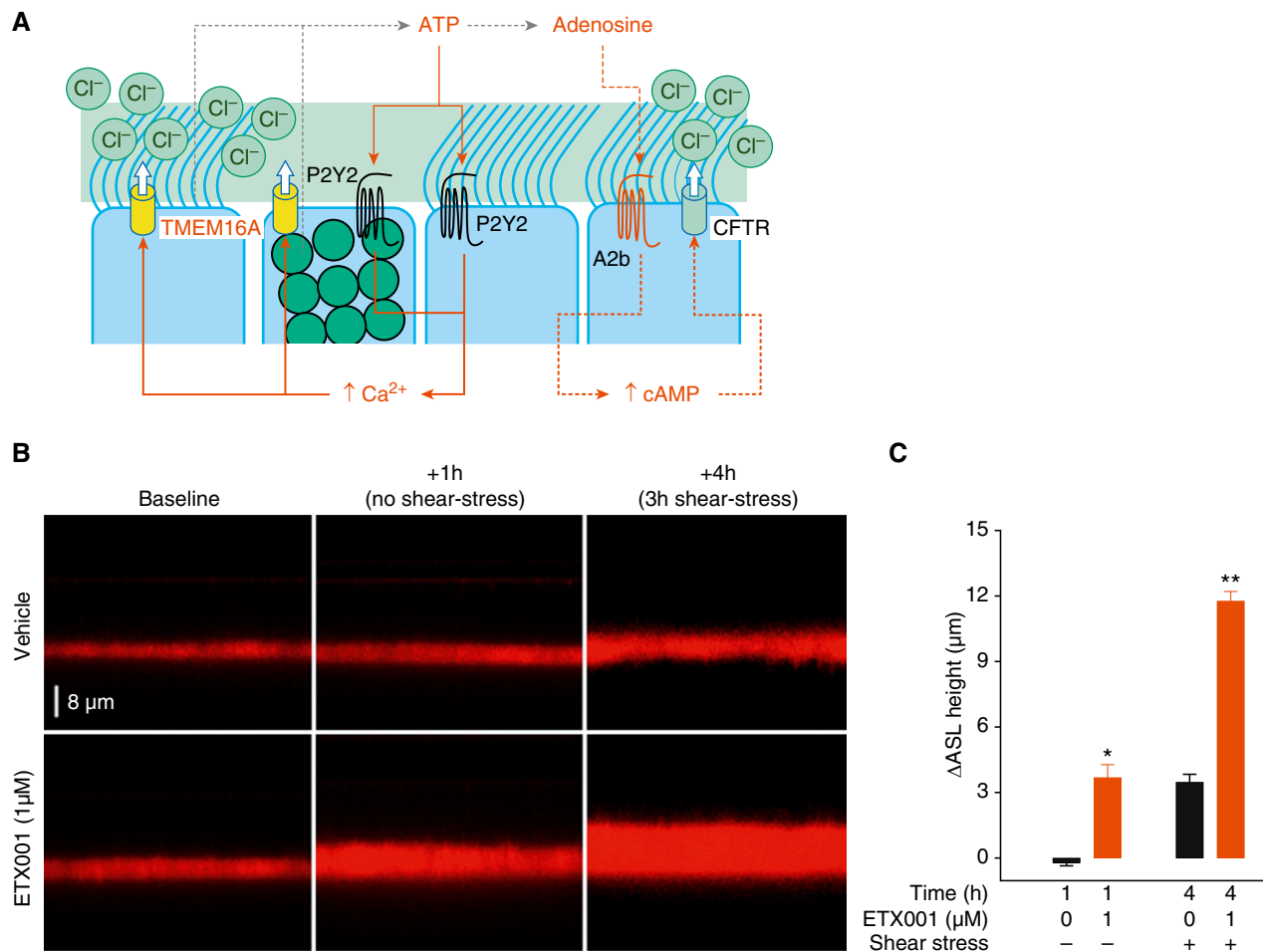
The identification of low-molecular-weight TMEM16A potentiator compounds such as ETX001 has enabled the physiological relevance of enhancing CaCC activity, with respect to anion and fluid

secretion in the airway epithelium, to be evaluated. ETX001 was identified as a potent potentiator of TMEM16A function in a patch-clamp assay using recombinantly expressed TMEM16A (Figure 1). The pharmacology of ETX001 translated from the whole-cell electrophysiology format into epithelial ion transport studies using either the same FRT-hTMEM16A cell line (see Figure E2) or CF-HBE cells (Figure 2). The correlation between the patch-clamp and the CF-HBE cell ion transport assays that was observed with more than 50 compounds from the ETX001 series (Figure 2C) builds confidence that the  $Ca^{2+}$ -dependent chloride secretory current in the native CF cells is dominated by a TMEM16A-mediated mechanism. This contrasts to a report that TMEM16A plays only a minor role in airway CaCC function (31). This study suggested that only a minor component of the CF-HBE cell CaCC-mediated response was sensitive to the TMEM16A inhibitor, T16A(inh)-A01. The reason for this inconsistency is likely due to only a partial inhibition of TMEM16A by T16A(inh)-A01 in patch-clamp studies (unpublished observations), as well as questions relating to the real target of this compound (32). In terms of selectivity versus other ion channels expressed in the airway epithelium, ETX001 was

without effect on either CFTR-mediated secretory responses in non-CF-HBE cells or on ENaC-mediated currents. In HBE cells, both CFTR and ENaC currents depend on accessory ion channels and transporters (e.g., NKCC1, ATP1A1, and Kv1.7) to maintain ion transport function (33), which are presumably likewise unaffected by ETX001 treatment at pharmacologically relevant concentrations.

Having established that ETX001 and related compounds could potentiate TMEM16A function under conditions in which  $[Ca^{2+}]_i$  was predicted to be static (i.e., buffered at 260 nM in patch-clamp or stable following stimulation with an EC<sub>20</sub> concentration of CPA in CF-HBE cells), it was important to establish whether the pharmacology was observed over a physiologically relevant range of  $[Ca^{2+}]_i$ . To address this, the P2Y2-receptor agonist UTP was added to IL-13 pretreated CF-HBE cells at increasing concentrations in the presence or absence of ETX001. ETX001 enhanced the exogenous UTP-induced secretory current response of CF-HBE cells (Figure 2D), causing both a leftward shift in the UTP concentration response and enhancing the maximum efficacy (see Figure 2E and additional donors in Figure E4), consistent with the ability of ETX001 to potentiate TMEM16A function across a physiologically relevant range of  $[Ca^{2+}]_i$ . In these studies, CF-HBE cells had been pretreated with IL-13 to increase the expression and function of TMEM16A and thereby maximize the assay window (17). The upregulation of TMEM16A function by IL-4 and IL-13 has been proposed to be a regulatory mechanism to ensure that mucin and fluid production by the airway remain in balance and the present data therefore support a potentiator activity of ETX001 under these inflammatory conditions. However, the potentiator activity of ETX001 was independent of an inflamed environment because efficacy was also observed in IL-13-naive CF-HBE cells (Figures 2F and 2G).

The use of P2Y2-receptor ligands in these studies is particularly relevant because the local release of ATP and UTP into the airway mucosa represents a key regulatory mechanism in the control of ion transport and fluid homeostasis (29, 30) (Figure 4). The release of these mediators is a kinetic process that is stimulated by shear and compressive forces during normal breathing (26, 34, 35) and is proposed to



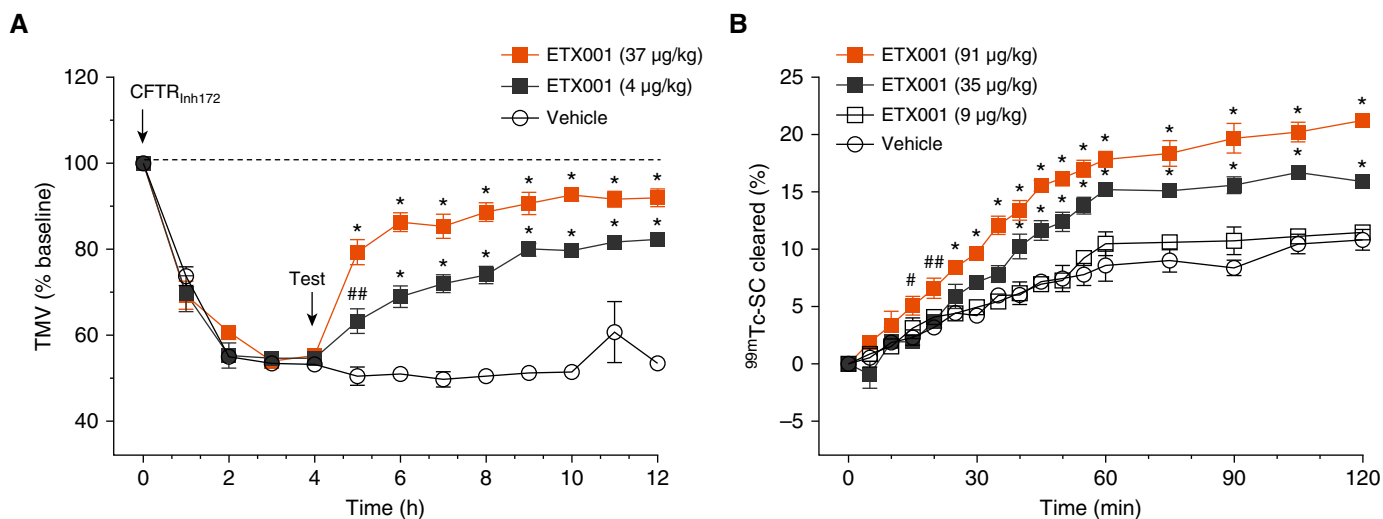
**Figure 4.** ETX001 enhances fluid secretion in cystic fibrosis–human bronchial epithelial (HBE) cells under static and shear stress–stimulated conditions. (A) A cartoon illustrating the physiological regulation of anion secretion in the human airway epithelium. (B) Sample XZ confocal images of airway surface liquid (ASL) visualized using Texas red staining in the absence and presence of ETX001 (1 μM). (C) The change in ASL height from baseline was measured following a 1-hour treatment with ETX001 or vehicle under static conditions (i.e., no shear stress) and after 3 hours of continual shear stress. Data are expressed as the mean change in ASL height (±SEM) for seven to eight inserts from a F508del/F508del HBE cells donor and is representative of three independent experiments. Cystic fibrosis–HBE cells had not been pretreated with IL-13. \* $P < 0.001$  and \*\* $P < 0.00001$  for ETX001-treated versus time-matched vehicle control using two-tailed Student's  $t$  test. TMEM16A=anoctamin-1.

adapt to maintain mucosal hydration as the respiration rate changes following the impaction of inhaled particulates on the epithelium and during cough (36, 37). Tarran and colleagues (26) illustrated that imposing cyclical shear stress to CF-HBE cells to mimic tidal breathing induced an ATP-dependent increase in fluid secretion, measured as an increase in height of ASL. Using a similar model system, ETX001 enhanced the fluid secretory response of CF-HBE cells (Figure 4). The ETX001-enhanced increase in fluid secretion was observed both before and after the initiation of shear stress, further supporting the conclusion that potentiation of TMEM16A could be achieved over the range of resting to physiologically stimulated  $[Ca^{2+}]_i$ .

Two *in vivo* sheep models were used to evaluate the potential for ETX001 to increase mucus clearance. Inhaled dosing of ETX001 to conscious sheep enhanced the rates of airway mucus clearance in the CFTR-inhibited TMV model (27) and in the whole-lung clearance model in which CFTR function is normal (21). Nominal doses in the range of 30 to 100 μg/kg provided a long-lasting enhancement of mucus clearance in both models. The efficacy observed in the whole-lung MCC model in which CFTR function is normal provides early data to support the concept that a TMEM16A potentiator may show benefit in patients with CF who are treated with CFTR potentiator and corrector therapies, as well as in patients without CF

who have alternative mucoobstructive lung disease.

To our knowledge, this is the first report of the discovery and efficacy of TMEM16A potentiator compounds. Compounds initially reported to be direct modulators of the channel (e.g., E<sub>act</sub>) (38) have subsequently been shown to act indirectly via elevation of  $[Ca^{2+}]_i$  (39, 40), negating their utility for validating CaCC function due to the numerous other cellular effects elevation of  $[Ca^{2+}]_i$  will trigger (e.g., goblet cell exocytosis and bronchospasm). In contrast, ETX001 potentiates the opening of the TMEM16A channel (i.e., it enhances the magnitude of the current in the presence of physiologically regulated  $[Ca^{2+}]_i$ ), but ETX001 does not



**Figure 5.** Inhaled ETX001 accelerates mucociliary clearance in conscious sheep models. (A) Dosing of conscious sheep with CFTR<sub>Inh172</sub> slowed the rate of tracheal mucus velocity (TMV). At 4 hours after dosing with CFTR<sub>Inh172</sub>, ETX001 or vehicle was administered as an inhaled nebulized, DMSO-based solution. TMV values are expressed as the mean ( $\pm$ SEM) % of the initial (pre-CFTR<sub>Inh172</sub>) rate ( $n=3$  sheep per group). (B) To assess whole-lung clearance, ETX001 or vehicle were dosed as an inhaled, nebulized suspension 4 hours before the inhaled administration of technetium-labeled sulfur colloid (<sup>99m</sup>Tc-SC). Mean ( $\pm$ SEM) clearance of <sup>99m</sup>Tc-SC in sheep is expressed as the percentage of the initial deposited lung dose ( $n=3-4$  sheep per group). Symbols denote a significant difference from the time-matched vehicle control animals using two-way ANOVA with a *post hoc* Dunnett's test. # $P < 0.01$ , ## $P < 0.001$ , and \* $P < 0.0001$ .

cause increases in  $[Ca^{2+}]_i$  *per se*. This conclusion is supported by two pieces of data. First, ETX001 enhances current in whole-cell patch-clamp studies when  $[Ca^{2+}]_i$  is clamped and heavily buffered with a combination of both fast (BAPTA) and slow (EGTA) chelators. Second, ETX001 was shown to have no effect on  $[Ca^{2+}]_i$  levels in fully differentiated CF-HBE cells *per se* nor any effect on the response to a calcium-mobilizing agonist, UTP (Figure 3). These findings are in contrast to others that have suggested positive TMEM16A modulation will lead to increases in  $[Ca^{2+}]_i$ , albeit those studies have used extrapolations from knockdown approaches and nonselective compounds that are proposed to be TMEM16A blockers (28). It has recently been suggested that inhibiting the function of TMEM16A may be of therapeutic benefit for the treatment of CF (41). This proposal is based on reports that nonselective regulators of TMEM16A function may have effects on

airway smooth muscle and mucin release from goblet cells (42, 43). To date, we have observed no effects of TMEM16A potentiator compounds on airway smooth muscle function (*in vitro*) or lung function (*in vivo*) (unpublished observations). Likewise, there were no effects of ETX001 or related compounds on the central to peripheral ratios of <sup>99m</sup>Tc in the lung scans from the sheep MCC model, changes that would have been expected were there a bronchospasm associated with TMEM16A potentiator administration (unpublished observations). Selective potentiators and blockers of TMEM16A will enable a thorough evaluation of the physiological role of this channel in a variety of biological systems.

### Conclusions

The concept that enhancing airway anion secretion will provide clinical benefit to many patients with CF is now well established with CFTR potentiator and

corrector therapies (1). Whether clinical efficacy can be established by enhancing the anion secretory function of TMEM16A in the airways remains to be established. The positive effects of ETX001 on both airway fluid secretion in CF-HBE cells and mucus clearance *in vivo*, together with the population of patients with CF who are not genetically matched to existing CFTR repair therapies, build a case for testing the hypothesis in the clinic. ■

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