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Inhibition of protease-ENaC signaling improves mucociliary function in cystic fibrosis airways

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Author contributions: S.L.M., B.H., M.J.S., J.S.E. and J.R. designed research. B.W. developed the concept of utilizing a broad spectrum inhibitor to provide un-biased profiling of potential CAPs. J.R., T.F., M.J.S., V.S-C., S.H. performed research. R.H. and B.W. contributed new analytic tools. S.L.M., B.H., M.J.S., J.S.E. and J.R. wrote the paper.

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At a Glance Commentary

Scientific knowledge on the subject

Previous studies highlight that epithelial sodium channel (ENaC) blockade may offset the development of CF (cystic fibrosis) lung disease in a manner independent of CFTR mutation (CF transmembrane conductance regulator). ENaC inhibition therefore provides significant opportunity for a therapeutic strategy that would be suitable for all patients.

What this study adds to the field

We report a rationally designed novel compound (QUB-TL1), restricted to the extracellular surface of airway epithelial cells (AECs), that selectively inhibits critical ENaC-activating proteases. QUB-TL1-mediated inhibition of protease-ENaC signaling in well-differentiated AECs obtained from CF patients (F508del homozygotes) improves ASL height and restores mucociliary function. This study provides evidence that protease inhibitor compounds such as QUB-TL1 have potential to ameliorate CF lung disease.

This article has on online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

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Abstract

Rationale: In cystic fibrosis (CF) a reduction in airway surface liquid (ASL) height compromises mucociliary clearance, favoring mucus plugging and chronic bacterial infection. Inhibitors of ENaC have therapeutic potential in CF airways to reduce the hyper-stimulated sodium and fluid absorption to levels which can restore airways hydration.

Objectives: To determine whether a novel compound (QUB-TL1) designed to inhibit protease/ENaC signaling in CF airways restores ASL volume and mucociliary function.

Methods: Protease activity was measured using fluorogenic activity assays. Differentiated primary airway epithelial cell cultures (F508del homozygotes) were used to determined ENaC activity (Ussing chamber recordings), ASL height (confocal microscopy) and mucociliary function (by tracking the surface flow of apically applied microbeads). Cell toxicity was measured by LDH assay.

Measurements and Results: QUB-TL1 inhibits extracellularly-located CAPs, including prostasin, matriptase and furin, the activities of which are observed at excessive levels at the apical surface of CF airway epithelial cells (AECs). QUB-TL1-mediated CAPs inhibition results in diminished ENaC-mediated Na^+ absorption in CF AECs due to internalization of a prominent pool of cleaved (active) ENaCγ from the cell surface. Importantly, diminished ENaC activity correlates with improved airway hydration status and mucociliary clearance. We further demonstrate QUB-TL1-mediated furin inhibition, which is in contrast to other serine protease inhibitors (camostat mesylate and aprotinin), affords protection against neutrophil elastase-mediated ENaC activation and *Pseudomonas aeruginosa* exotoxin A-induced cell death.

Conclusions: QUB-TL1 corrects aberrant CAP activities providing a mechanism to delay or prevent the development of CF lung disease in a manner independent of CFTR mutation.

Abstract word count: 248

Keywords: channel activating proteases, prostasin, furin, pseudomonas aeruginosa exotoxin A, airway surface liquid

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Introduction

Cystic fibrosis (CF) is the most common life-limiting autosomal recessive genetic disease in Europe, North America and Australia; with lung disease characterized by chronic infection and inflammation, accounting for the majority of morbidity and mortality [1]. The conducting airways are lined by a thin film of fluid known as the airway surface liquid (ASL) which facilitates mucociliary clearance; a critical component of innate immune defense facilitating the removal of inhaled pathogens [2]. The ASL volume is regulated by oppositely directed Cl^- and Na^+ flux (due to osmotic gradients established by active ion transport) across the airway epithelium [3]. In CF airways this regulation is lost due to the absence of CFTR-mediated Cl^- secretion and concomitant hyperabsorption of Na^+ via the epithelial sodium channel (ENaC) [4;5]. ENaC hyperabsorption contributes to dehydration of the ASL layer, the depletion of which is regarded as an initiating factor for CF lung pathophysiology [6].

A seminal study in the late 1990's demonstrated aprotinin (which inhibits trypsin-like proteases) reduces ENaC activity in kidney epithelial cells, which was subsequently restored by trypsin application [7]. It is now apparent that endoproteolysis of ENaC subunits (α and γ), catalyzed by CAPs (channel activating proteases); many of which belong to the trypsin-like subfamily of serine proteases, increases channel conductance which otherwise remains low due to Na^+ self-inhibition [8]. In CF airways this fundamental regulatory mechanism is abnormal with imbalance between CAPs and their natural inhibitors. Furin and prostasin are well characterized CAPs, shown to work in sequential manner to fully activate ENaC that have been reported at excessive levels in CF airway epithelial cells [9-12]. siRNA-mediated knockdown of prostasin reduces ENaC currents by ~70% in CF AECs [13] and this effect is replicated when camostat mesylate, an inhibitor of the CAPs prostasin and matriptase [14], is delivered to the nasal epithelium of CF patients [15]. The furin inhibitor decanoyl-RVKR-

CMK (furin I) decreases basal ENaC currents and augments the inhibitory action of aprotinin when added to CF AECs [16].

In this study we sought to evaluate a novel rationally designed compound (QUB-TL1) postulated to inhibit trypsin-like CAPs. We subsequently investigate the hypothesis QUB-TL1-mediated correction of aberrant CAPs-ENaC signaling restores CF airways hydration and mucociliary function.

Some of the results of these studies have been previously reported in the form of an abstract [17;18].

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Methods

Full details are available in the online supplement.

Synthesis of QUB-TL1

The synthesis of QUB-TL1 was achieved by adopting and modifying previously reported synthetic protocols developed by our group [19;20] and shown in Scheme S1.

Primary airway epithelial cell cultures

Primary airway epithelial cells obtained from CF patients (F508del/F508del homozygotes) were isolated were cultured on Transwell inserts under air-liquid interface conditions, and studied when fully reaching polarized confluency and a high stable transepithelial electrical resistance (3-5 weeks).

Evaluation of proteolytic activity

Kinetic evaluation of QUB-TL1 (using recombinant trypsin-like proteases) and evaluation of AEC surface proteolytic activity was performed using fluorogenic substrate based assays.

Measurement of ENaC activity

Amiloride-sensitive ENaC current was measured in primary humans AECs using an Ussing chamber (Physiologic Instruments).

ASL height measurement

ASL height was measured using live-cell confocal fluorescence microscopy as published previously [21]; and described further in the online supplement.

Assessment of mucociliary clearance

Mucociliary clearance was quantified by measuring the velocity of microbeads apically applied to differentiated CF AECs.

Statistics

The Mann-Whitney *U*-test was used to compare two unpaired groups, whereas a Kruskal-Wallis test followed by a Dunn's test was used for multiple comparisons. Results are expressed as the means ± s.e.m. with $p<0.05$ deemed statistically significant. Data analysis was performed using GraphPad Prism software (version 5.03).

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Results

Synthesis and characterization of QUB-TL1

Biotin-PEG-Suc-Arg^{P}(OPh)$_2$ (QUB-TL1) (Scheme E1) consists of an arginine-derived diphenylphosphonate moiety that reacts with the active site serine residues of trypsin-like proteases affording a stable phosphonylated and irreversibly inhibited enzyme. QUB-TL1 inhibits the serine proteases tested with a rank order of effectiveness, as assessed by the second order rate constant (k_3/K_i), of: trypsin> HAT (human airway trypsin-like protease)> matriptase> furin> prostasin (Table E1). The selectivity of QUB-TL1 for trypsin-like serine proteases is indicated by a lack of effect against the non-trypsin-like serine proteases neutrophil elastase and chymotrypsin (Table E1), which have a primary specificity for valine and phenylalanine, respectively, at P_1; in contrast to the trypsin-like proteases which have a preference for basic amino acids such as arginine at P_1. The action of QUB-TL1 is irreversible as demonstrated by detection of recombinant trypsin under reducing conditions (Fig. E1). Currently available inhibitors used to inhibit trypsin-like CAPs such as aprotinin and camostat mesylate are cell permeable thus unwanted intracellular protease inhibition is unavoidable. A highly polar pegylated sequence in QUB-TL1, in addition to an arginine residue at P_1 restricts QUB-TL1 to the extracellular surface demonstrated by a lack of effect of the compound on intracellular trypsin-like activity in intact cells (Fig. E2).

QUB-TL1 inhibits trypsin-like activity detected at the extracellular surface of NuLi-1 and CuFi-1 cells

Prostasin, which is sensitive to QUB-TL1 (Table S1), has been reported as the dominant tryptic activity at the apical surface of AECs [9]. Herein we have employed the substrate Boc-Gln-Ala-Arg-NH_2Mec, commonly used to assess prostasin activity, to assess CAP activity in CF airway samples (Fig. 1). As trypsin-like enzymes, in addition to prostasin, are

present in these complex biological samples and may potentially activate this substrate any observed activity in these studies is taken as a measure of general tryptic activity. We observed significantly elevated levels of both cell-attached and soluble tryptic activity in CuFi-1 (CF cell line) compared with NuLi-1 (non-CF) controls (Fig. 1 A and C). We further established cell-attached and secreted tryptic activity was significantly inhibited in the presence of QUB-TL1 in these cells, with a similar effect elicited by aprotinin or camostat mesylate treatment (Fig. 1 B and D). The reduction in enzymatic activity upon QUB-TL1 treatment is not attributable to any toxic effect on airway epithelial cell lines as evidenced by LDH cytotoxicity assay (Fig. E3).

QUB-TL1 inhibits cell surface furin-like activity and affords protection from *Pseudomonas aeruginosa* exotoxin A.-induced cytotoxicity

Cell-attached furin-like activity was readily detectable and moreover found at significantly elevated levels in CuFi-1 cultures compared with NuLi-1 controls (Fig. 2A). As expected the observed furin-like activity was highly sensitive to treatment with inhibitors of furin (QUB-TL1 or furin I) (Fig. 2B). It has been reported elsewhere [14;22], and further demonstrated here using recombinant human enzyme (Fig. 2C), that aprotinin and camostat mesylate do not alter furin activity. Consistent with these findings neither aprotinin nor camostat mesylate had any inhibitory action on cell surface furin-like activity when added to NuLi-1 or CuFi-1 cultures (Fig. 2B). Furin activates a virulence factor released from *Pseudomonas aeruginosa* (*Pseudomonas aeruginosa* exotoxin A (PE)), highly relevant in CF airways, that is 10,000 times more toxic to mice than endotoxin [23-26]. As QUB-TL1 inhibits cell surface furin-like activity we examined the impact of QUB-TL1 on PE-induced cytotoxicity. Initial experiments were performed using submerged airway epithelial cell lines (NuLi-1 and CuFi-1 cells) wherein we confirmed cell viability decreased in a PE dose-dependent manner (Fig.

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2D). QUB-TL1 and furin I significantly rescued PE-induced cytotoxicity, in contrast to camostat mesylate and aprotinin which failed to do so (Fig. 2E). The ability of QUB-TL1 to reduce PE-induced cytotoxicity in differentiated AECs was subsequently confirmed (Fig. 2F).

QUB-TL1 reduces the levels of ENaCγ at the apical surface of primary CF AECs

We visualized the impact of QUB-TL1 on apically located ENaCγ. In the absence of treatment we observed a pool of full-length unprocessed ENaCγ (~90 kDa) at the apical surface of non-CF AECs, which was degraded by the addition of exogenous trypsin (Fig. 3A). Contrastingly, in CF cultures, this inactive form of ENaCγ was absent, but rather these cells exhibited a pronounced pool of processed ENaCγ (~60 kDa), that was not further degraded by trypsin application (Fig. 3A). Together these findings are indicative of elevated CAP-driven proteolysis and activation of ENaC in CF AECs under basal conditions. Importantly when CF cells were treated with QUB-TL1 (for 2 h) the prominent processed form of ENaCγ (~60 kDa) was diminished by ~70% (Fig. 3C). We did not discern any obvious changes with regards ENaCγ status when QUB-TL1 was added to non-CF AECs (Fig. 3A). Moreover when we examined the intracellular fraction of proteins we did not observe any difference between non-CF and CF AECs in the presence or absence of QUB-TL1 (Fig. 3B).

Activated ENaCγ is internalized from the apical surface of primary CF AECs upon QUB-TL1 treatment

We next examined the effect of QUB-TL1 over an extended treatment period (24 h). Similar to the results described in Fig. 3A full length ENaCγ was not identifiable in at the apical surface of CF AECs, yet a processed pool at ~60 kDa was apparent (Fig. 4A). Treatment of CF AECs with QUB-TL1 for 24 h resulted in a sustained reduction this processed pool of

ENaCγ protein at the apical cell surface (Fig. 4A). When we analyzed intracellular ENaCγ in these samples we observed an accumulation of the processed form of ENaCγ over time which was readily visible by 24 h (Fig. 4B). Consistent with this finding when we analyzed ENaCγ levels by confocal microscopy we also observed increased intracellular levels post QUB-TL1 treatment (24 h) in primary CF AECs (Fig. 4C).

QUB-TL1 reduces ENaC-mediated Na^+ absorption in primary CF AECs

We investigated the effect of QUB-TL1 on ENaC-mediated Na^+ transport by assessing the short circuit current (I_{sc}) across primary CF human AECs mounted in an Ussing chamber. When QUB-TL1 (10 μM) was added to these cells a steady decline in amiloride-sensitive ENaC current was observed; similar to that elicited by 10 μg/ml aprotinin (Fig. 5 A and B). Forskolin stimulates insertion of ENaC at the apical cell surface [27]; which is then subject to CAP-mediated activation. CAP-inhibition mediated by QUB-TL1 was found to reduce forskolin-stimulated ENaC activation (Fig. 5C). Neutrophil elastase, which is present in CF airways, is a known activator of ENaC. Intact furin activity is necessary for full elastase-induced ENaC activation [16]. As QUB-TL1 inhibits furin (Table S1 and Fig. 2 A-C) we examined whether QUB-TL1 reduces elastase-mediated ENaC activation. Aprotinin which does not inhibit furin (Fig. 2C) was included as a control. In QUB-TL1 pre-treated cultures elastase could only induce a fractional response (~50% induction of I_{sc}); as defined against the total trypsin inducible pool (Fig. 5 A and D). In contrast aprotinin did not alter elastase-induced ENaC activation which was found to be maximal and not augmented by trypsin application (Fig. 5 A and D). We also examined the impact of QUB-TL1 on primary non-CF AECs wherein the compound was demonstrated to reduce ENaC activity (τ=1.36 minutes); with a slower rate of inhibition observed in CF AECs (τ=3.29 minutes) (Fig. 5E).

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QUB-TL1 increases ASL height and mucociliary clearance rates in primary CF AECs

As QUB-TL1 reduces ENaC activity we proceeded to assess the impact of the compound on ASL height and mucociliary clearance rates. Addition of 50 μM QUB-TL1 to primary CF AECs for a period of 24 h resulted in a ~2-fold increase in ASL height (from a mean value of 8.51 μM to 16.04 μM, N=3) (Fig. 6 A and B). In CF airways mucus stasis occurs due to airway dehydration. As QUB-TL1 improved hydration status of CF AECs we examined whether the compound improved mucociliary clearance also. Mucociliary flow was determined by tracking and measuring the velocity of apically applied microbeads across the CF AEC surface. After addition of QUB-TL1 (50 μM) to primary CF AECs for 24 h we observed markedly improved mucociliary clearance (from 2.88 to 7.68 μM/sec, N=6) (Fig. 6C).

Discussion

The successful use of the CFTR corrector (Kalydeco) as a treatment for CF patients with the G551D mutation demonstrates the considerable therapeutic benefit of small molecules which target the basic defect in CF [28]. Kalydeco is, however, only suitable for a small subset of CFTR mutations (~5% of CF patients), thus the development of additional therapies, in addition to those that target CFTR, is necessary. ENaC is a major pathway for fluid absorption from the ASL compartment leading to compromised innate immunity in the lung. We have developed a compound that selectively targets extracellularly located CAPs, excessively active in CF airways resulting in internalization of active ENaC with concomitant reduction in channel activity. We demonstrate QUB-TL1 markedly improves both ASL height and mucociliary clearance (Fig. 6). As impaired mucociliary clearance represents a key initiating factor for CF pulmonary pathogenesis compounds such as QUB-TL1 may represent valuable tools to improve CF lung health independent of CFTR mutation. We have not yet assessed the impact of QUB-TL1 on lung function in an *in vivo* system due to a lack of a suitable animal model. The lower airways of *cftr*$^{-/-}$ mice do not display CF-like pathology [29]. A transgenic mouse model overexpressing βENaC was subsequently developed that exhibits CF-like disease [30] and was shown later to respond to amiloride treatment with morbidity and mortality reduced by preventive treatment [31]. However in these animals ENaC activity is not modulated by extracellular proteases/protease inhibition likely reflecting the presence of a population of αβ ENaC channels at the surface of the airway epithelial membrane that lack the γ subunit and that are constitutively active [32].

Our observation that CAP activity is increased in CuFi-1 cells (compared with NuLi-1 controls) (Fig. 1 and 2) is consistent with previous studies [9;11;33] including work conducted using primary CF AECs wherein excessive prostasin levels are reported [10].

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Whilst this phenomenon of CAP-antiprotease imbalance in CF airways is increasingly evident the underlying molecular mechanisms remain unclear and warrant further investigation. Speculatively, inflammation-driven changes may be of importance as active matriptase is detected at the site of inflammation in various human skin disorders but not in normal epidermis [34], whilst HAT is found in the sputum of patients with chronic inflammatory respiratory diseases [35]. TGFβ1 (which is associated with pulmonary dysfunction in CF [36]) has been shown to increase furin gene expression in rat synovial cells [37].

Although primarily localized in the trans-Golgi network (TGN), furin cycles to the cell surface where it is implicated in various pathologies via the processing of certain pathogenic proteins to maturity [38]. Here we demonstrate for the first time a surface furin-like activity that is resistant to commercially available trypsin-like protease inhibitors (camostat mesylate and aprotinin) yet sensitive to QUB-TL1 and furin I. This activity is significantly elevated at the apical surface of a CF cell line (CuFi-1), raising the possibility that furin may contribute to the modulation of ENaC at this site. Indeed electrophysiological experiments (discussed further below) conducted using primary AECs indicate QUB-TL1-mediated furin inhibition confers a degree of protection to ENaC upon neutrophil elastase application (Fig. 5A and D). *P. aeruginosa* is the most common pathogen that chronically infects the lungs of adult CF patients, significantly contributing to morbidity and mortality [39]. PE is recognized as a major toxic component of *P. aeruginosa* secreted from ~80% of isolates that contributes to mortality in experimental animals and is detected in the respiratory secretions of patients with CF [25;40;41]. As the intoxication process consists of a furin-mediated processing step [24] we investigated whether QUB-TL1 would elicit any protection from PE. QUB-TL1 significantly rescued PE-induced airway epithelial cell toxicity, in contrast to camostat

mesylate and aprotinin (Fig. 2E and 2F). The ability of QUB-TL1 to inhibit furin, in addition to targeting trypsin-like enzymes, may therefore confer additional benefit.

Proteolysis of ENaCγ at the apical membrane of AECs is the dominant component with regards overall channel activity [42]. Under basal conditions we observe degradation of ENaCγ in primary CF cultures that was not evident in non-CF controls that could be elicited by exogenous trypsin application (Fig. 3A). This observation is consistent with numerous reports which demonstrate in CF cells: (i) protease (CAP)-antiprotease imbalance [11;33] (ii) increased susceptibility of ENaC to proteolysis due to defective regulatory systems i.e. SPLUNC1 and CFTR [43;44] and (iii) hyperactivation of ENaC [5]. Overall this observation supports a mechanism whereby protease-antiprotease imbalance, potentially coupled with a susceptible substrate (ENaC), promotes ENaC hyperabsorption in CF AECs.

Studies using heterologous expression systems demonstrate processed ENaCγ at ~75 kDa [45;46], whereas we observe cleaved ENaCγ (influenced by QUB-TL1 and trypsin application) at an apparent molecular weight of ~60 kDa (Fig. 3A). Although not all cleavage products are functionally relevant our observation is consistent with another recent study, also conducted using normal and CF primary human bronchial epithelial cells, wherein processed ENaCγ is detected at the same molecular weight, and moreover shown to be modulated by cathepsin B activity [47]. As such additional studies are warranted to fully understand the regulation and functional role of this pool of ENaCγ in CF airways. In addition to visualizing surface ENaCγ we also sought to examine ENaC α and β subunits, which are required along with γ to assemble a functional channel. To date, we have been unable to detect these subunits (α and β) at the surface of primary AECs. This is likely due to a lack of suitable antibodies capable of detecting the low levels of endogenous ENaC

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subunits (α and β) present at the cell surface. Net ENaC-mediated Na^+ transport depends not only on the open probability of the channel (Po), controlled by proteolytic activation, but also on the number/density of channels at the cell surface (N) regulated by ubiquitination, internalization and degradation [16]. We demonstrate QUB-TL1 internalizes an active form of ENaCγ, prominent at the apical surface of primary CF AECs (Figs 3 and 4). The internalization of ENaCγ from the apical cell surface represents an intriguing observation. When one considers, not only host-derived, but those proteases released from infecting pathogens, potentially there may be a wide repertoire of CAPs capable of influencing ENaC activity. Feasibly it may prove challenging to inhibit all enzymes capable of stimulating ENaC. As such an intervention which enables "shepherding" of ENaC away from the apical surface may confer channel protection regardless of the composition of extracellularly located CAPs.

The reduction in active ENaCγ at the surface of primary CF AECs (Fig. 3A and 3C) correlates with a steady decline in ENaC-mediated Na^+ transport (Fig. 5A and B) likely reflecting the removal of a pool of ENaC irreversibly activated at the surface of CF AECs by endogenous surface CAPs (prior to compound addition). Newly inserted cell surface ENaC is subsequently subject to protection from proteolysis by QUB-TL1. Consistent with this model, forskolin application, which stimulates insertion of ENaC at the apical cell surface [27], results in a limited induction of I_{sc} in the presence of QUB-TL1 compared with untreated controls (Fig. 5C). Neutrophil elastase, which is found in microgram quantities in the CF lung, is a known activator of ENaC via cleavage of the γ subunit [48;49]. Previous studies demonstrate intact furin activity (using furin I) is required for complete elastase-induced activation of ENaC [16]. QUB-TL1 treatment reduced elastase-induced ENaC activation; in contrast to aprotinin, which does not inhibit furin (Fig. 5A and D).

As CF AECs exhibit elevated levels of surface CAPs (Fig. 1 and 2) one may have expected QUB-TL1 to have a pronounced effect in CF cultures (compared with non-CF); yet this was not the case (Fig. 5E). This may occur as ENaC arriving at the apical surface of CF AECs is partially activated (to a greater degree than in non-CF cells) due to intracellular processing events that are unaffected by cell impermeable QUB-TL1 [11;12;50]. Importantly, the ability of QUB-TL1 to reduce ENaC activity in non-CF cell cultures highlights potential efficacy in indications other than CF characterized by mucus plugging; including chronic obstructive pulmonary disease (COPD), which is a major cause of death worldwide [51;52]. Moreover inactivation of trypsin-like protease activity in chronic airways disease may be of benefit considering HAT, which is readily identifiable in sputum collected from such patients [35], has been demonstrated to enhance mucin gene expression (MUC5AC) and mucus hypersecretion in human airway epithelial cells [53;54], as well as modulating inflammation and fibrosis of the airways due to elevated IL-8 levels [55] and via stimulation of human bronchial fibroblast proliferation [56].

In conclusion, our data is consistent with a mechanism whereby CAPs-antiprotease imbalance provides a molecular bridge between CFTR dysfunction and ENaC hyperactivation in CF AECs. QUB-TL1 acts to restore CAPs-antiprotease balance at the extracellular surface of CF AECs causing a reduction in the amount of active ENaC at this location resulting in improved airways hydration and enhanced mucociliary function. A reduction in infection-related tissue damage is also achieved with regards prevention of PE-induced cell death. Together these finding suggest QUB-TL1 may be efficacious with regards offsetting the development of CF lung disease.

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Figure legends

Fig. 1. QUB-TL1 inhibits trypsin-like activity at the extracellular surface of NuLi-1 and CuFi-1 cells. Tryptic activity was assayed using BOC-QAR-NH_2Mec. (A) Progress curves for cell-attached (N=5) and (C) secreted (N=5) trypsin-like activity. Quantitative data demonstrating the effect of protease inhibitors on these activities is shown for cell-attached (N=5) (B) and secreted samples (N=5) (D). Data represent mean ± s.e.m.; *p<0.05, **p<0.01.

Fig. 2. QUB-TL1 inhibits furin-like activity at the extracellular surface of NuLi-1 and CuFi-1 cells. (A) Progress curves for basal cell attached furin-like activity (N=5). (B) Cell attached furin-like activity in the presence or absence of protease inhibitors (N=5). (C) Recombinant human furin activity in the presence of protease inhibitors (QUB-TL1, 50 μM aprotinin and 50 μM camostat mesylate) (N=4). (D) Cytotoxicity was measured in epithelial cell cultures treated over a range of PE doses. Data is presented as % cell viability based on control cells being assigned as 100% viable and cells exposed to lysis solution taken as maximal LDH release (100% toxicity control) (N=4). Protease inhibitors (50 μM) were added to NuLi-1 and CuFi-1 submerged cultures (E) (N=4) or differentiated cultures (F) (N=3) for 1 h prior to the addition of 2000 ng/ml PE for a further 23 h and cellular cytotoxicity determined. Data represent mean ± s.e.m.; *p<0.05, **p<0.01.

Fig. 3. Assessment of the impact of QUB-TL1 on ENaCγ visualized at the surface of primary CF AECs. ENaCγ protein at the apical cell surface (A) and within the intracellular compartment (B) after cultures were treated in the presence or absence of 50 μM QUB-TL1 (2 h) or 2 μg ml^{-1} trypsin (15 min). (C) Quantification of ENaCγ (~60 kDa pool) at the apical surface of CF AECs as calculated using NIH software (ImageJ) (N=3). Primary AECs (ΔF508/ΔF508) were derived from 3 individual patients. Data represent mean ± s.e.m..

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Fig. 4. QUB-TL1 internalizes active ENaCγ from the apical surface of primary CF AECs. ENaCγ at the apical cell surface (A) and within the intracellular compartment of CF AECs (B) after cultures had been treated in the presence or absence of 50 μM QUB-TL1 (24 h) or 2 μg ml^{-1} trypsin (15 min). Western blots are representative of 2 independent experiments (primary AECs (ΔF508/ΔF508) were derived from 2 individual patients) (C) Confocal microscopy using immunofluorescence labeling of ENaCγ subunit (using the same antibody as the Western blotting experiments in Fig 3. and 4; Abcam #3468) in primary CF AECs post QUB-TL1 treatment (10 μM, 24 h).

Fig. 5. QUB-TL1 decreases ENaC-mediated Na^+ transport across primary CF AECs. A representative I_{sc} recording is shown in (A). After a 10-15 minute equilibration period cultures were incubated with protease inhibitors (either 10 μM QUB-TL1 or 10 μg/ml aprotinin) which were added to the apical chamber followed by the sequential application of 10 μM forskolin, 2.59 μg/ml neutrophil elastase, 20 μg/ml trypsin and 10 μM amiloride. (B) Quantification of the decline in amiloride-sensitive I_{sc} upon protease inhibitor application (for ~45 minutes) (N=2). Primary AECs (ΔF508/ΔF508) were derived from 2 individual patients. (C) Mean amiloride-sensitive forskolin inducible I_{sc} measured in the presence or absence of 10 μM QUB-TL1 (N=4). Primary AECs (ΔF508/ΔF508) were derived from 4 individual patients. (D) Quantification of the % change in I_{sc} as a function of the total trypsin-inducible pool (taken as maximal (100%)) when elastase was added to aprotinin or QUB-TL1 pre-treated cell cultures (N=2). Primary AECs (ΔF508/ΔF508) were derived from 2 individual patients. (E) Summary traces comparing the impact of 10 μM QUB-TL1 in primary non-CF and CF AECs (primary AECs (ΔF508/ΔF508) were derived from 3 individual patients). To isolate ENaC currents, cultures were treated with 10 μM CFTR inhibitor 172 after mounting. Data represent mean ± s.e.m.; *$p<0.05$.

Fig. 6. Effect of QUB-TL1 on ASL height and mucociliary clearance in primary CF AECs. (A) Height of the fluorescently labeled ASL layer in primary CF AECs determined by confocal microscopy. Control data represents initial ASL height before 24 h treatment with QUB-TL1 (N=3). Experiments were performed on two passages (p2 and p3) of primary AECs (ΔF508/ΔF508) obtained from two individual patients (4 measurements overall). One data set from a p2 culture (dashed line) exhibited an unexpectedly large initial ASL height and was treated as an outlier. (B) Representative image of fluorescently labeled ASL in the presence or absence of QUB-TL1. A white bar has been included to denote the measurement of ASL. (C) Mucociliary clearance rates (velocity of microbead movement) in CF primary AECs measured after a 24 h treatment with vehicle control (DMF) or 50 μM QUB-TL1 (N=6). Ø denotes no treatment added to cultures. Data represent mean ± s.e.m. The asterisks denotes significantly different (**$p<0.01$) than the corresponding control value. Primary AECs were obtained from six individual CF patients (ΔF508/ΔF508).

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Fig.1

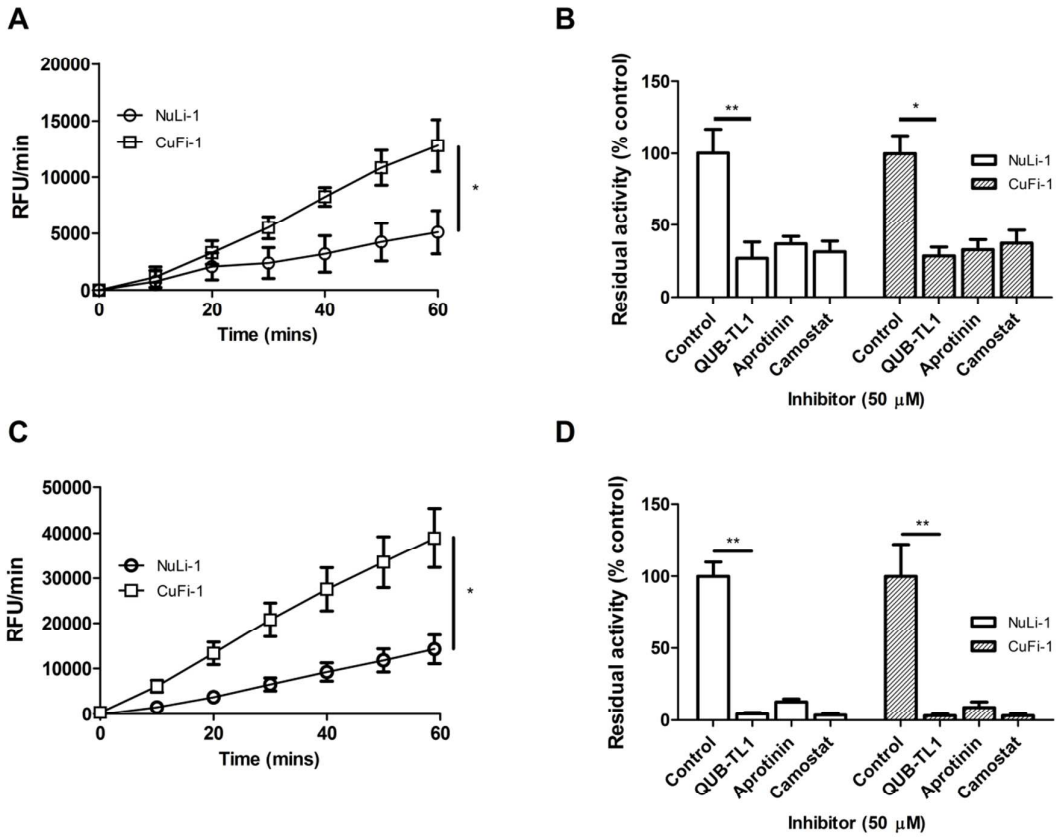
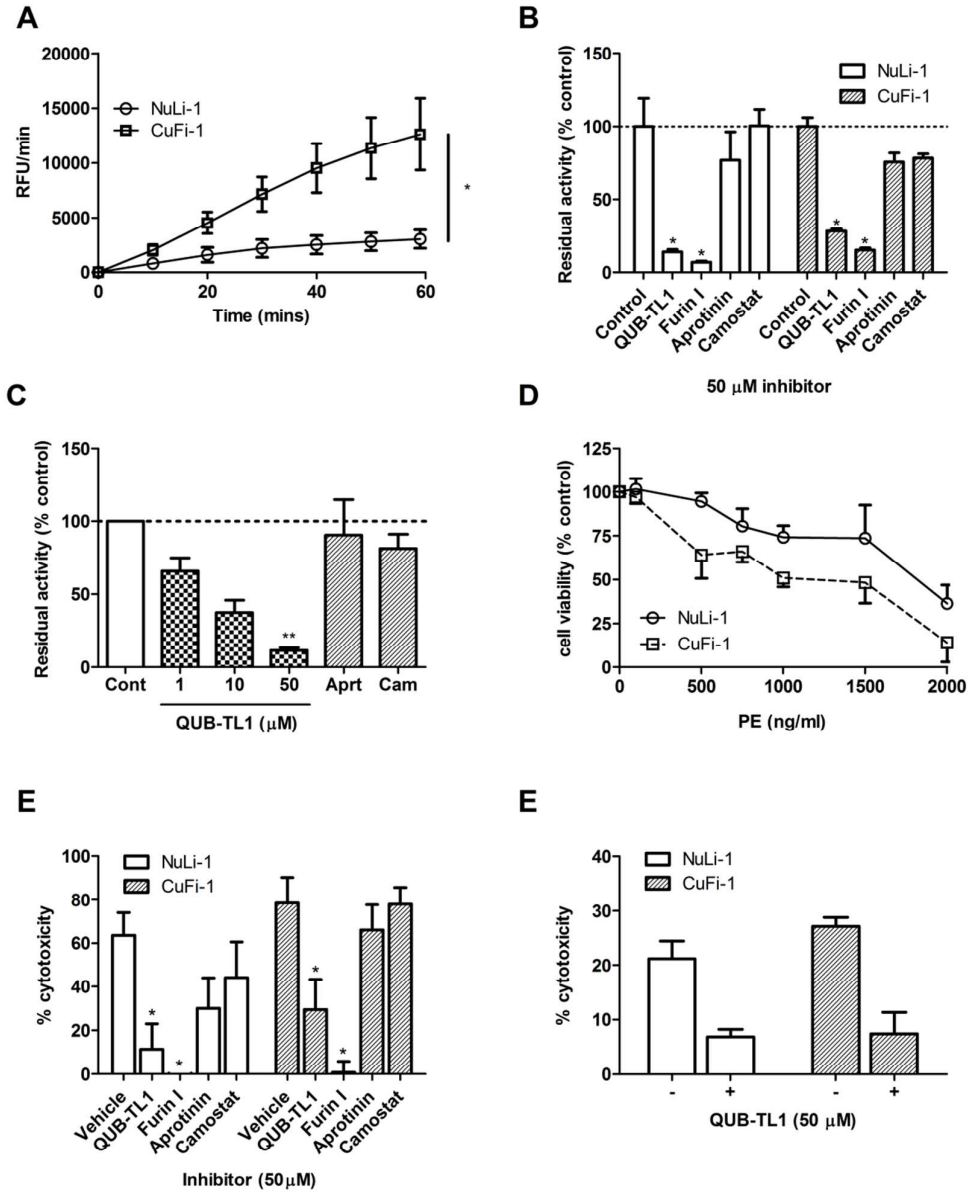


Fig. 2



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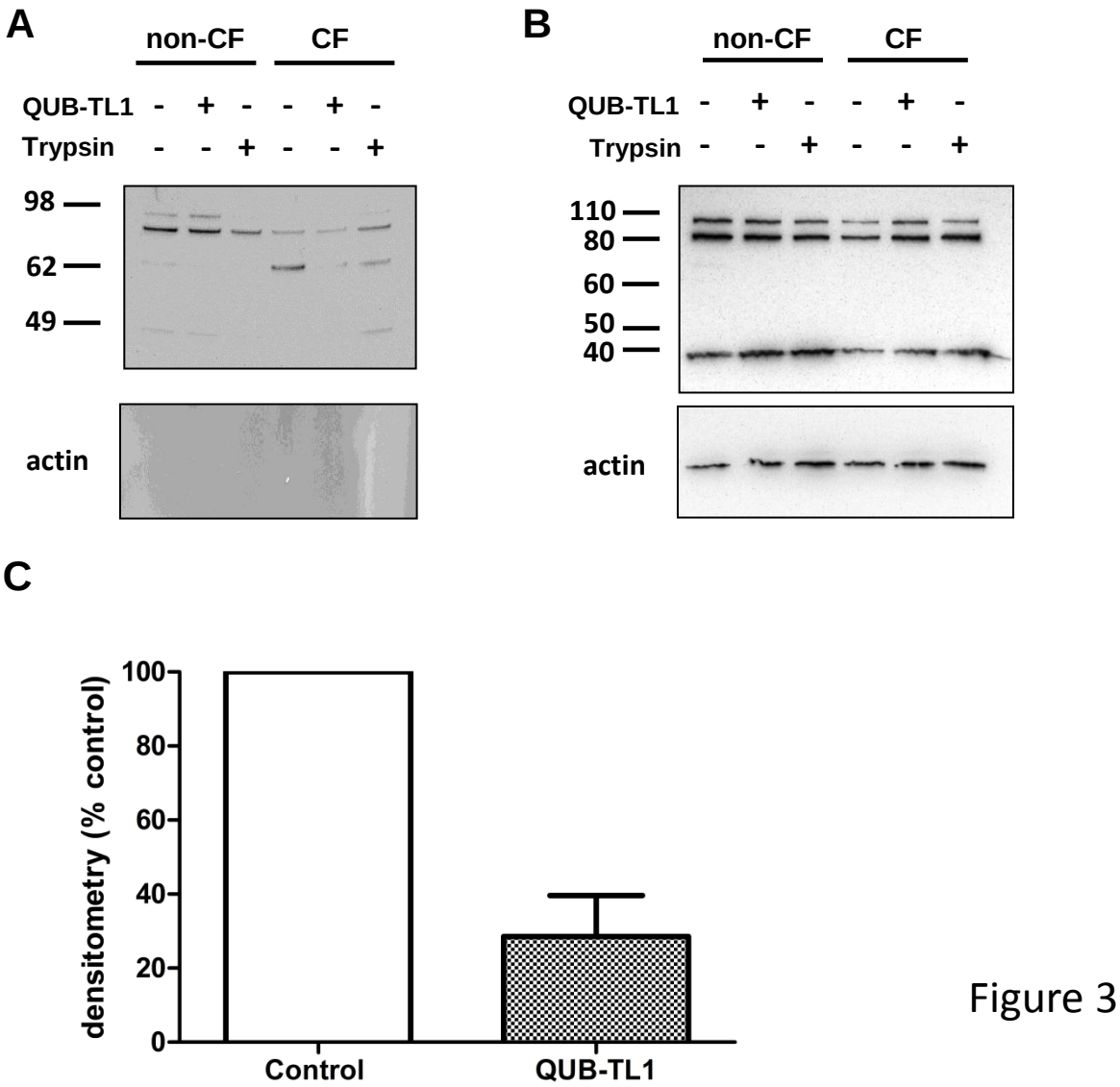

Figure 3

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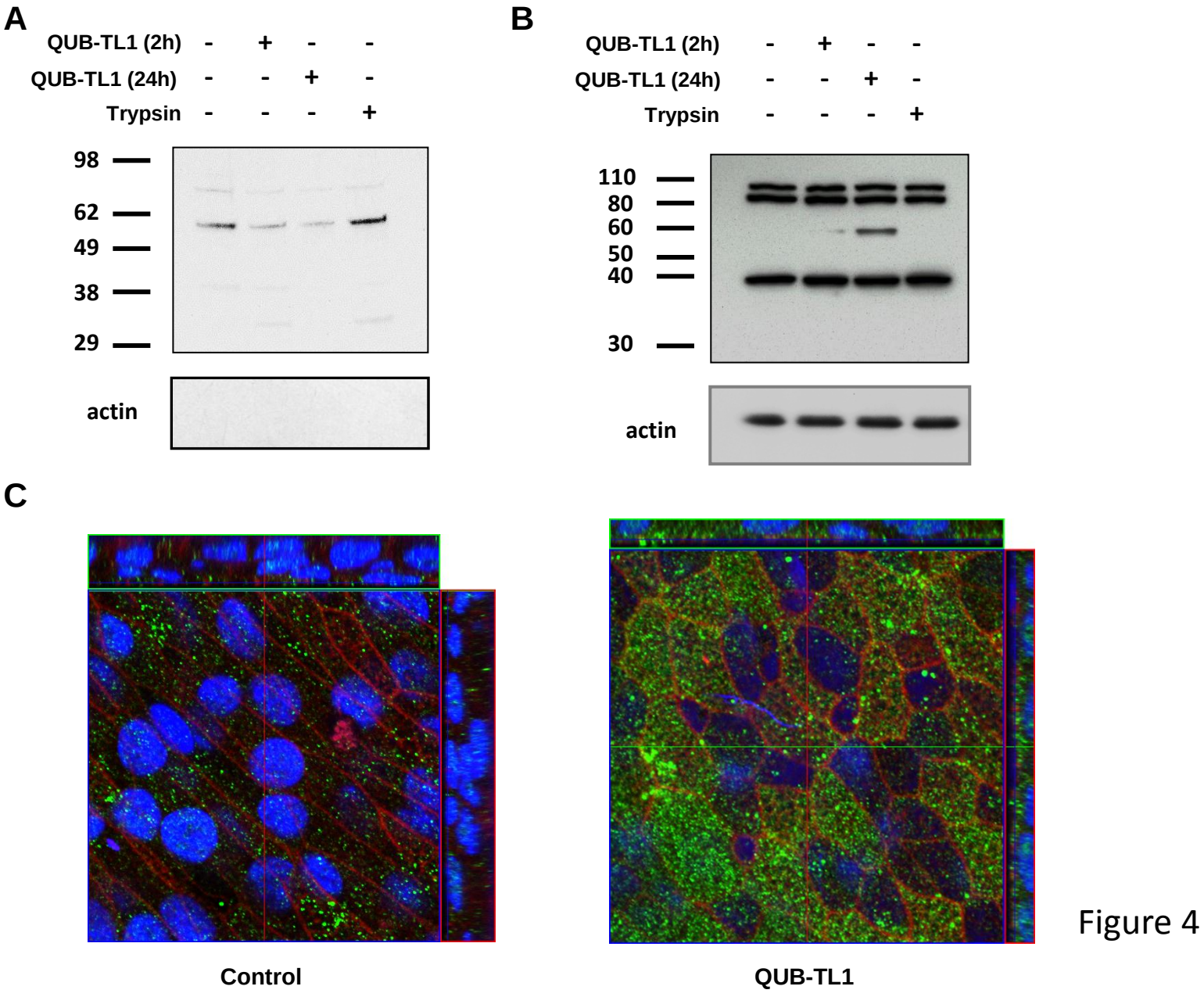

Figure 4

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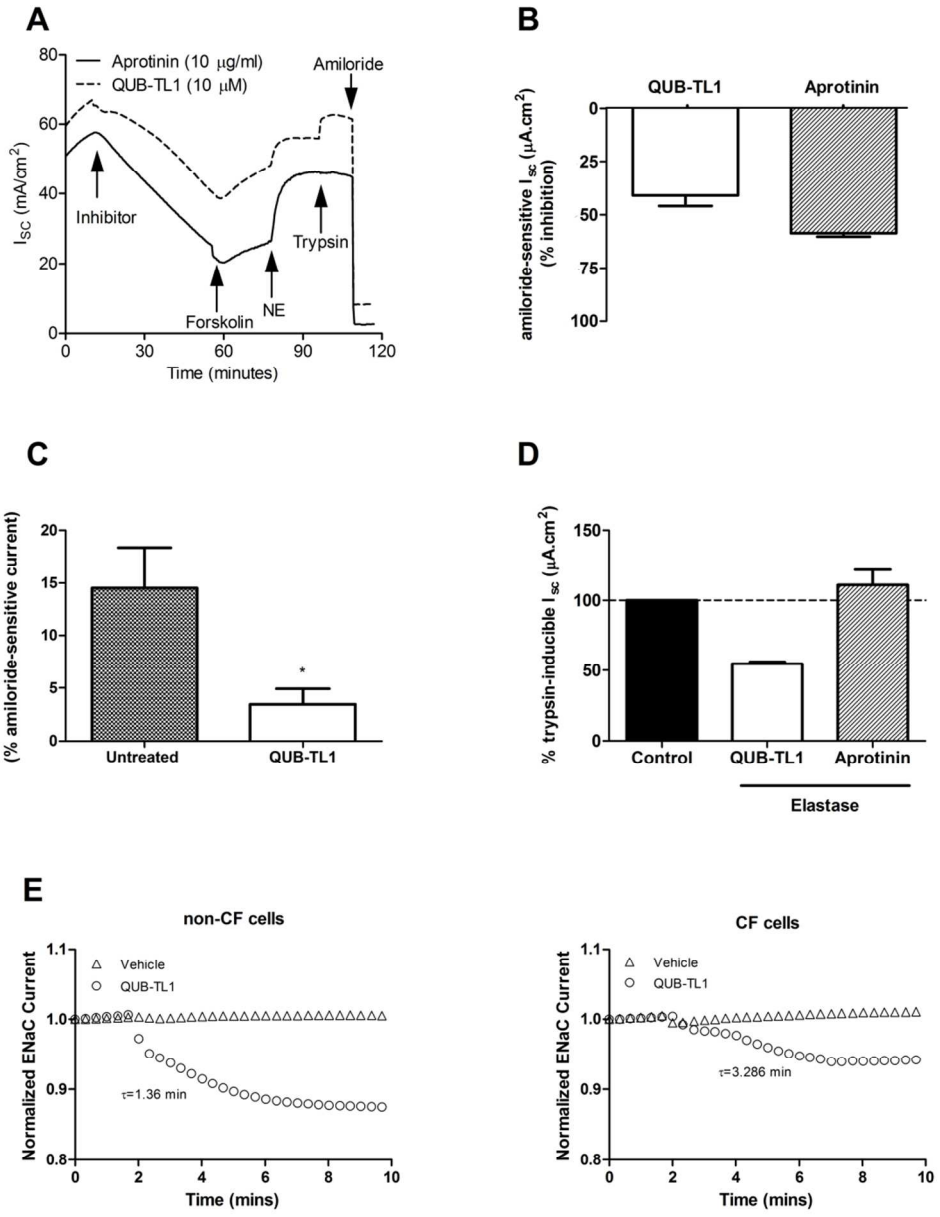
Fig. 5



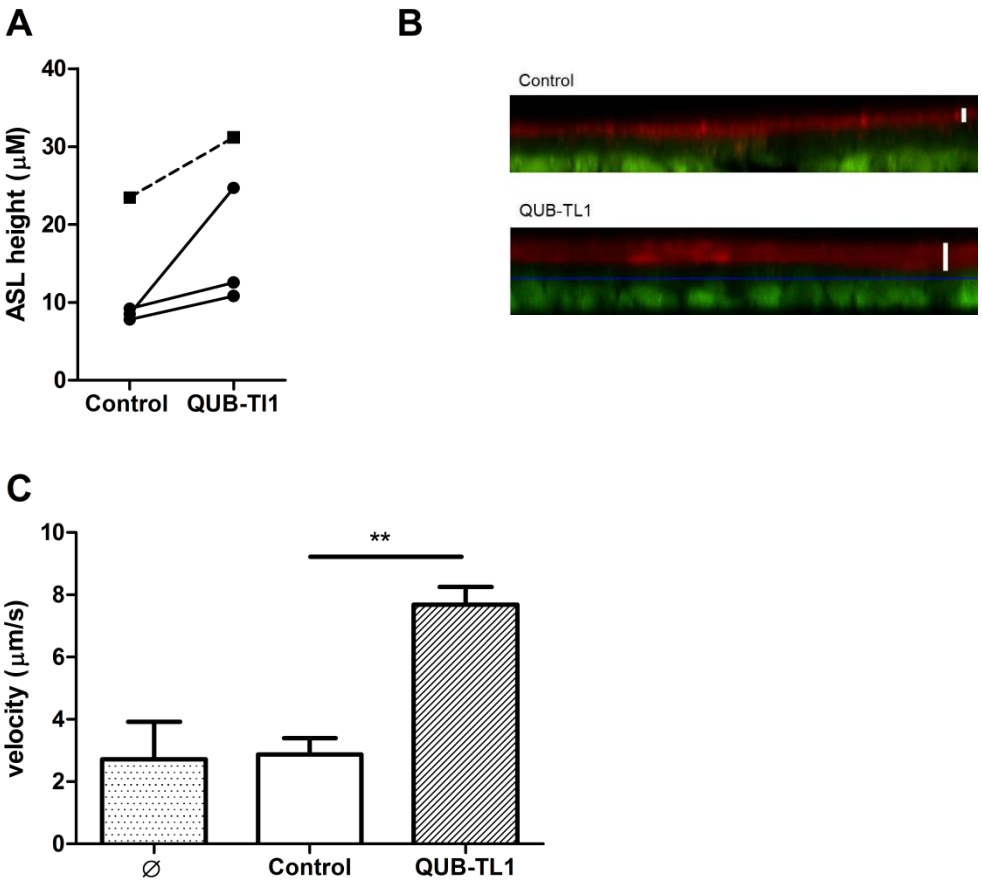


Figure 6

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Inhibition of protease-ENaC signaling improves mucociliary function in cystic fibrosis airways

James A. Reihill, Brian Walker, Robert A. Hamilton, Timothy E. G. Ferguson, J. Stuart Elborn, M. Jackson Stutts, Brian J. Harvey, Vinciane Saint-Criq, Siobhan Hendrick and S. Lorraine Martin

Online Data Supplement

Supplemental Methods

Kinetic evaluation of QUB-TL1

Recombinant trypsin and the fluorogenic substrates Z-Gly-Gly-Arg-NH_2Mec and MeOSuc-Ala-Ala-Pro-Val-NH_2Mec were sourced from Sigma-Aldrich (Poole, Dorset, UK). Recombinant prostasin, matriptase, human airway trypsin and furin, along with the substrates Boc-Gln-Ala-Arg-NH_2Mec and p-Glu-Arg-Thr-Lys-Arg-NH_2Mec were obtained from R&D systems (Abingdon, UK). Recombinant neutrophil elastase was obtained from Calbiochem (Nottingham, UK) whereas Boc-Phe-Ser-Arg-NH_2Mec was from PeptaNova (Sandhausen, Germany). A 10 mM stock solution of inhibitor (in DMF) was diluted along with 50 μM (final concentration) fluorogenic substrate in assay buffer (25 mM Tris/HCl pH 7.4, (for determination of furin activity the assay buffer additionally contained 1 mM $CaCl_2$) to give a range of final inhibitor concentrations (at least 5). Samples along with appropriate solvent (DMF) controls were assayed in microtitre plates maintained at 37°C. The reaction was initiated by the addition of recombinant enzymes and the rate of substrate hydrolysis continuously recorded using λ_{ex} 360 nm, λ_{em} 480 nm, over a period of 90 minutes using a FLUOROSTAR Optima microplate reader (BMG Labtech). The resultant inhibition progress curves for QUB-TL1 generated for each protease were then analyzed according to the kinetic models developed by Tian and Tsou [1] and Walker and Elmore [2], for irreversible inhibitors, using GRAFIT (Erithacus Software).

Cell culture

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NuLi-1 (normal lung) cell line derived from human airway epithelium of normal genotype and CuFi-1 derived from a CF patient (F508del/F508del) were transformed with a reverse transcriptase component of telomerase, hTERT and human papillomavirus type 16 E6 and E7 genes [3]. Cells were grown to form a polarized confluent monolayer on Transwell Permeable Supports (0.4 μM polyester membrane) (Corning Costar) as described previously [4]. These cells were used to assess extracellular trypsin- (Fig. 1) and furin-like activity (Fig. 2), cellular permeability (Fig. E2) and cytotoxicity experiments (Fig. E3). All other experiments were performed using normal or CF primary cultures (homozygous for the F508del mutation) using well differentiated primary airway epithelial cell cultures grown in an air-liquid interface. Immunocytochemistry studies and ASL height measurements were conducted at the Royal College of Surgeons in Ireland (Dublin) using pediatric bronchial epithelial cells obtained by bronchoscopy from patients recruited under the SHIELD (Study of Host Immunity and Early Lung Disease in Children with Cystic Fibrosis). Study directed by Dr Paul McNally, National Children's Hospital Crumlin, Dublin. After collection, cells were washed and seeded in flasks in tobramycin (80 μg/ml, Calbiochem, Nottingham, UK) containing BEGM. When cells reached 70% confluence, they were split and seeded onto semi-permeable support in a mixture (50:50) of tobramycin containing BEGM and retinoic acid (1 μM, Sigma-Aldrich) containing DMEM. After approximately 28 days, cells formed a ciliated well-differentiated polarized epithelium. ENaCγ subunit processing experiments utilized differentiated bronchial epithelial cell cultures provided by Epithelix (Sàrl Epithelix Sàrl, Plan-Les-Ouates, Switzerland) which were maintained with serum free MucilAir culture medium (EP-04MM). Electrophysiological experiments were conducted at the University of North Carolina using bronchial epithelial cells obtained from human donor lung as described previously [5]. In brief, human donor lungs were processed by the UNC CF Center Tissue Core (Chapel Hill, NC, USA) using procedures approved by the UNC Institutional

Committee for the Protection of the Rights of Human Subjects. Epithelial cells were isolated from bronchial segments and cultured on 12 mm Snapwell culture inserts (Corning, Manassas, VA, USA) under air-liquid interface conditions, and studied when fully reaching polarized confluency and a high stable transepithelial electrical resistance (3-5 weeks).

Evaluation of AEC proteolytic activity

The apical surface of polarized epithelial cell cultures was washed for 15 minutes with 80 µl of PBS. This PBS wash was retained and subsequently incubated (15 µg protein) with fluorogenic substrates (Boc-Gln-Ala-Arg-NH_2Mec for tryptic activity and p-Glu-Arg-Thr-Lys-Arg-NH_2Mec for furin-like activity) in order to analyze soluble (secreted) enzymatic activity. Cell surface (cell-attached) proteolytic activity was measured in stringently washed cultures by directly adding the respective fluorogenic peptide substrates (50 µM) to the apical compartment of differentiated cultures and monitoring the formation of –NH_2Mec at λ_{ex} 360 nm and λ_{em} 480 nm using a 5 mm orbital scan every 60 s over a 1 h period.

Lactate dehydrogenase (LDH) (cytotoxicity) assay:

Cytotoxicity was determined using the LDH-Cytotoxicity Colorimetric Assay Kit II (Bio Vision, California, USA) in accordance with the instructions provided by the manufacturer. Addition of the supplied cell lysis solution to control wells was used to determine 100% cytotoxicity in each set of experiments.

Surface biotinylation and Western blotting

AECs were washed with ice-cold PBS prior to the addition of 1 mg ml^{-1} Sulfo-NHS-SS-Biotin (Pierce) (diluted in PBS pH 8) to the apical compartment. Quench buffer consisting of PBS + 100 mM glycine was added to the basolateral compartment at this time. AECs were

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incubated on ice for 30 min at which point the Sulfo-NHS-SS-Biotin solution in the apical compartment was replaced with 500 µl of quench buffer. Cells were washed three times in ice-cold PBS then solubilized in lysis buffer consisting of 150 mM NaCl, 50 mM Tris, 1 mM EGTA, 1 % v/v NP-40 supplemented with Halt™ protease inhibitor cocktail (Pierce). Protein concentrations were subsequently determined using the BCA assay (Pierce). Biotinylated proteins in 100 µg of cellular lysate were recovered by incubation with high capacity NeutrAvidin beads (Pierce) overnight at 4°C in lysis buffer (as described above). Samples were centrifuged at 13,000 x g for 10 min at 4°C and the supernatant fraction removed into a fresh tube (intracellular protein fraction). Biotinylated proteins (apical surface fraction) were eluted from NeutrAvidin beads in Laemmli reducing treatment treatment. Intracellular and apical surface proteins were resolved using standard SDS-PAGE and transferred to PVDF. Membranes were probed with ENaCγ (Abcam #ab3468) or β-actin antibodies (NEB cell signaling).

Immunocytochemistry

Antibodies used were: ENaCγ (Abcam #ab3468). Secondary conjugated antibodies: chicken anti-rabbit 488 nm, chicken anti-goat 568 nm, goat anti-rabbit 488 nm (Invitrogen), goat anti-rabbit horse-radish peroxidase (Abcam). Following treatment, cell monolayers were washed twice in cold PBS and fixed at room temperature in 4 % PFA/PBS. Cells were blocked and permeabilized in 2 % gelatin from cold water fish skin/0.1 % TritonX-100/PBS and incubated with primary antibodies as indicated in the figure legends, for 2 h room temperature (RT) followed by secondary antibodies in a dilution of 1:500 for 30 min RT. Semi-permeable membranes were carefully excised from plastic casing, and embedded apical side up under a glass cover-slip, using Vectashield hardest mounting medium containing DAPI. Plasma membrane staining using WGA Alexa633 nm (200 µg ml^{-1} for 5 min) was performed on ice

before fixation. TRITC-phalloidin to stain intracellular actin was added in combination with the secondary antibodies at a dilution of 1:2000, for 30 min RT. Images were captured using a LSM 710 laser scanning confocal microscope (Zeiss) with 63× oil objective.

Ussing chamber recordings

Cultured bronchial epithelia were mounted in Ussing chamber devices (Physiologic Instruments). The submucosal and mucosal bathing solution was low-Cl^- (3 mm) Krebs bicarbonate Ringer's solution with gluconate. Bioelectric properties were digitally recorded (no. VCC600; Physiologic Instruments, San Diego, CA) using ACQUIRE software (Physiologic Instruments). Voltage was clamped to 0 mV except for 3-s pulses to ±10 mV every 60 s. Short circuit current (I_{SC}) under these conditions is >95% amiloride-inhibited and therefore accurately reflects ENaC-mediated Na^+ absorption. Test compounds (inhibitors, proteases, amiloride, as indicated) were added to the luminal bath. Forskolin (10 μM) was applied bilaterally.

ASL height measurement

Briefly the ASL was labeled with 8 μl PBS containing 1 mg/ml Texas red®-dextran (10 kD; Invitrogen, Auckland, New Zealand) by apical application the day prior to the experiment and cells treated with 50 μM QUB-TL1 for 24 h. Prior to the experiment AECs were stained using Calcein-AM (5 μM, Invitrogen, Auckland, New Zealand) dissolved in medium culture for 60 min and introduced to the basolateral compartment of the insert. The Fluorinet™ electronic fluid Perfluorocarbon-72 (FC-72, 3M, St Paul, USA) was added to the apical compartment of the insert at a volume of 0.5 ml on order to prevent ASL evaporation. Epithelia were Z-scanned using a Zeiss LSM 510 Meta using a 40X objective. For each culture insert, 3 different microscope fields randomly chosen were XZ scanned. In each

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microscope field, the ASL height was measured using the Zeiss LSM Image analyzer software (Carl Zeiss Microlmaging GmbH, Germany) in 9 separate regions randomly determined and then averaged.

Assessment of mucociliary clearance

QUB-TL1 (5 μl of 50 μM solution in PBS) was added to AECs for a period of 24 h at which point mucociliary clearance was monitored using a high speed acquisition camera (Sony) connected to an Axiovert 200M microscope (Zeiss). This involved tracking microbeads (30 μm) that were added onto the apical surface of AECs over a 1 min period. Microbead movement was analysed using ImagePro Plus software (Mediacy) with the velocity of each particle calculated in order to determine the rate of mucociliary clearance.

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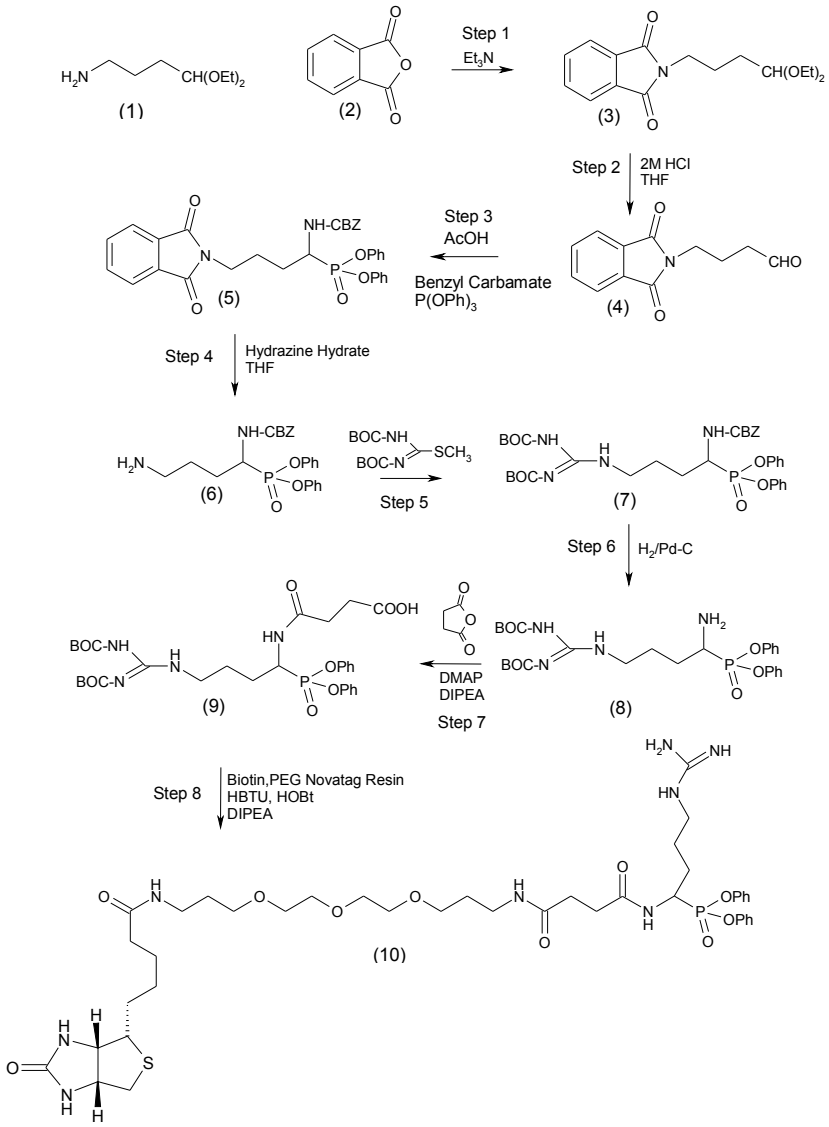
Supplemental Scheme



Scheme E1. QUB-TL1 synthetic scheme.

In essence, 4-(*N*-phthaloyl)butyraldehyde (3), {prepared from phthalic anhydride (2) and 4-aminobutyraldehyde diethylacetal (1)} was heated with triphenyl phosphite and benzyl carbamate in AcOH to give N^{ω}-Pth-N^{α}-Cbz-Orn^{P}(OPh)$_2$ (5). Removal of the phthaloyl group using hydrazine hydrate followed by reaction with *N,N'*-bis-Boc-*S*-methylthiourea yielded

the arginine derivative (7), which was deprotected at the α-nitrogen by catalytic hydrogenation using H_2 and 10% Pd/C (w/w). The deprotected material (8) was then reacted with succinic anhydride and coupled to Biotin-PEG-Novatag Resin using HATU/DIPEA mediated coupling, and the product (10) was released from the resin by treatment with TFA/H_2O/TIPS (95%/2.5%/2.5%; v/v/v). The identity of QUB-TL1 was confirmed using Electro- Spray Ionization Mass Spectrometry (ESI-MS).

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Supplemental Table

Proteinase	Fluorogenic substrate	K_i (M)	k_3 (min^{-1})	k_3/K_i ($M^{-1}.min^{-1}$)
Trypsin	Gly-Gly-Arg-NH_2Mec	1.735×10^{-7}	0.284	1.64×10^{6}
HAT	Phe-Ser-Arg-NH_2Mec	8.46×10^{-6}	1.18	1.39×10^{5}
Matriptase	Gln-Ala-Arg-NH_2Mec	3.17×10^{-6}	0.17	5.3×10^{4}
Furin	Glu-Arg-Thr-Lys-Arg-NH_2Mec	3.48×10^{-6}	0.024	6.9×10^{3}
Prostasin	Gln-Ala-Arg-NH_2Mec	2.3×10^{-6}	0.0103	4.48×10^{3}
Neutrophil elastase	Ala-Ala-Pro-Val-NH_2Mec	NI	-	-
Chymotrypsin	Ala-Ala-Pro-Phe-NH_2Mec	NI	-	-

Table E1. Kinetic evaluation of QUB-TL1. A summary of determined overall second order rate constants (k_3/Ki) for the range of serine proteases tested against QUB-TL1.

Supplemental Figures

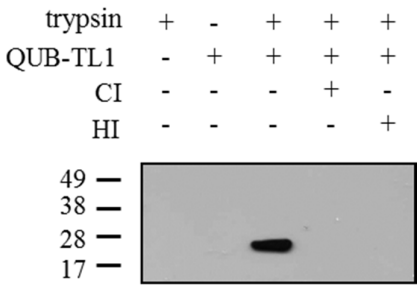

Fig. E1. Irreversible inhibition of recombinant trypsin by QUB-TL1**.** Western blot performed under reducing conditions demonstrating QUB-TL1 is irreversibly bound to trypsin visualized using streptavidin-HRP which detects the biotin reporter group on the compound. Controls include pre-treatment with a competitive inhibitor (CI) (50 μM aprotinin) or heat inactivation (HI) of trypsin (65°C, 10 minutes).

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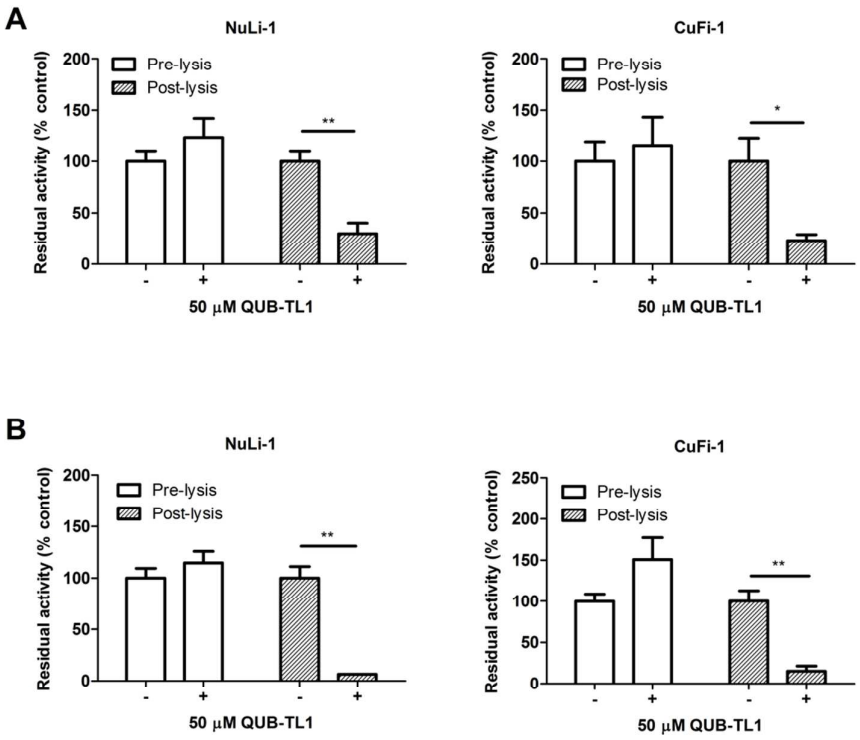

Fig. E2. QUB-TL1 is cell impermeable. QUB-TL1 (50 μM) or vehicle control was added to the apical compartment of differentiated NuLi-1 and CuFi-1 epithelial cell cultures for 2 h, washed (X3) with PBS, prior to solubilization in lysis buffer (denoted Pre-Lysis). Retained untreated lysate was subsequently probed in the presence or absence of QUB-TL1 (denoted Post-Lysis). Enzymatic activity in pre- and post-lysis treated samples was assessed by incubating 15 μg of protein (determined by BCA assay) with (A) 50 μM Boc-Gln-Ala-Arg-NH_2Mec (N=4) or (B) 50 μM p-Glu-Arg-Thr-Lys-Arg-NH2Mec (N=4). Data represent mean ± s.e.m. The asterisks denotes significantly different (*P<0.05, **P<0.01) than the corresponding control value.

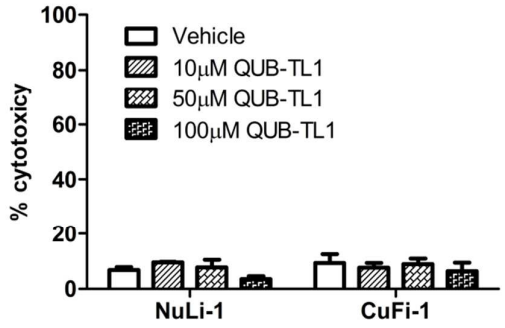

Fig. E3. QUB-TL1 is non-toxic when added to AECs. LDH release from QUB-TL1-treated cells (submerged cultures) was used to determine % cytotoxicity (N=3).