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Correction of ΔF508-CFTR trafficking defect by the bioavailable compound glafenine

Renaud Robert*, Graeme W. Carlile*, Jie Liao, Haouaria Balghi, Pierre Lesimple, Na Liu, Bart Kus, Daniela Rotin, Martina Wilke, Hugo R. de Jonge, Bob J. Scholte, David Y. Thomas & John W. Hanrahan

Departments of Physiology (R.R., J.L., H.B., P.L., N.L., J.W.H.) and Biochemistry (G.W.C., D.Y.T.), McGill University, Montréal, Québec, Canada, H3G 1Y6

The Hospital for Sick Children, Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada, M5G 1X8 (B.K., D.R.)

Departments of Biochemistry (M.W., H. R. de J) and Cell Biology (B.J.S.), Erasmus University Medical Centre, Rotterdam, the Netherlands

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Running title: Glafenine corrects ΔF508-CFTR trafficking

Address correspondence to: Dr. John W. Hanrahan or Dr. Renaud Robert, McIntyre Building, Physiology Department, 3655 Promenade Sir William Osler, Montreal, Quebec, H3G 1Y6, Canada Phone: 514-398-8320; Fax: 514-398-7452; E-mail: john.hanrahan@mcgill.ca or renaud.robert@mcgill.ca

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Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; HTS, high throughput screening; ER, endoplasmic reticulum; 3HA, three haemaglutinin-epitope tags; BHK, baby hamster kidney

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Abstract

Cystic Fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, which encodes a cAMP-activated anion channel expressed in epithelial cells. The most common mutation ΔF508 leads to protein misfolding, retention by the endoplasmic reticulum, and degradation. One promising therapeutic approach is to identify drugs that have been developed for other indications but which also correct the CFTR trafficking defect, thereby exploiting their known safety and bioavailability in humans and reducing the time required for clinical development. We have screened approved, marketed and off-patent drugs with known safety and bioavailability using a ΔF508-CFTR trafficking assay. Among the confirmed hits was glafenine, an anthranilic acid derivative with analgesic properties. Its ability to correct the misprocessing of CFTR was confirmed by *in vitro* and *in vivo* studies using a concentration that is achieved clinically in plasma (10 μM). Glafenine increased the surface expression of ΔF508-CFTR in BHK cells to ~ 40% of that observed for wt-CFTR, comparable to the known CFTR corrector VRT-325. Partial correction was confirmed by the appearance of mature CFTR in Western blots and by two assays of halide permeability in unpolarized BHK and HEK cells. Incubating polarized CFBE41o$^-$ monolayers and intestines isolated from ΔF508-CFTR mice (treated *ex-vivo*) with glafenine increased the short-circuit current (I_{sc}) response to forskolin + genistein, and this effect was abolished by 10 μM $CFTR_{inh}172$. *In vivo* treatment with glafenine also partially restored total salivary secretion. We conclude that the discovery of glafenine as a CFTR corrector validates the approach of investigating existing drugs for the treatment of CF, although localized delivery or further medicinal chemistry may be needed to reduce side-effects.

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Introduction

Cystic fibrosis (CF) is a common autosomal recessive disorder characterized by pulmonary disease and exocrine gland dysfunction (O'Sullivan and Freedman, 2009). It is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, which encodes a cAMP-activated anion channel that is expressed in epithelia of the lung, pancreas, intestine, liver, sweat glands and reproductive tract (Rommens et al., 1989; Riordan et al., 1989; Hanrahan et al., 2003). Among the >1600 known mutations of the CFTR gene (www.genet.sickkids.on.ca/cftr/), the most common is a deletion of phenylalanine at position 508 in the first nucleotide-binding domain (NBD1). This mutation (ΔF508), which is present on at least one chromosome in ~90% of people with CF, impairs the folding, trafficking, membrane stability, and gating of the ΔF508-CFTR channel protein (Cheng et al., 1990; Lukacs et al., 1993; Hwang and Sheppard, 2009). Although the mutant is retained in the endoplasmic reticulum (ER) and rapidly degraded in the proteasome (Kopito et al., 1999) the trafficking defect can be reversed by incubation at ≤30°C or by chemical chaperones such as glycerol or phenylbutyrate (Denning et al., 1992; Sato et al., 1996; Rubenstein et al., 1997). The rescued protein has reduced metabolic stability and is less responsive to stimulation by cAMP agonists compared to wild-type CFTR (Dalemans et al., 1991; Swiatecka-Urban et al., 2005). Nevertheless, recovery of a small fraction of CFTR function may be sufficient to alleviate CF symptoms (Johnson et al., 1992).

Some small molecules, named CFTR correctors, have been reported to partially restore ΔF508-CFTR function, including 4-phenylbutyrate and curcumin (Rubenstein et al., 1997; Egan et al., 2004), the quinazoline CFTR corrector VRT-325, and diverse compound families such as benzo[c]quinoliziniums, aminoarylthiazoles, bis-aminomethylbithiazoles and phosphodiesterase Type 5 inhibitors (Dormer et al., 2001; Dormer et al., 2005; Loo et al., 2005 ; Pedemonte et al., 2005 ; Van Goor et al., 2006 ; Carlile et al., 2007; Robert et al., 2008). Moreover, clinically

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available drugs such as sildenafil and the alpha-glucosidase inhibitor miglustat also partially correct ΔF508-CFTR processing (Dormer et al., 2005; Norez et al., 2006). One strategy for CF drug development is to exploit the known safety and bioavailability of clinically available drugs to reduce the time needed for pre-clinical development. For this reason we have screened the Prestwick Chemical Library using a high throughput screening (HTS) assay that identifies ΔF508-CFTR trafficking correctors (Carlile et al., 2007). This collection contains 1120 pure and structurally diverse compounds of which 90% are drugs that have been marketed for a broad spectrum of therapeutic actions in neuropsychiatry, cardiology, immunology, and the treatment of pain and inflammation. The remaining 10% are bioactive alkaloids or related substances with drug-like characteristics.

One hit from this HTS campaign was glafenine, which has been used previously in the treatment of pain (Ginsberg et al., 1983). Here we evaluated the potency of glafenine as a corrector of ΔF508-CFTR processing in several *in vitro* model systems including non-polarized epithelial cells, human airway epithelial cell monolayers, and freshly isolated intestines from CF mice. We found partial correction of ΔF508-CFTR trafficking in these preparations and also *in vivo* using transgenic CF mice, suggesting that glafenine may be useful in the development of therapeutics for the treatment of CF.

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Materials and Methods

High throughput screening assay

Screening was performed using BHK cells that stably express ΔF508-CFTR bearing three tandem haemagglutinin-epitope tags (3HA) and linker sequences in the fourth extracellular loop after amino acid 901 (Carlile et al., 2007; Robert et al., 2008). Rescue of the mutant by test compounds was monitored by measuring antibody binding to cells that had been fixed with paraformaldehyde (for details see Carlile et al., 2007).

YFP fluorescence assay

Strongly adhesive Human Epithelial Kidney cells stably expressing both the human macrophage scavenger receptor (HEK293 Griptite) and ΔF508-CFTR were plated in 96-well plates and transiently transfected with pcDNA3 plasmid encoding a halide sensitive variant of eYFP (H148Q/I152L). After 24h cells were exposed to 10μM test compound in triplicate and incubated for an additional 24h. Cells were then stimulated with 25μM forskolin, 45μM IBMX and 50μM genistein for 20 minutes and the high content screening assay was performed using a Cellomics platform as described by Trzcinska-Daneluti et al. (2009). 50mM iodide was added robotically and the resulting decrease in fluorescence was measured. Images were taken at time 0, stored and used later to calculate a mask that selected cells which expressed YFP at time 0 for halide flux measurements. Quenching was detected in 15 images taken over the course of an experiment lasting 40 seconds. Results were generated from 150-300 cells per well.

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Immunoblot analysis

Total protein was quantified in cell lysates using the Bradford assay (BioRad), separated using SDS-PAGE (6% polyacrylamide gels), and analyzed by Western blotting as described previously (Robert et al., 2008). Western blots were blocked with 5% skimmed milk in PBS and probed overnight at 4^{o}C with a monoclonal primary anti-CFTR antibody (clone M3A7, Chemicon) diluted 1:1000. The blots were washed four times in PBS before adding the secondary HRP-conjugated anti-mouse antibody at a dilution of 1:5000 (Amersham) for one hour at room temperature, then washed again five times in PBS and visualized using chemiluminescence (Pierce). The relative intensity of each CFTR glycoform (band B or band C) was estimated by densitometry using ImageJ software and reported as a percentage of wild-type CFTR after normalization to the amount of tubulin in the same lane.

Immunostaining

BHK and CFBE cells were seeded onto 25-mm diameter glass coverslips and incubated at 37°C overnight. Another aliquot of CFBE cells was seeded onto 12-mm fibronectin-coated Snapwell inserts (Corning Incorporated) and the apical medium was removed after 24h to establish an air-liquid interface. Cells were then treated with 0.1% DMSO (vehicle control), 10µM glafenine in DMSO, or incubated at 29°C for 24h. Cells grown on coverslips were rinsed in TBS, fixed with 3% paraformaldhehyde (PFA) in TBS for 20 min at room temperature, then rinsed in TBS, permeabilized with 0.1% Triton X-100 in TBS for 10 min, and rinsed again with TBS. CFBE monolayers grown on Snapwell filters were fixed in 4% PFA in PBS for one hour, rinsed with PBS, extirpated, and imbedded in paraffin. Sections (5µm) were laid on microscopy slides, re-hydrated by successive immersion in xylene (3 baths), ethanol (2 baths), 70% ethanol, 50%

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ethanol and water. The samples were then stained by indirect immunofluorescence as follows: After fixation, non-specific binding sites were blocked with TBS containing 0.5% BSA for 1h at room temperature, then the cells were stained with primary antibody (anti-CFTR C-terminal monoclonal, 1:100, clone 24–1, R&D Systems) for 90 min at room temperature and rinsed in TBS containing 0.5% BSA. Next, samples were incubated either with the goat anti-mouse FITC or Cy3-conjugated secondary antibody to detect CFTR (1:1000; Jackson ImmunoResearch Laboratories). Samples were mounted in Prolong Gold anti-fade solution (Invitrogen) with or without DAPI for observation with a confocal laser scanning microscope (Carl Zeiss, Confocor LSM 510 META, ×63/numerical aperture 1.4, oil).

Halide efflux assay

Assays were performed using a robotic liquid handling system (BioRobot 8000, Qiagen) and Qiagen 4.1 software. Cells were cultured to confluence in 24-well plates. Cells were treated for 24h with vehicle (DMSO, 1:1000), with the test compounds glafenine, VRT-325 (Van Goor et al., 2006) or corr-4a (Pedemonte et al., 2005) or incubation at 29^{o}C. The medium in each well was then replaced with 1 ml of iodide loading buffer: 136 mM NaI, 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 11 mM glucose, 20 mM Hepes, pH 7.4 with NaOH) and incubated for 1h at 37^{o}C. At the beginning of each experiment, the loading buffer was removed by aspiration and cells were washed eight times with 300 µl efflux buffer (same as loading buffer except that NaI was replaced with 136 mM $NaNO_3$) to remove extracellular I^-. Efflux was measured by replacing the medium with 300 µl fresh efflux buffer at 1 min intervals for up to 11 min. The first four aliquots were used to establish a stable baseline, then buffer containing 10µM forskolin + 50µM genistein was used to stimulate CFTR activity. Iodide concentration was measured in each aliquot (300 µl) using an iodide-sensitive electrode. Relative iodide efflux rate was calculated using the difference

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between maximum (peak) iodide concentration during stimulation and minimum iodide concentration before stimulation (in $\mu M.min^{-1}$). Data are presented as means ± SEM.

Voltage-clamp of CFBE41o- cell monolayers

Short-circuit current (I_{sc}) was measured across monolayers in modified Ussing chambers. CFBE41o$^-$ cells (10^6) were seeded onto 12-mm fibronectin-coated Snapwell inserts (Corning Incorporated) and the apical medium was removed after 24h to establish an air-liquid interface. Transepithelial resistance was monitored using an EVOM epithelial volt-ohmmeter and cells were used when the transepithelial resistance was 300-400 $\Omega.cm^2$. ΔF508-CFBE41o$^-$ monolayers were treated on both sides with optiMEM medium containing 2% (v/v) FBS and one of the following compounds: 0.1% DMSO (negative control), 10μM glafenine, 10μM VRT-325, or cells were incubated at 29°C (positive control) for 24h before being mounted in EasyMount chambers and voltage clamped using a VCCMC6 multichannel current-voltage clamp (Physiologic Instruments). The apical membrane conductance was functionally isolated by permeabilizing the basolateral membrane with 200 μg/ml nystatin and imposing an apical-to-basolateral Cl^- gradient. The basolateral bathing solution contained (in mM) 1.2 NaCl, 115 Na-gluconate, 25 $NaHCO_3$, 1.2 $MgCl_2$, 4 $CaCl_2$, 2.4, KH_2PO_4, 1.24 K_2HPO_4 and 10 glucose (pH 7.4). The $CaCl_2$ concentration was increased to 4mM to compensate for the chelation of calcium by gluconate. The apical bathing solution contained (in mM) 115 NaCl, 25 $NaHCO_3$, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 2.4 KH_2PO_4, 1.24 K_2HPO_4 and 10 mannitol (pH 7.4). The apical solution contained mannitol instead of glucose to eliminate currents mediated by Na^+-glucose co-transport. Successful permeabilization of the basolateral membrane was obvious from the reversal of I_{sc} under these conditions. Solutions were continuously gassed and stirred with 95% O_2-5% CO_2 and maintained at 37°C. Ag/AgCl reference electrodes were used to measure transepithelial voltage and pass

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current. Pulses (1mV amplitude, 1s duration) were delivered every 90s to monitor resistance. The voltage clamps were connected to a PowerLab/8SP interface for data collection. CFTR was activated by adding 10µM forskolin + 50µM genistein to the apical bathing solution.

Ex-vivo *experiments*

Glafenine was tested ex-vivo using ileum from homozygous ΔF508-CFTR mice (backcrossed on the FVB genetic background for more than 12 generations, *Cftr*$^{tm1\ Eur}$; van Doorninck et al., 1995) and wild-type littermates controls. Only female mice, 14–17 weeks old and weighing 24-30g were used in this assay, and were genotyped by standard PCR methods using tail DNA. The mice were kept in the animal facility at McGill University and fed a high protein diet (SRM-A, Hope Farms, Woerden, NL) modified to contain pork instead of beef. All procedures followed Canadian Institutes of Health Research (CIHR) guidelines and were approved by the faculty Animal Care Committee. For ex-vivo experiments, the last third of the ileum was stripped of muscle and several pieces were mounted immediately in Ussing chambers. After equilibration for 10-15 minutes, I_{sc} was measured at time 0h and 10µM forskolin + 50µM genistein were added (0h). Following this stimulation, each piece of ileum was rinsed and incubated in William's E-Glutamax medium supplemented with insulin (10µg/ml), 100U/ml penicillin, and 100µg/ml streptomycin and dexamethasone (20µg/ml). Each piece was exposed to 10µM glafenine dissolved in DMSO or to vehicle alone (0.1% DMSO) for 4h, then the I_{sc} response to forskolin + genistein was measured again. Tissue viability was confirmed by adding 10mM glucose to stimulate electrogenic Na^+-glucose co-transport (the apical solution normally contained mannitol instead of glucose). Results are expressed as the mean ± SEM of n pieces of ileum from N mice.

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Salivary secretion

The procedure followed those described by Best & Quinton (Best & Quinton, 2005). Only male homozygous Δ508-CFTR (*Cftr*$^{tm1\ Eur}$) and wild-type mice of the same strain, 10-12 weeks old and weighing 20-25g were used in this assay. A micro osmotic pump (Alzet Model 1003D) was fixed under the skin on the back of each mouse to deliver glafenine or vehicle for 48h. The micro osmotic pumps were filled with 90µl of solution containing glafenine in DMSO (50mg/ml) or 90µl of DMSO (controls). The mean pump rate was 1µl per hour, representing a delivery rate of 50µg of glafenine per hour. Mouse body weight and behaviour were monitored to assess the well-being of the mice. After 48h, mice were anaesthetized with ketamine and diazepam and treated with a subcutaneous injection of 1mM atropine into the left cheek. Small strips of Whatman filter paper were placed inside the previously injected cheek for ~4 min to absorb any saliva. Solution containing 100µM isoprenaline and 1mM atropine was then injected into the left cheek at the same site to induce secretion at time zero and the filter paper was replaced every minute for 30 minutes. Each piece of filter paper was immediately placed and sealed in a pre-weighed vial and the time of removal was recorded. The total amounts of salivary secretion were normalized to the mass of the mouse in grams. Results are expressed as the mean ± SEM of n mice.

Statistics

All results are expressed as the mean ± SEM of *n* observations. Data sets were compared by analysis of variance (ANOVA) or Student's t-tests using GraphPad Prism version 4. Differences were considered statistically significant when $p<0.05$. ns: non significant difference, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

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Results

To test the hypothesis that drugs developed for other indications might correct ΔF508-CFTR trafficking, we identified and validated hit compounds as outlined in Figure 1A. For the first step we used a protein trafficking assay based on immunodetection of HA epitopes in the fourth extracellular loop of ΔF508-CFTR (Carlile et al., 2007). A primary screen of 1120 compounds yielded 61 positive compounds having fluorescence values >1 standard deviation above the mean for the plate. These hits were selected (cherry picked) and re-tested in duplicate. Positive compounds with intrinsic fluorescence were not considered further. From the original 61 positives, 50 were confirmed by immunodetection of surface HA epitopes at the cell surface and counter screened for functional correction using an eYFP fluorescence quenching assay in which halide permeability is detected by iodide entry using a high content screening platform (see methods). Of the 50 confirmed hits in the trafficking assay, 15 were also positive according to the functional assay, leading to 25 – 70% quenching of the YFP signal of the positive control (cells incubated at low temperature). Thus, based on the sequential use of trafficking and functional assays we identified a small number of known drugs or drug-like molecules with ΔF508-CFTR corrector activity.

One of the hits was glafenine hydrochloride (2-[(7-chloro-4-quinolinyl)amino]benzoic acid 2,3-dihydroxypropyl ester; Figure 1B), a non-steroidal anti-inflammatory drug which has been used previously as an analgesic although it is not presently on the market in most countries. According to the trafficking assay glafenine increased ΔF508-CFTR surface expression by 40% when compared with ΔF508-CFTR cells treated with vehicle alone and normalized to BHK cells expressing wild-type CFTR (Figure 1C). We compared the effects of glafenine with those of the established corrector VRT-325 under identical conditions (Van Goor et al., 2006). VRT-325,

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which has some toxicity in cell culture, caused a similar increase in ΔF508-CFTR cell surface expression (36%), although the level of surface expression was still less than following temperature correction at 29°C, or when compared with a representative cell line expressing 3HA-tagged WT-CFTR, which was taken as 100% when normalizing the values obtained for each experiment under different condition (Figure 1C).

Glafenine effects on trafficking were further confirmed using the YFP functional assay (Figure 1D). Treating cells with 10μM glafenine for 24h enhanced the cAMP-stimulated iodide influx into cells expressing ΔF508-CFTR and YFP, indicating that functional CFTR at the plasma membrane was increased (Figure 1D), although glafenine and VRT-325 both caused less rescue than incubation at low temperature (Figure 1C and D).

To further validate glafenine as a CFTR corrector, its effect on protein expression and maturation was analyzed by immunoblotting (Figure 2A). Maturation was confirmed by the appearance of the complex glycosylated (band C) form of ΔF508-CFTR in BHK cells after treatment with 10μM glafenine for 24h, consistent with results from the screening assay (Figure 2A). The extent of correction is shown semi-quantitatively in Figure 2B. Glafenine increased the expression of the immature CFTR glycoform (band B) by 8-fold and the mature CFTR glycoform (band C) by 3-fold when compared to DMSO treatment (vehicle; Figure 2B). This increase represents 30% (immature form) and 8% (mature form) of the CFTR signal in BHK cells expressing wild-type CFTR (wt; Figure 2B). Thus immunoblotting results confirmed that glafenine enhances the maturation of ΔF508-CFTR protein, although it was less efficacious than low temperature (29°C) .

The cellular localization of ΔF508-CFTR protein was examined in BHK and CFBE cells after glafenine treatment by indirect immunofluorescence staining and confocal microscopy. In

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control cells (DMSO), ΔF508-CFTR protein was predominantly located around the nucleus in both BHK and CFBE cells cultured at 37°C (Figure 2C). Treatment with glafenine (10μM) or low temperature (29°C) for 24h caused redistribution of ΔF508-CFTR protein toward the periphery, causing the margins of BHK and CFBE cells to become more distinct (Figure 2C), consistent with trafficking to the plasma membrane. In polarized CFBE monolayers that had been treated with vehicle alone, CFTR staining was intracellular with no CFTR detectable at the apical (a) surface (Figure 2C, CFBE ΔF508 polarized), however when incubated with glafenine or at low temperature, CFTR staining became evident along the apical surface (Figure 2C, see arrows) indicating partial correction of the trafficking defect.

In view of the increased ΔF508 CFTR expression at the plasma membrane induced by glafenine, we tested halide permeability using an automated iodide efflux assay and compared its activity with the known correctors VRT-325 (10μM) and corr-4a (10μM) (Figure 3). Treatment with glafenine (10μM) for 24h partially restored iodide efflux responses to 10μM forskolin + 50μM genistein when compared with control cells treated with vehicle alone (Figure 3A). Consistent with the YFP-quenching results, glafenine increased the cAMP-stimulated response 3.3-fold, as compared to 4.9-fold and 7-fold changes obtained with other correctors and low temperature, respectively (Figure 3B). We also examined different concentrations of glafenine and found that 1-10μM glafenine was required to restore ΔF508-CFTR iodide efflux significantly in BHK cells (Figure 3C). These results suggest that glafenine increases ΔF508-CFTR activity at concentrations in the 1-10μM range in BHK cells with efficacy that is about half that of low temperature.

Since BHK cells are non-polarized and trafficking might differ from that in epithelial cells, glafenine was also tested by monitoring CFTR-dependent I_{sc} across polarized CFBE41o$^-$ cell

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monolayers in Ussing chambers. The basolateral membrane was permeabilized using nystatin to ensure that apical Cl^- conductance was rate-limiting for the I_{sc} stimulation. Figure 4A shows representative recordings of the I_{sc} obtained from ΔF508-CFBE41o$^-$ monolayers that had been incubated with vehicle alone, 10μM glafenine or 10μM VRT-325 at 37^{o}C for 24h. In the condition used, forskolin and genistein stimulated a small current response even in control monolayers maintained at 37^{o}C (Figure 4A, vehicle), and the current was sensitive to the CFTR channel blocker $CFTR_{inh}$-172 (10μM) indicating some residual ΔF508-CFTR activity in these cells. Glafenine and VRT-325 (10μM for 24h) increased the forskolin + genistein-stimulated I_{sc} by about 1.25 and 2-fold compared with DMSO controls (n = 5), respectively (Figure 4B). The corrected I_{sc} was blocked by $CFTR_{inh}$-172, indicating that the stimulated-current was mediated by ΔF508-CFTR (Figure 4A). Functional rescue by glafenine (n = 6) was 3.7% of that induced by low-temperature (29°C; n = 10), representing ~1.8% of the wild-type CFTR current (Figure 4B, note scale). These results confirm that glafenine causes a modest but significant correction of ΔF508-CFTR activity in polarized CFBE airway epithelial cell monolayers. Because certain small-molecule modulators may exhibit dual corrector and potentiator activities (Dormer et al., 2001), we examined the potentiating effect of glafenine to exclude the possibility that it acutely stimulates pre-existing chloride channels rather than trafficking correction. For these studies, polarized CFBE airway epithelial cell monolayers were first incubated at 29°C for 24h to rescue ΔF508-CFTR, then mounted in Ussing chambers to assess the potentiating effect of 10μM glafenine after forskolin stimulation (Figure 4C). Unlike genistein, glafenine did not further increase I_{sc}, indicating that it is not a ΔF508-CFTR potentiator (Figure 4C and D).

Transgenic mice that are homozygous for the ΔF508 mutation have reduced cAMP-stimulated secretion in some tissues, which can be partially rescued by low temperature (French et al., 1996). To investigate glafenine effects in native tissue (see Figure 1A), pieces of intestine

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were isolated from homozygous ΔF508-CFTR mice and from non-CF littermate controls (wt) and their I_{sc} responses to forskolin and genistein were recorded. Responses were measured twice, at time 0 (0h) and then again after 4h incubation *ex-vivo* in William's E-Glutamax medium containing 10μM glafenine or 0.1% DMSO (4h) to assess inter-tissue variation and to control for any time-dependent changes (Figures 5A and 5B). Incubation with 10μM glafenine for 4h increased the response to 10μM forskolin + 50μM genistein by 38% (1.5-fold; grey bar) relative to those at time 0, and this increase was statistically significant at $p<0.05$ (Figures 5A and 5B; n = 20). By contrast, incubation with vehicle alone (white bar) under identical conditions did not affect the I_{sc} response to forskolin + genistein (Figures 5A and 5B; n = 18). This increase represents restoration of ~5% of the response observed using tissues from wild-type mice (Figures 5A and 5B; n = 11). Genistein had a weak effect on I_{sc} across mouse intestinal epithelium consistent with a less severe impact of ΔF508 on mouse CFTR (Ostedgaard et al., 2007) and hence less dependence on potentiators compared to human ΔF508-CFTR.

CF mice provide a unique opportunity to test the efficacy of correctors *in vivo*, therefore we tested glafenine *in-vivo* using an assay developed by Best & Quinton (Best & Quinton, 2005) that measures β-adrenergic-stimulated salivary secretion. Mice received either glafenine (50μg / hour) or vehicle alone (DMSO) for 48h by continuous micro osmotic pump delivery. When stimulated with isoprenaline (in the presence of atropine), total salivary secretion in the CF mice treated with glafenine increased ~3-fold compared to the controls, which corresponds to ~6.6% restoration of normal salivary secretion evoked in the WT control mice (Figure 5D). There were no changes in behaviour or body weight when mice received glafenine by osmotic pump as compared to those receiving vehicle alone.

Taken together, the results from *ex-* and *in-vivo* experiments indicate that glafenine partially corrects defective processing of ΔF508-CFTR in mouse ileum and salivary glands, consistent

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with the gain of function observed in BHK, HEK293 and CFBE41o- human airway epithelial cells.

Discussion

Identifying small molecules that correct the processing of CFTR mutants is a promising approach for the development of effective pharmacotherapies for cystic fibrosis (Loo et al., 2005; Pedemonte et al., 2005; Van Goor et al., 2006 ; Carlile et al., 2007; Robert et al., 2008), however developing hits into quality leads and ultimately drugs is time-consuming and expensive, and requires extensive preclinical studies of absorption, distribution, metabolism, excretion and toxicology in addition to clinical trials. Since much of this information would have already been collected when the drug was approved for other indications, finding a ΔF508-CFTR corrector amongst drugs that have already been approved would therefore be advantageous. Our screen identified glafenine, an anthranilic acid derivative as such a drug. Although no longer prescribed in most countries, it has analgesic properties and has been used to relieve pain since the 1960's, particularly in dentistry. We found that it partially corrects the misprocessing of ΔF508-CFTR, and this effect was most pronounced *in vitro* using concentrations (10μM) that are achieved clinically in plasma.

Glafenine corrected ΔF508-CFTR mislocalization across all pharmacological assays tested, however the level of correction varied greatly, from 40% of wild-type in the cell surface assay in BHK cells to 6.5% in *in-vivo* mouse salivary assays and 2% in polarized CFBE cells. The reasons for this variation are not known but may be related to the cell type and/or ΔF508-CFTR expression level. Although some variation in the processing and function of CFTR-ΔF508 has been noted amongst species (eg Ostedgaard et al.2008), the mice used in this study nevertheless had a strong CF phenotype. ΔF508 homozygotes died of intestinal obstruction if fed regular diet,

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and ß-adrenergic-stimulated salivary secretion was robust in WT mice but was negligible in littermates homozygous for ΔF508. The ΔF508 mutation may have slightly less impact on the processing of mouse CFTR when studied in transfected cells, however the CF phenotype in these mice was still severe (i.e. lethal). Moreover the mechanisms by which ΔF508 reduce channel activity and induce protein misprocessing may be distinct, as indicated by the fact that most potentiators have little corrector activity and vice versa. We observed much more correction in BHK than in CFBE cells when both cell types expressed human CFTR, therefore the CFTR orthologue (i.e. human vs rodent) could not explain different responses in transfected cells. Similar variation between cell types has been observed after knock down of the Hsp90 cochaperone Aha1, which increases ΔF508-CFTR maturation in HEK293 but not in CFBE cells (Wang et al., 2006), and also after low temperature, which corrects ΔF508-CFTR more efficiently in HEK293 than BHK cells, presumably due to differences in the chaperone folding environment (Wang X et al., 2008).

Glafenine caused a modest 2-6% increase in CFTR function in various cell types, however the amount of correction needed to provide significant clinical benefit remains uncertain. Estimates range from 6-10% of wild-type CFTR (Johnson. et al., 1992), to more recent studies that indicate overexpression in 25% of surface airway epithelial cells would be sufficient (Zhang et al., 2009). The impact of low levels of correction in patients may depend on genetic background and other factors that influence the severity of CF in the population.

Glafenine use has been associated with increased risk of nephrotoxicity, hepatotoxicity, gastrointestinal disturbances and anaphylaxis (Parfitt, 1999). These adverse effects are not well understood however glafenine may damage the stomach and kidney directly (Van Kolfschoten et al., 1983) and through its major metabolite glafenic acid, as occurs with other non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit cyclooxygenases (Wolfe et al., 1999). Medicinal

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chemistry or altered formulation or route of administration could be useful for minimizing these problems. Alternatively, hybrid molecules might be developed that combine a non-selective cyclooxygenase inhibitor and nitric oxide donor to prevent stomach ulceration by NSAIDs (Abadi et al., 2005). Glafenine inhibits prostaglandin biosynthesis and its analgesic activity is correlated with this inhibition (Deraedt et al., 1976) but whether its ability to partially correct ΔF508-CFTR processing occurs via prostenoids remains to be determined. Many studies have highlighted the importance of inflammation in CF and it is conceivable that glafenine improves ΔF508-CFTR trafficking by acting on inflammatory signalling pathways. Interestingly, clinical trials have shown that the anti-inflammatory drug ibuprofen slows progression of CF lung disease when taken for 2-4 years (eg Konstan et al., 2007). Its beneficial effect was presumed to be due to the management of inflammation rather than improved trafficking of ΔF508-CFTR, however the latter possibility has not been excluded.

NSAIDs such as ibuprofen, salicylic acid and niflumic acid are open-channel blockers of CFTR (Devor & Schultz, 1998; Scott-Ward et al., 2004), which may explain the inhibition of iodide efflux by high concentrations of glafenine in the present study. Glafenine increased steady-state CFTR protein expression without affecting the level of CFTR mRNA (data not shown), however further studies are needed to assess whether this reflects an increase in the rate of CFTR translation or its protein stability.

Glafenine is on a small, but growing list of correctors that include sodium 4-phenylbutyrate, curcumin, sildenafil and its analogue KM11060, corrector 4a, miglustat and VRT-325 (Rubenstein et al., 1997; Egan et al., 2004; Dormer et al., 2001; Dormer et al., 2005; Loo et al., 2005; Pedemonte et al., 2005; Norez et al., 2006; Van Goor et al., 2006; Carlile et al., 2007; Robert et al., 2008). A potential advantage of clinically available compounds such as glafenine is

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the large body of data that has already been collected about it, which may shorten the time to clinical and provide hints regarding its mode of action.

The relative potency of glafenine compared to other reported small molecule correctors remains to be established, however glafenine increased ΔF508-CFTR surface expression in BHK cells to ~40% of that observed for wild-type CFTR, which is comparable to the known corrector VRT-325 (Van Goor et al., 2006) and superior to some others in this assay including corrector 4a, sildenafil and miglustat (Carlile et al., 2007; Robert et al. 2008; unpublished data). By contrast, VRT-325 was more effective in correcting ΔF508-CFTR trafficking than glafenine in polarized CFBE cells in Ussing chamber assays, and miglustat gave more functional correction than glafenine in mouse ileum *ex-vivo* (Norez et al., 2006). The modest correction provided by all correctors reported thus far leaves much room for improvement however the present results provide further evidence that existing drugs may be useful as chemical probes for the development of CF therapeutics. Investigating why corrector potencies vary dramatically in different cell types may provide insights into their mechanisms of action and identify potential drug targets.

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Footnotes

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Figure legends

Figure 1. Identification of glafenine as a ΔF508-CFTR corrector. (A) Schematic of high-throughput screening and hit validation. (B) Chemical structure of glafenine hydrochloride. (C) Effect of glafenine hydrochloride on the surface expression of ΔF508-CFTR. BHK cells expressing ΔF508-CFTR were pre-treated for 24h with either 0.1% DMSO (vehicle, n = 4), 10μM glafenine (glafenine, n = 4), 10μM VRT-325 (VRT-325, n = 4), or incubated at low temperature (29°C, n = 4) prior to monitoring surface expression in the high-throughput assay. A representative BHK cell line expressing wild-type CFTR (wt, n = 4) is also shown for comparison. Data are presented as mean ± SEM and compared to the control. (D) Traces showing fluorescence quenching by iodide influx in HEK293 cells that co-express ΔF508-CFTR and a halide-sensitive YFP. Cells were pre-treated for 24h with 0.1% DMSO (vehicle, n = 3), 10μM glafenine (glafenine, n = 3), 10μM VRT-325 (VRT-325, n = 3) or low temperature (29°C, n = 3). Correction of ΔF508-CFTR function was assayed in a plate reader as quenching of YFP fluorescence by iodide in the presence of 25μM forskolin, 45μM IBMX and 50μM genistein.

Figure 2. Effect of glafenine on the surface expression of ΔF508-CFTR. (A) Immunoblot showing ΔF508-CFTR in lysates of BHK cells treated with 10μM glafenine for 24h. Control ΔF508-CFTR cells were treated with vehicle (0.1% DMSO; negative control) or incubated at 29^{o}C for 24h (positive control). BHK cells expressing the wild type-CFTR (wt) are also shown for comparison. Band C corresponds to mature, complex-glycosylated CFTR and band B to core-glycosylated CFTR. Tubulin was used as internal control. (B) Quantification of immunoblots by densitometry from four independent experiments monitoring the relative amounts of band C and band B normalized to the background and the wild-type control (wt). The optical density of all

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the bands and background were measured then the two bands for wt were designated 100% and the background 0%. The percentage for each band is then calculated separately. (C) Representative confocal images of four independent experiments showing the localization of ΔF508-CFTR in BHK and in non-polarized and polarized CFBE cells after 24h incubation with vehicle alone (DMSO, 1:1000), 10μM glafenine (glafenine), or at low temperature (29°C). Nuclei are stained in blue and CFTR in green. Note the perinuclear localization of ΔF508-CFTR in BHK and CFBE in DMSO controls and the spreading induced by glafenine or low temperature. Also, apical staining is observed in glafenine and low temperature treated polarized CFBE cells (a, apical; b, basolateral) but not in DMSO controls. Scale bars are 5μm, 10μm and 40μm for BHK, non-polarized CFBE and polarized CFBE cells, respectively.

Figure 3. Functional rescue of ΔF508-CFTR by glafenine in BHK and CFBE41o$^-$. (A) Iodide efflux assay of corrected ΔF508-CFTR at the plasma membrane of BHK cells after treatment with 10μM glafenine for 24h (n = 16). Stimulation was evoked by 10μM forskolin (Fsk) + 50μM genistein (Gst). Control cells received vehicle alone (0.1% DMSO, n = 32). (B) Bar graph comparing stimulation of the largest peak iodide efflux from BHK cells expressing ΔF508-CFTR after subtracting the basal rate prior to stimulation. Cells were treated 24h at 37°C with vehicle alone (0.1% DMSO, n = 32), glafenine (n = 16), or various positive controls including corrector corr-4a (corr-4a; 10μM; n = 14,), VRT-325 (VRT-325; 10μM; n = 32), and at low temperature (29°C; n = 16). Data are presented as the mean ± SEM. Significance compared to vehicle alone was determined using an unpaired t-test. (C) Dependence of functional rescue on glafenine concentration. BHK cells expressing ΔF508-CFTR were treated for 24h prior to measuring iodide efflux (n = 4 for each concentration). Results are also shown for control cells receiving vehicle

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alone (0.1% DMSO) and cells that were pre-treated with 10µM VRT-325. BHK expressing wild-type CFTR (wt) were used as positive control. Data are presented as the mean ± SEM. Significance compared to vehicle alone was determined using an unpaired t-test.

Figure 4. Rescue of ΔF508-CFTR in human bronchial epithelia (CFBE41o$^-$). (A) Representative traces of the short-circuit current (I_{sc}) responses to 10µM forskolin, 50µM genistein and 10µM CFTRinh-172 after 24 hour exposure of CFBE41o$^-$ cells to 0.1% DMSO (vehicle), 10µM glafenine or 10µM VRT-325 . CFTR-mediated current are shown as upward reflections due to the membrane permeabilization and the apical-to-basolateral chloride gradient.

(B) Bar graph showing the change in I_{sc} (ΔI_{sc}) after adding forskolin + genistein, defined as the difference between the sustained phase of the current response after genistein and the baseline before stimulation. Stimulation of I_{sc} across permeabilized CFBE41o-monolayers with apical-to-basolateral Cl^- gradient after pre-treatment with vehicle (DMSO; vehicle), glafenine or VRT-325 for 24h. Also shown is the result obtained with CFBE41o$^-$ cells treated at low temperature (29°C) or expressing wild-type CFTR (wt). Data for each condition are presented as the mean ± SEM (n = 5 for control, n = 6 for glafenine, n = 6 for VRT-325, n = 10 for 29°C and n = 4 for wt) and significance compared to vehicle alone was determined using an unpaired t-test. Note break in y axis. (C) Representative traces of the short-circuit current (I_{sc}) responses to 10µM forskolin plus 0.1% DMSO (vehicle), 50µM genistein or 10µM glafenine tested as potentiators on CFBE41o$^-$ cells treated 24h at 29°C. (D) Bar graph showing the change in I_{sc} (ΔI_{sc}) after adding DMSO, glafenine or genistein after the forskolin-stimulated I_{sc} defined as the difference between the sustained phase of the current response after DMSO, glafenine or genistein and the sustained

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phase of the current response after forskolin. Significance compared to vehicle alone was determined using an unpaired t-test.

Figure 5. *Ex-vivo* and *in-vivo* rescue of ΔF508-CFTR in mouse by glafenine. (A) Representative trace of the short-circuit current (I_{sc}) response to 10μM forskolin (Fsk) and 50μM genistein (Gst) on the same piece of ΔF508-CFTR mouse ileum before (0h) and after (4h) an incubation *ex-vivo* with 10μM glafenine versus a piece of ileum from wild-type mice (wt). (B) Bar graph showing the change in I_{sc} (ΔI_{sc}) after adding forskolin + genistein. Stimulation of I_{sc} across several pieces of ileum from different mice before (0h) and after (4h) pre-treatment with vehicle (vehicle; n = 18 ileum from N = 6 mice), glafenine (glafenine; n = 20 ileum from N = 6 mice) or wild-type mice (wt; n = 11 ileum from N = 4 mice) for 4h. Data are presented as the mean ± SEM and significance compared to their respective control at time 0h was determined using an unpaired t-test. Note break in y axis. (C) Total saliva secreted by wild-type mice (wt; n = 6), CF mice treated with vehicle alone (vehicle; n = 6) or CF mice treated with glafenine by micro osmotic pump (glafenine; n = 6). Data for each condition are presented as the mean ± SEM. An unpaired t-test was used to compare CF mice treated with vehicle alone and CF mice treated with glafenine.

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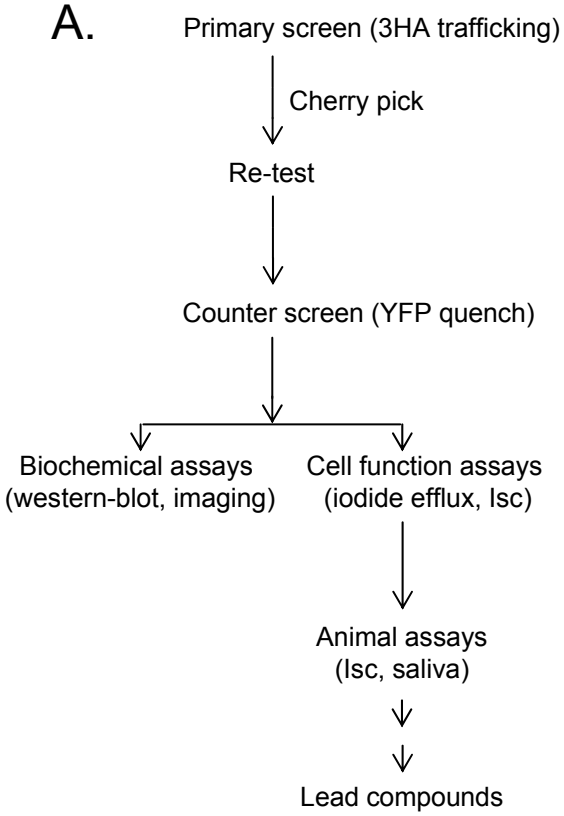

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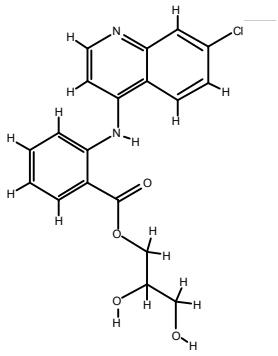

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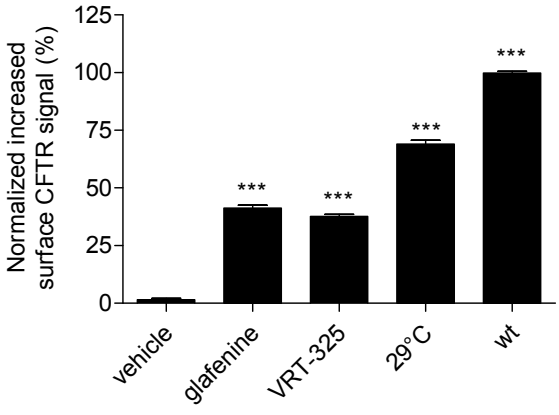
2-[(7-chloro-4-quinolinyl)amino]benzoic acid 2,3-dihydroxypropyl ester

C.



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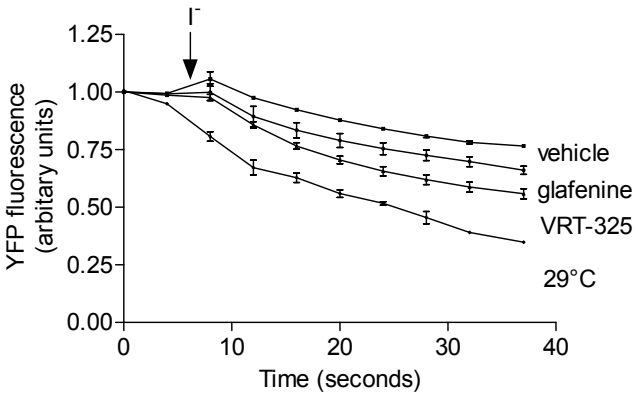

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Figure 2

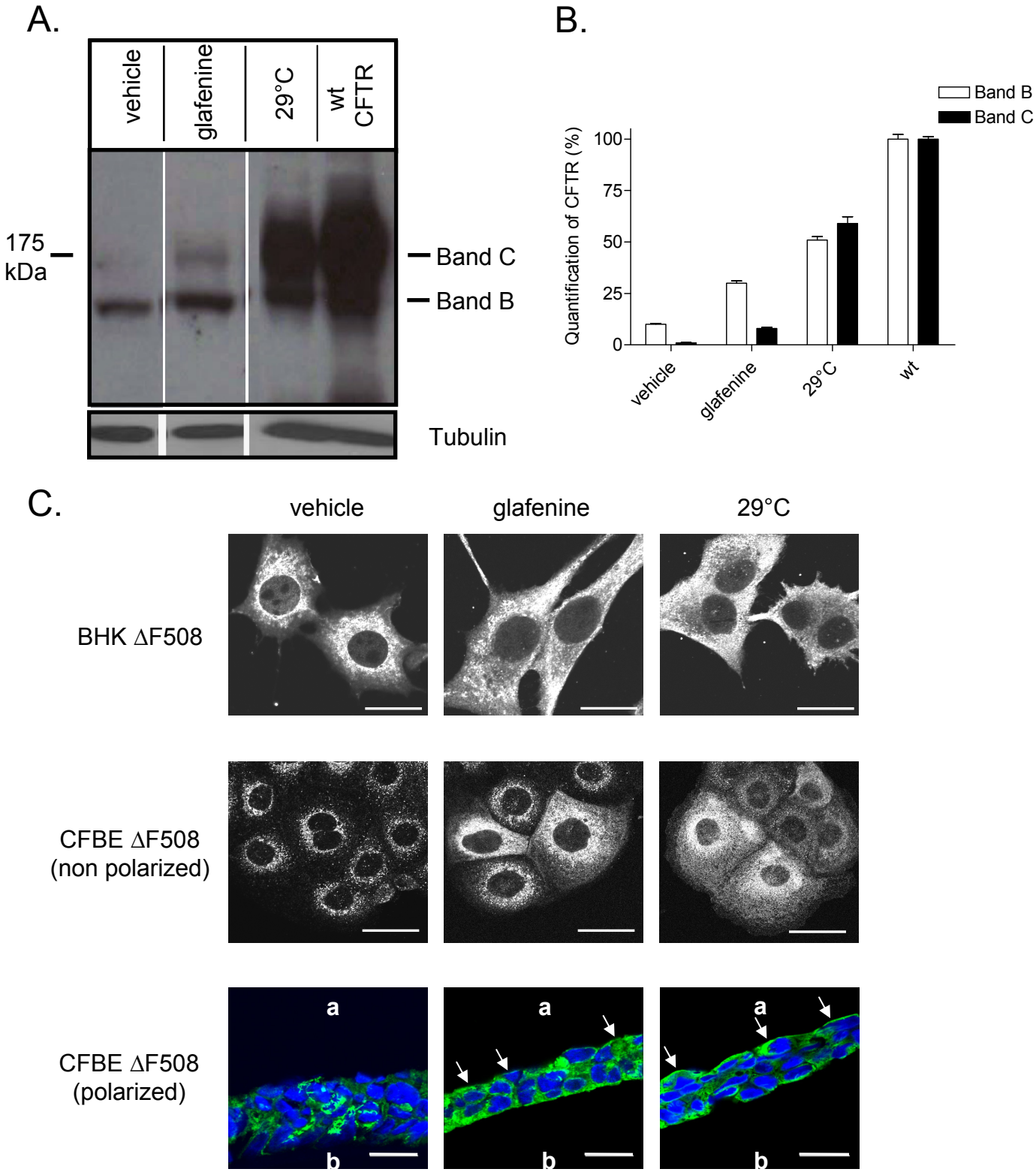

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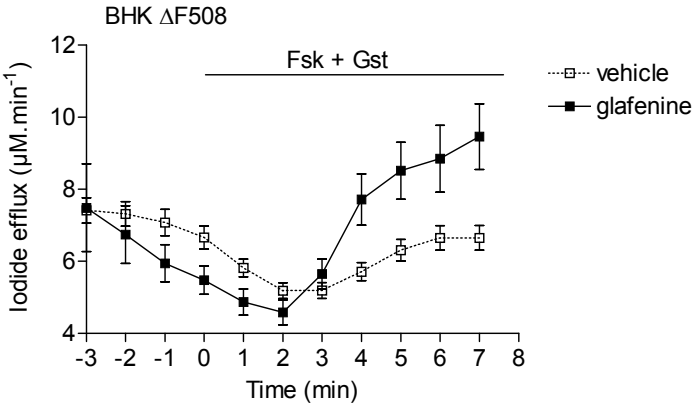
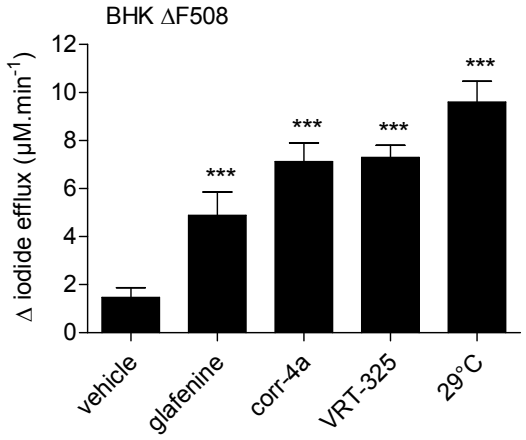
Figure 3

A.



B.



C.

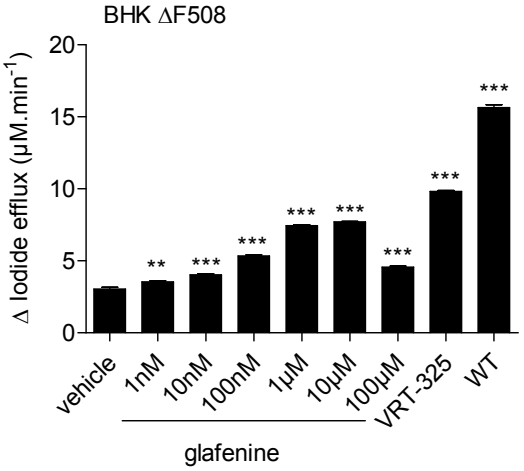


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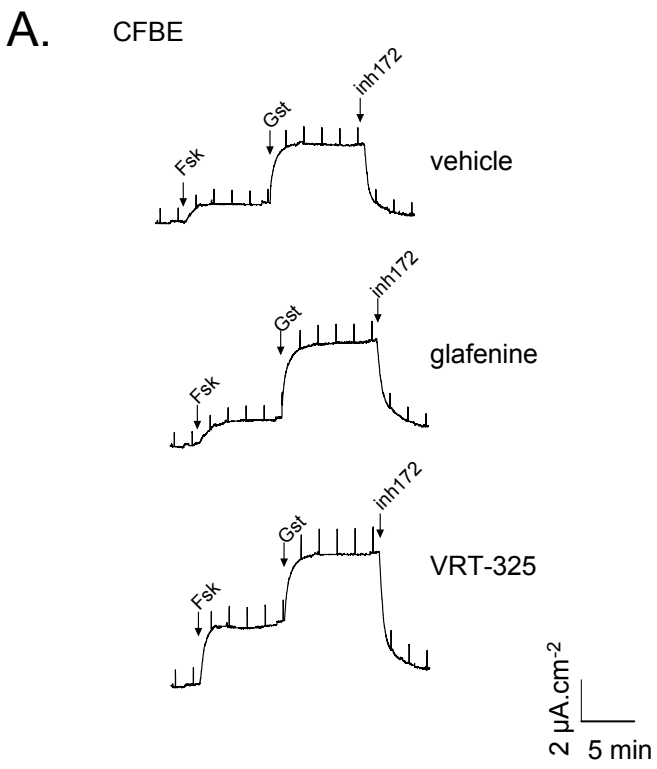
Figure 4



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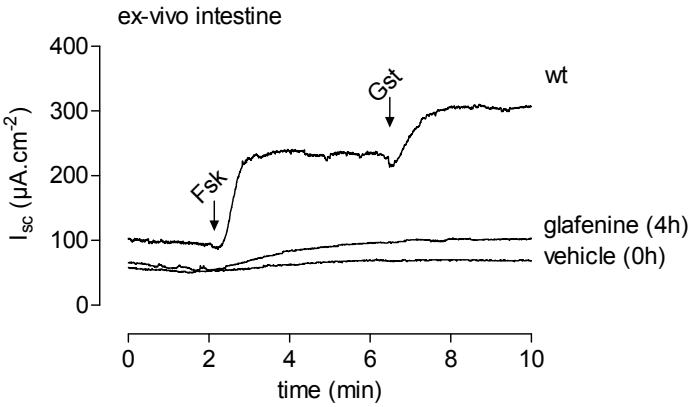
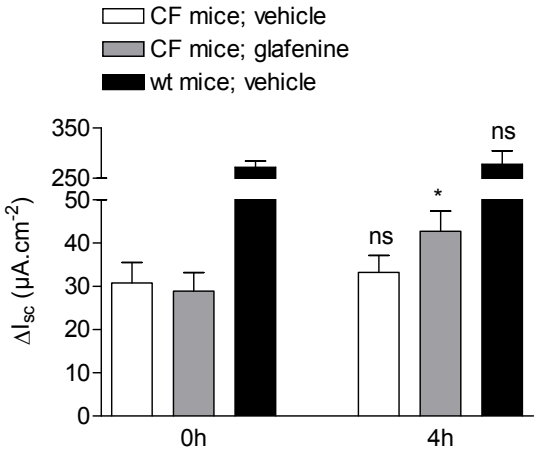
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5

A.



B.



C.