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# Correction of the ΔPhe508 Cystic Fibrosis Transmembrane Conductance Regulator Trafficking Defect by the Bioavailable Compound Glafenine

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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 ΔPhe508 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 that 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 ΔPhe508-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 ΔPhe508-CFTR in baby hamster kidney (BHK) cells to ~40% of that observed for wild-type CFTR, comparable with the known CFTR corrector 4-cyclohexyloxy-2-{1-[4-(4-methoxybenzensulfonyl)-piperazin-1-yl]-ethyl}-quinazoline (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 human embryonic kidney cells. Incubating polarized CFBE41o$^-$ monolayers and intestines isolated from ΔPhe508-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.





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 the epithelia of the lung, pancreas, intestine, liver, sweat glands, and reproductive tract (Riordan et al., 1989; Rommens et al., 1989; Hanrahan et al., 2003). Among the >1600 known mutations of the CFTR gene (available at http://www.genet.sickkids.on.ca/cftr/), the most common is a deletion of phenylalanine at position 508 in the first nucleotide binding domain. This mutation (ΔPhe508), which is present on at least one chromosome in ~90% of people with CF, impairs the folding, trafficking, membrane stability, and gating of the ΔPhe508-CFTR channel protein (Cheng et al.,

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**ABBREVIATIONS:** CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; BHK, baby hamster kidney; YFP, yellow fluorescent protein; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; TBS, Tris-buffered saline; $I_{sc}$, short-circuit current; HEK, human embryonic kidney; WT, wild type; NSAID, nonsteroidal anti-inflammatory drug; KM11060, 7-chloro-4-[4-[4-chlorophenyl)sulfonyl]-1-piperazinyl]quinoline; VRT-325, 4-cyclohexyloxy-2-{1-[4-(4-methoxybenzensulfonyl)-piperazin-1-yl]-ethyl}-quinazoline.

1990; Lukacs et al., 1993; Hwang and Sheppard, 2009). Although the mutant is retained in the endoplasmic reticulum and rapidly degraded in the proteasome (Kopito, 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 with 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 ΔPhe508-CFTR function, including 4-phenylbutyrate and curcumin (Rubenstein et al., 1997; Egan et al., 2004), the quinazoline CFTR corrector 4-cyclohexyloxy-2-{1-[4-(4-methoxybenzensulfonyl)-piperazin-1-yl]-ethyl}-quinazoline (VRT-325) and diverse compound families such as benzo[*c*]quinoliziniums, aminoarylthiazoles, bis-aminomethylbithiazoles, and phosphodiesterase type 5 inhibitors (Dormer et al., 2001, 2005; Loo et al., 2005; Pedemonte et al., 2005; Van Goor et al., 2006; Carlile et al., 2007; Robert et al., 2008). Moreover, clinically available drugs such as sildenafil and the α-glucosidase inhibitor miglustat also partially correct ΔPhe508-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 preclinical development. For this reason, we have screened the Prestwick Chemical Library using a high-throughput screening assay that identifies ΔPhe508-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 high-throughput screening 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 ΔPhe508-CFTR processing in several in vitro model systems, including nonpolarized epithelial cells, human airway epithelial cell monolayers, and freshly isolated intestines from CF mice. We found partial correction of ΔPhe508-CFTR trafficking in these preparations and in vivo using transgenic CF mice, suggesting that glafenine may be useful in the development of therapeutics for the treatment of CF.

## Materials and Methods

**High-Throughput Screening Assay.** Screening was performed using BHK cells that stably express ΔPhe508-CFTR bearing three tandem hemagglutinin-epitope tags 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 (Carlile et al., 2007).

**YFP Fluorescence Assay.** Strongly adhesive human embryonic kidney cells stably expressing both the human macrophage scavenger receptor (HEK293 GripTite cells; Invitrogen, Carlsbad, CA) and ΔPhe508-CFTR were plated in 96-well plates and transiently transfected with pcDNA3 plasmid encoding a halide sensitive variant of enhanced YFP (H148Q/I152L). After 24 h, cells were exposed to 10 μM test compound in triplicate and incubated for an additional 24 h. Cells were then stimulated with 25 μM forskolin, 45 μM 3-isobutyl-1-methylxanthine, and 50 μM genistein for 20 min and the high-content screening assay was performed using a high-content screening platform (Cellomics, Inc., Pittsburgh, PA) as described by Trzcinska-Daneluti et al. (2009). Iodide (50 mM) 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 that expressed YFP at time 0 for halide flux measurements. Quenching was detected in 15 images taken over the course of an experiment lasting 40 s. Results were generated from 150 to 300 cells/well.

**Immunoblot Analysis.** Total protein was quantified in cell lysates using the Bradford assay (Bio-Rad Laboratories, Hercules, CA), separated using SDS-polyacrylamide gel electrophoresis (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°C with a monoclonal primary anti-CFTR antibody (clone M3A7; Millipore Bioscience Research Reagents, Temecula, CA) diluted 1:1000. The blots were washed four times in PBS before adding the secondary horseradish peroxidase-conjugated anti-mouse antibody at a dilution of 1:5000 (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) for 1 h at room temperature, then washed again five times in PBS and visualized using chemiluminescence (Pierce Chemical, Rockford, IL). The relative intensity of each CFTR glycoform (band B or 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 Life Sciences, Lowell, MA), and the apical medium was removed after 24 h to establish an air-liquid interface. Cells were then treated with 0.1% DMSO (vehicle control), 10 μM glafenine in DMSO, or incubated at 29°C for 24 h. Cells grown on coverslips were rinsed in TBS, fixed with 3% paraformaldehyde 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% paraformaldehyde in PBS for 1 h, rinsed with PBS, extirpated, and embedded in paraffin. Sections (5 μm) were laid on microscopy slides and rehydrated by successive immersion in xylene (three baths), ethanol (two baths), 70% ethanol, 50% ethanol, and water. The samples were then stained by indirect immunofluorescence as follows: after fixation, nonspecific binding sites were blocked with TBS containing 0.5% bovine serum albumin for 1 h at room temperature, then the cells were stained with primary antibody (anti-CFTR C-terminal monoclonal, 1:100, clone 24-1; R&D Systems, Minneapolis, MN) for 90 min at room temperature and rinsed in TBS containing 0.5% bovine serum albumin. Next, samples were incubated either with the goat anti-mouse fluorescein isothiocyanate or with Cy3-conjugated secondary antibody to detect CFTR (1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA). Samples were mounted in Prolong Gold anti-fade solution (Invitrogen) with or without 4,6-diamidino-2-phenylindole for observation with a confocal laser-scanning microscope (Confocor LSM 510 META, ×63, numerical aperture 1.4, oil; Carl Zeiss, Thornwood, NY).

**Halide Efflux Assay.** Assays were performed using a robotic liquid handling system (BioRobot 8000; QIAGEN, Valencia, CA) and QIAGEN 4.1 software. Cells were cultured to confluence in 24-well plates. Cells were treated for 24 h 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°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, and 20 mM HEPES, pH 7.4 with NaOH) and incubated for 1 h at 37°C. At the beginning of each experiment, the loading buffer was removed by aspiration, and cells were washed eight times with 300 $\mu$l of 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 $\mu$l of fresh efflux buffer at 1-min intervals for up to 11 min. The first four aliquots were used to establish a stable baseline, and then buffer containing 10 $\mu$M forskolin + 50 $\mu$M genistein was used to stimulate CFTR activity. Iodide concentration was measured in each aliquot (300 $\mu$l) using an iodide-sensitive electrode. Relative iodide efflux rate was calculated using the difference between maximum (peak) iodide concentration during stimulation and minimum iodide concentration before stimulation (measured in micromoles per minute). Data are presented as means ± S.E.M.

**Voltage-Clamp of CFBE41o$^-$ Cell Monolayers.** We used the CFBE41o$^-$ airway epithelial cell line subsequently transduced with TranzVector lentivectors containing WT or ΔPhe508-CFTR that was generously provided by Dr. J. P. Clancy (University of Alabama at Birmingham, Birmingham, AL) 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 Life Sciences), and the apical medium was removed after 24 h to establish an air-liquid interface. Transepithelial resistance was monitored using an epithelial Volt Ohm Meter, and cells were used when the transepithelial resistance was 300 to 400 Ω · $cm^2$. ΔPhe508-CFBE41o$^-$ monolayers were treated on both sides with Opti-MEM medium containing 2% (v/v) fetal bovine serum and one of the following compounds: 0.1% DMSO (negative control), 10 $\mu$M glafenine, 10 $\mu$M VRT-325, or cells were incubated at 29°C (positive control) for 24 h before being mounted in EasyMount chambers and voltage-clamped using a VCCMC6 multichannel current-voltage clamp (Physiologic Instruments, San Diego, CA). The apical membrane conductance was functionally isolated by permeabilizing the basolateral membrane with 200 $\mu$g/ml nystatin and imposing an apical-to-basolateral $Cl^-$ gradient. The basolateral bathing solution contained 1.2 mM NaCl, 115 mM sodium gluconate, 25 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 4 mM $CaCl_2$, 2.4 mM $KH_2PO_4$, 1.24 mM $K_2HPO_4$, and 10 mM glucose, pH 7.4. The $CaCl_2$ concentration was increased to 4 mM to compensate for the chelation of calcium by gluconate. The apical bathing solution contained 115 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 2.4 mM $KH_2PO_4$, 1.24 mM $K_2HPO_4$, and 10 mM mannitol, pH 7.4. The apical solution contained mannitol instead of glucose to eliminate currents mediated by $Na^+$-glucose cotransport. 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 current. Pulses (1-mV amplitude, 1-s duration) were delivered every 90 s to monitor resistance. The voltage clamps were connected to a PowerLab/8SP interface for data collection (ADInstruments Inc., Colorado Springs, CO). CFTR was activated by adding 10$\mu$M forskolin plus 50 $\mu$M genistein to the apical bathing solution.

**Ex Vivo Experiments.** Glafenine was tested ex vivo using ileum from homozygous ΔPhe508-CFTR mice (backcrossed on the FVB genetic background for more than 12 generations, *Cftr$^{tm1\ Eur}$*; van Doorninck et al., 1995) and wild-type littermate controls. Only female mice, 14 to 17 weeks old and weighing 24 to 30 g, were used in this assay and were genotyped by standard polymerase chain reaction methods using tail DNA. The mice were kept in the animal facility at McGill University and were fed a high-protein diet (SRM-A; Hope Farms, Woerden, the Netherlands) modified to contain pork instead of beef. All procedures followed the Canadian Institutes of Health Research 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 to 15 min, $I_{sc}$ was measured at time 0 h, and 10 $\mu$M forskolin + 50 $\mu$M genistein was added (0 h). After this stimulation, each piece of ileum was rinsed and incubated in William's E-GlutaMAX medium supplemented with insulin (10 $\mu$g/ml), 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin and dexamethasone (20 $\mu$g/ml). Each piece was exposed to 10 $\mu$M glafenine dissolved in DMSO or to vehicle alone (0.1% DMSO) for 4 h, and then the $I_{sc}$ response to forskolin + genistein was measured again. Tissue viability was confirmed by adding 10 mM glucose to stimulate electrogenic $Na^+$-glucose cotransport (the apical solution normally contained mannitol instead of glucose). Results are expressed as the mean ± S.E.M. of $n$ pieces of ileum from $N$ mice.

**Salivary Secretion.** The procedure followed those described by Best and Quinton (2005). Only male homozygous ΔPhe508-CFTR (*Cftr$^{tm1\ Eur}$*) and wild-type mice of the same strain, 10 to 12 weeks old and weighing 20 to 25 g were used in this assay. A micro-osmotic pump (Alzet, Cupertino, CA) was fixed under the skin on the back of each mouse to deliver glafenine or vehicle for 48 h. The micro-osmotic pumps were filled with 90 $\mu$l of solution containing glafenine in DMSO (50 mg/ml) or 90 $\mu$l of DMSO (controls). The mean pump rate was 1 $\mu$l/h, representing a delivery rate of glafenine 50 $\mu$g/h. Mouse body weight and behavior were monitored to assess the well being of the mice. After 48 h, mice were anesthetized with ketamine and diazepam and treated with a subcutaneous injection of 1 mM 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 100 $\mu$M isoprenaline and 1 mM atropine was then injected into the left cheek at the same site to induce secretion at time 0, and the filter paper was replaced every minute for 30 min. Each piece of filter paper was immediately placed and sealed in a preweighed 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 ± S.E.M. of $n$ mice.

**Statistics.** All results are expressed as the mean ± S.E.M. of $n$ observations. Data sets were compared by analysis of variance or Student's $t$-tests using Prism software (vers. 4; GraphPad Software Inc., San Diego, CA). Differences were considered statistically significant when $p < 0.05$: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

## Results

To test the hypothesis that drugs developed for other indications might correct ΔPhe508-CFTR trafficking, we identified and validated hit compounds as outlined in Fig. 1A. For the first step, we used a protein trafficking assay based on immunodetection of hemagglutinin epitopes in the fourth extracellular loop of ΔPhe508-CFTR (Carlile et al., 2007). A primary screen of 1120 compounds yielded 61 positive compounds having fluorescence values >1 S.D. greater than the mean for the plate. These hits were selected (cherry-picked) and retested in duplicate. Positive compounds with intrinsic fluorescence were not considered further. From the original 61 positives, 50 were confirmed by immunodetection of surface hemagglutinin epitopes at the cell surface and were counterscreened for functional correction using an enhanced YFP fluorescence-quenching assay in which halide-permeability is detected by iodide entry using a high-content screening platform (see *Materials and Methods*). Of the 50 confirmed hits in the trafficking assay, 15 were also positive according to the functional assay, leading to 25 to 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 ΔPhe508-CFTR corrector activity.

One of the hits was glafenine hydrochloride (2-[(7-chloro-4-quinolinyl)amino]benzoic acid 2,3-dihydroxypropyl ester; Fig. 1B), a nonsteroidal anti-inflammatory drug that has been used previously as an analgesic, although it is not presently available on the market in most countries. According to the trafficking assay, glafenine increased ΔPhe508-CFTR surface expression by 40% compared with ΔPhe508-CFTR cells treated with vehicle alone and normalized to BHK cells expressing wild-type CFTR (Fig. 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, which has some toxicity in cell culture, caused a similar increase in ΔPhe508-CFTR cell surface expression (36%), although the level of surface expression was still less than after temperature correction at 29°C or compared with a representative cell line expressing three hemagglutinin-epitope-tagged WT-CFTR, which was taken as 100% when normalizing the values obtained for each experiment under different conditions (Fig. 1C).

Glafenine effects on trafficking were further confirmed using the YFP functional assay (Fig. 1D). Treating cells with 10 μM glafenine for 24 h enhanced the cAMP-stimulated iodide influx into cells expressing ΔPhe508-CFTR and YFP, indicating that functional CFTR at the plasma membrane was increased (Fig. 1D), although glafenine and VRT-325 both caused less rescue than incubation at low temperature (Fig. 1, C and D).

To further validate glafenine as a CFTR corrector, its effect on protein expression and maturation was analyzed by immunoblotting (Fig. 2A). Maturation was confirmed by the appearance of the complex glycosylated (band C) form of ΔPhe508-CFTR in BHK cells after treatment with 10 μM glafenine for 24 h, consistent with results from the screening assay (Fig. 2A). The extent of correction is shown semiquantitatively in Fig. 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 compared with DMSO treatment (vehicle; Fig. 2B). This increase represents 30% (immature form) and 8% (mature form) of the CFTR signal in BHK cells expressing wild-type CFTR (wt; Fig. 2B). Thus immunoblotting results confirmed that glafenine enhances the maturation of ΔPhe508-CFTR protein, although it was less efficacious than low temperature (29°C).

The cellular localization of ΔPhe508-CFTR protein was examined in BHK and CFBE cells after glafenine treatment by indirect immunofluorescence staining and confocal microscopy. In control cells (DMSO), ΔPhe508-CFTR protein was predominantly located around the nucleus in both BHK and CFBE cells cultured at 37°C (Fig. 2C). Treatment with glafenine (10 μM) or low temperature (29°C) for 24 h caused redistribution of ΔPhe508-CFTR protein toward the periphery, causing the margins of BHK and CFBE cells to become more distinct (Fig. 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 (Fig. 2C, CFBE ΔPhe508 polarized); however, when incubated

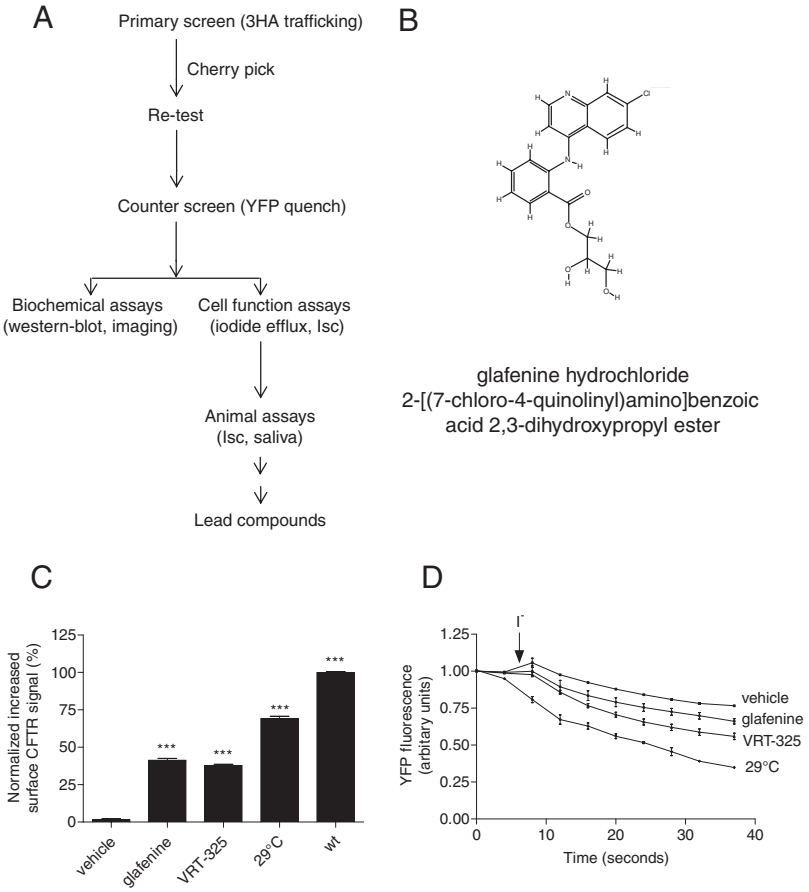


**Fig. 1.** Identification of glafenine as a ΔPhe508-CFTR corrector. A, schematic of high-throughput screening and hit validation. B, chemical structure of glafenine hydrochloride. C, effect of glafenine hydrochloride on the surface expression of ΔPhe508-CFTR. BHK cells expressing ΔPhe508-CFTR were pretreated for 24 h with either 0.1% DMSO (vehicle, $n = 4$), 10 μM glafenine (glafenine, $n = 4$), 10 μM VRT-325 (VRT-325, $n = 4$), or incubated at low temperature (29°C, $n = 4$) before 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 ± S.E.M. and are compared with the control. D, traces showing fluorescence quenching by iodide influx in HEK293 cells that coexpress ΔPhe508-CFTR and a halide-sensitive YFP. Cells were pretreated for 24 h with 0.1% DMSO (vehicle, $n = 3$), 10 μM glafenine (glafenine, $n = 3$), 10 μM VRT-325 (VRT-325, $n = 3$), or low temperature (29°C, $n = 3$). Correction of ΔPhe508-CFTR function was assayed in a plate reader as quenching of YFP fluorescence by iodide in the presence of 25 μM forskolin, 45 μM 3-isobutyl-1-methylxanthine, and 50 μM genistein.

with glafenine or at low temperature, CFTR staining became evident along the apical surface (Fig. 2C, see arrows), indicating partial correction of the trafficking defect.

In view of the increased ΔPhe508-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 (10 $\mu$M) and corr-4a (10 $\mu$M) (Fig. 3). Treatment with glafenine (10 $\mu$M) for 24 h partially restored iodide efflux responses to 10 $\mu$M forskolin + 50 $\mu$M genistein compared with control cells treated with vehicle alone (Fig. 3A). Consistent with the YFP-quenching results, glafenine increased the cAMP-stimulated response 3.3-fold, compared with 4.9- and 7-fold changes obtained with other correctors and low temperature, respectively (Fig. 3B). We also examined different concentrations of glafenine and found that 1 to 10 $\mu$M glafenine was required to restore ΔPhe508-CFTR iodide efflux significantly in BHK cells (Fig. 3C). These results suggest that glafenine increases ΔPhe508-CFTR activity at concentrations in the 1 to 10 $\mu$M range in BHK cells with efficacy that is approximately half that of low temperature.

Because BHK cells are nonpolarized and trafficking might differ from that in epithelial cells, glafenine was also tested by monitoring CFTR-dependent $I_{sc}$ across polarized CFBE41o$^-$ cell 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 ΔPhe508-CFBE41o$^-$ monolayers that had been incubated with vehicle alone, 10 $\mu$M glafenine, or 10 $\mu$M VRT-325 at 37°C for 24 h. In the condition used, forskolin and genistein stimulated a small current response even in control monolayers maintained at 37°C (Fig. 4A, vehicle), and the current was sensitive to the CFTR channel blocker $CFTR_{inh}$-172 (10 $\mu$M), indicating some residual ΔPhe508-CFTR activity in these cells. Glafenine and VRT-325 (10 $\mu$M for 24 h) increased the forskolin + genistein-stimulated $I_{sc}$ by approximately 1.25- and 2-fold compared with DMSO controls ($n$ = 5), respectively (Fig. 4B). The corrected $I_{sc}$ was blocked by $CFTR_{inh}$-172, indicating that the stimulated current was mediated by ΔPhe508-CFTR (Fig. 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 (Fig. 4B, note scale). These results confirm that glafenine causes a modest but significant correction of

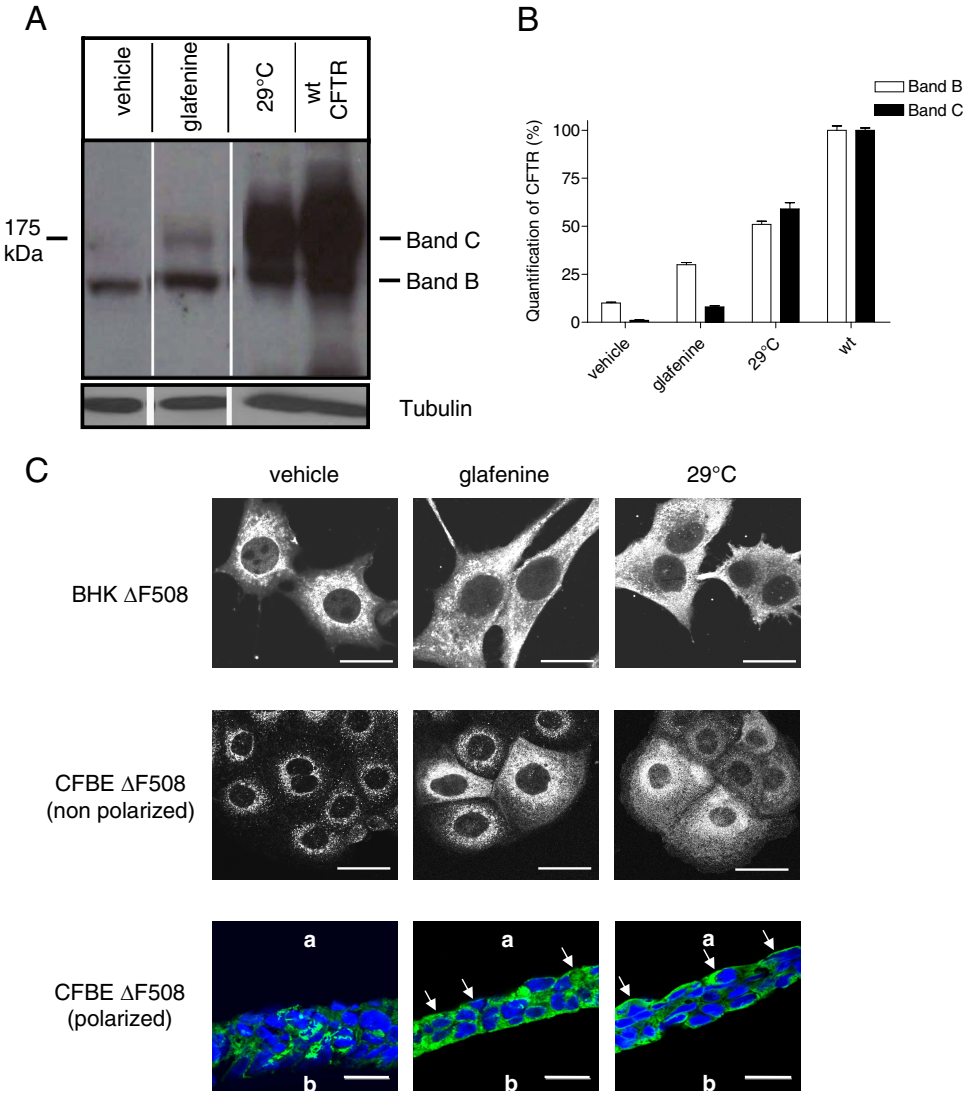


**Fig. 2.** Effect of glafenine on the surface expression of ΔPhe508-CFTR. A, immunoblot showing ΔPhe508-CFTR in lysates of BHK cells treated with 10 $\mu$M glafenine for 24 h. Control ΔPhe508-CFTR cells were treated with vehicle (0.1% DMSO; negative control) or incubated at 29°C for 24h (positive control). BHK cells expressing the wild type-CFTR (wt) are also shown for comparison. Band C corresponds to mature, complex-glycosylated CFTR, and band B corresponds 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 bands C and B normalized to the background and the wild-type control (wt). The optical density of all the bands and background were measured, and then the two bands for wt were designated 100% and the background as 0%. The percentage for each band is then calculated separately. C, representative confocal images of four independent experiments showing the localization of ΔPhe508-CFTR in BHK and in nonpolarized and polarized CFBE cells after 24 h incubation with vehicle alone (DMSO, 1:1000), 10 $\mu$M glafenine (glafenine), or at low temperature (29°C). Nuclei are stained in blue and CFTR in green. Note the perinuclear localization of ΔPhe508-CFTR in BHK and CFBE in DMSO controls and the spreading induced by glafenine or low temperature. In addition, 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 5, 10, and 40 $\mu$m for BHK, nonpolarized CFBE, and polarized CFBE cells, respectively.

ΔPhe508-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 immediately 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 24 h to rescue ΔPhe508-CFTR and then were mounted in Ussing chambers to assess the potentiating effect of 10 μM glafenine after forskolin stimulation (Fig. 4C). Unlike genistein, glafenine did not further increase $I_{sc}$, indicating that it is not a ΔPhe508-CFTR potentiator (Fig. 4, C and D).

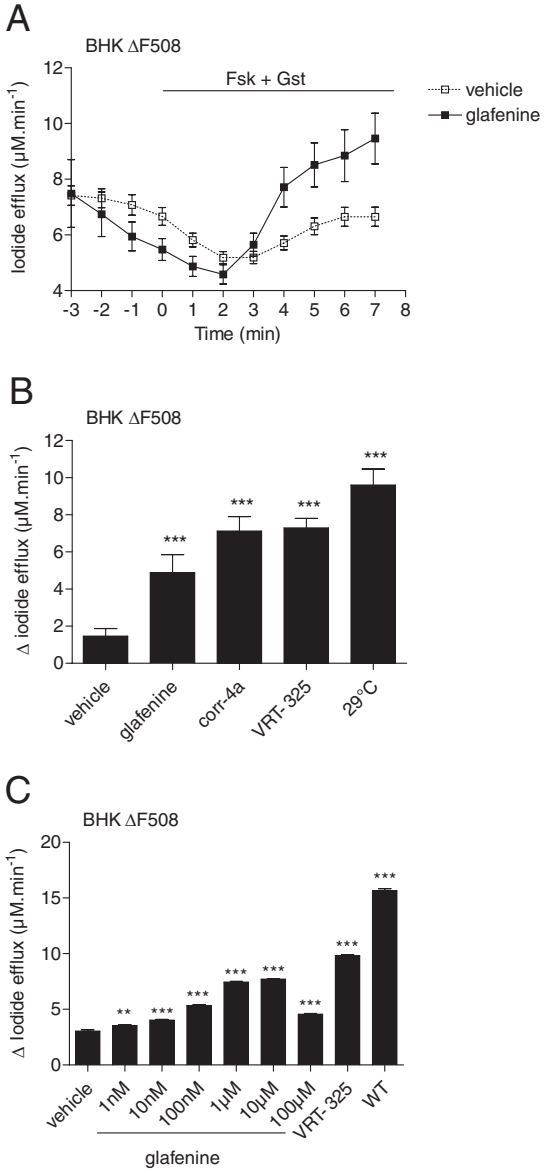


**Fig. 3.** Functional rescue of ΔPhe508-CFTR by glafenine in BHK. A, iodide efflux assay of corrected ΔPhe508-CFTR at the plasma membrane of BHK cells after treatment with 10 μM glafenine for 24 h ($n$ = 16). Stimulation was evoked by 10 μM forskolin (Fsk) + 50 μM genistein (Gst). Control cells received vehicle alone (0.1% DMSO, $n$ = 32). B, bar graph comparing stimulation of the largest peak iodide efflux from BHK cells expressing ΔPhe508-CFTR after subtracting the basal rate before stimulation. Cells were treated for 24 h at 37°C with vehicle alone (0.1% DMSO, $n$ = 32), glafenine ($n$ = 16), or various positive controls including corrector corr-4a (10 μM; $n$ = 14), VRT-325 (10 μM; $n$ = 32), and at low temperature (29°C; $n$ = 16). Data are presented as the mean ± S.E.M. Significance compared with vehicle alone was determined using an unpaired $t$ test. C, dependence of functional rescue on glafenine concentration. BHK cells expressing ΔPhe508-CFTR were treated for 24 h before measuring iodide efflux ($n$ = 4 for each concentration). Results are also shown for control cells receiving vehicle alone (0.1% DMSO) and cells that were pretreated with 10 μM VRT-325. BHK expressing wild-type CFTR (wt) were used as positive control. Data are presented as the mean ± S.E.M. Significance compared with vehicle alone was determined using an unpaired $t$ test.

Transgenic mice that are homozygous for the ΔPhe508 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 (Fig. 1A), pieces of intestine were isolated from homozygous ΔPhe508-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 (0 h) and then again after 4 h of incubation ex vivo in William's E-GlutaMAX medium containing 10 μM glafenine or 0.1% DMSO (4h) to assess intertissue variation and to control for any time-dependent changes (Figs. 5A and 5B). Incubation with 10 μM glafenine for 4 h increased the response to 10 μM forskolin + 50 μM genistein by 38% (1.5-fold; gray bar) relative to those at time 0, and this increase was statistically significant at $p < 0.05$ (Fig. 5, A and B; $n$ = 20). By contrast, incubation with vehicle alone (open bar) under identical conditions did not affect the $I_{sc}$ response to forskolin + genistein (Fig. 5, A and B; $n$ = 18). This increase represents restoration of ~5% of the response observed using tissues from wild-type mice (Fig. 5, A and B; $n$ = 11). Genistein had a weak effect on $I_{sc}$ across mouse intestinal epithelium, consistent with a less severe impact of ΔPhe508 on mouse CFTR (Ostedgaard et al., 2007) and hence less dependence on potentiators compared with human ΔPhe508-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 and Quinton (2005) that measures β-adrenergic-stimulated salivary secretion. Mice received either glafenine (50 μg/h) or vehicle alone (DMSO) for 48 h 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 with the controls, which corresponds to ~6.6% restoration of normal salivary secretion evoked in the WT control mice (Fig. 5C). There were no changes in behavior or body weight when mice received glafenine by osmotic pump compared with those receiving vehicle alone.

Taken together, the results from ex and in vivo experiments indicate that glafenine partially corrects defective processing of ΔPhe508-CFTR in mouse ileum and salivary glands, consistent 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. Because much of this information would have already been collected when the drug was approved for other indications, finding a ΔPhe508-CFTR corrector among 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 1960s, particularly in dentistry. We found that it partially corrects the misprocessing of ΔPhe508-CFTR, and this effect was most pronounced in vitro using concentrations (10 μM) that are achieved clinically in plasma.

Glafenine corrected ΔPhe508-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 ΔPhe508-CFTR expression level. Although some variation in the processing and function of CFTR-ΔPhe508 has been noted among species (Ostedgaard et al., 2007), the mice used in this study nevertheless had a strong CF phenotype. ΔPhe508 homozygotes died of intestinal obstruction if fed a regular diet, and β-adrenergic-stimulated salivary secretion was robust in WT mice but was negligible in littermates homozygous for ΔPhe508. The ΔPhe508 mutation may have slightly less impact on the processing of mouse CFTR when

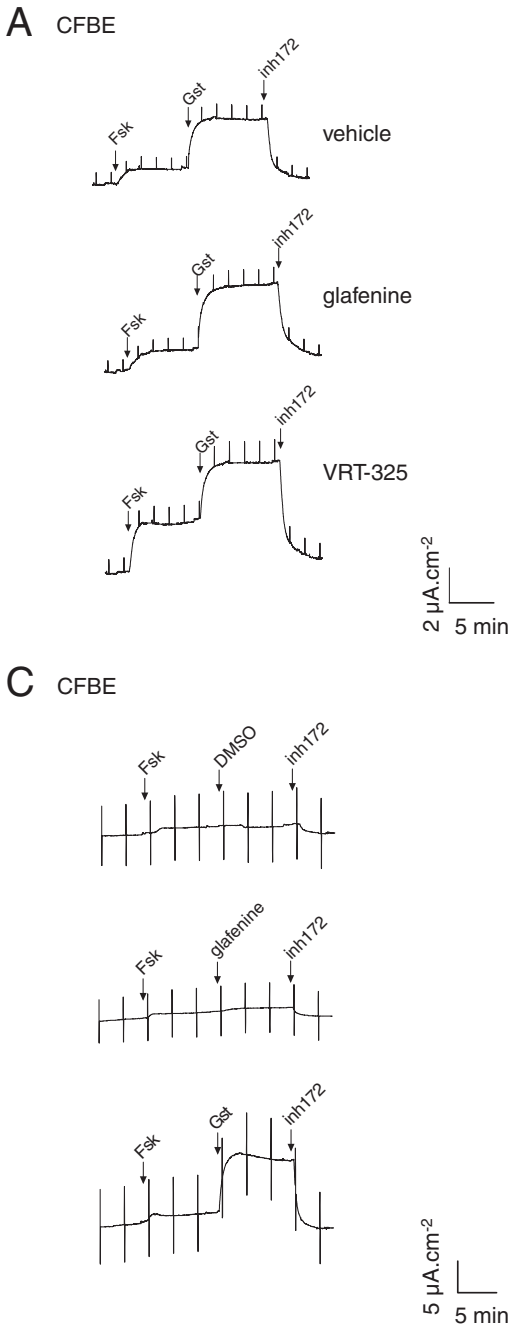


**Fig. 4.** Rescue of ΔPhe508-CFTR in human bronchial epithelia (CFBE41o⁻). A, representative traces of the short-circuit current ($I_{sc}$) responses to 10 μM forskolin, 50 μM genistein, and 10 μM $CFTR_{inh}$-172 after 24-h exposure of CFBE41o⁻ cells to 0.1% DMSO (vehicle), 10 μM glafenine, or 10 μM VRT-325. B, bar graph showing the change in $I_{sc}$ ($\Delta I_{sc}$) after adding forskolin + genistein, defined as the difference between the sustained phase of the current response after genistein and the baseline before stimulation. 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 ± S.E.M. ($n$ = 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 with vehicle alone was determined using an unpaired $t$ test. Note the break in $y$-axis. C, representative traces of the $I_{sc}$ responses to 10 μM forskolin plus 0.1% DMSO (vehicle), 50 μM genistein, or 10 μM glafenine tested as potentiators on CFBE41o⁻ cells treated for 24 h at 29°C. D, bar graph showing the change in $I_{sc}$ ($\Delta I_{sc}$) 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 phase of the current response after forskolin. Significance compared with vehicle alone was determined using an unpaired $t$ test; ns, nonsignificant difference.

studied in transfected cells; however, the CF phenotype in these mice was still severe (i.e., lethal). Moreover, the mechanisms by which ΔPhe508 reduce channel activity and induce protein misprocessing may be distinct, as indicated by the fact that most potentiators have little corrector activity

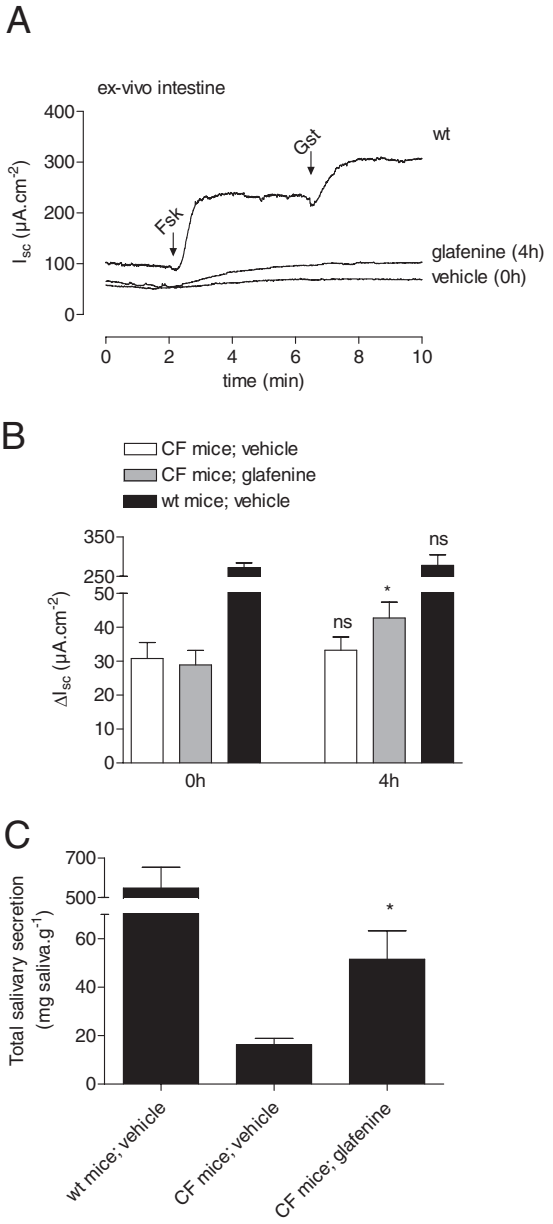


**Fig. 5.** Ex vivo and in vivo rescue of ΔPhe508-CFTR in mouse by glafenine. A, representative trace of the short-circuit current ($I_{sc}$) response to 10 μM forskolin (Fsk) and 50 μM genistein (Gst) on the same piece of ΔPhe508-CFTR mouse ileum before (0 h) and after (4 h) an incubation ex vivo with 10 μM glafenine versus a piece of ileum from wild-type mice (wt). B, bar graph showing the change in $I_{sc}$ ($\Delta I_{sc}$) after adding forskolin + genistein. Stimulation of $I_{sc}$ across several pieces of ileum from different mice before (0 h) and after (4 h) pretreatment with vehicle ($n$ = 18 ileum from $N$ = 6 mice), glafenine ($n$ = 20 ileum from $N$ = 6 mice), or wild-type mice (wt; $n$ = 11 ileum from $N$ = 4 mice) for 4 h. Data are presented as the mean ± S.E.M., and significance compared with their respective control at time 0 h 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 ($n$ = 6) or CF mice treated with glafenine by micro-osmotic pump (glafenine; $n$ = 6). Data for each condition are presented as the mean ± S.E.M. An unpaired $t$ test was used to compare CF mice treated with vehicle alone and CF mice treated with glafenine; ns, nonsignificant difference.

and vice versa. We observed much more correction in BHK than in CFBE cells when both cell types expressed human CFTR; therefore, the CFTR ortholog (i.e., human versus rodent) could not explain different responses in transfected cells. Similar variation between cell types has been observed after knock down of the 90-kDa heat shock protein cochaperone Aha1, which increases ΔPhe508-CFTR maturation in HEK293 but not in CFBE cells (Wang et al., 2006), and after low temperature, which corrects ΔPhe508-CFTR more efficiently in HEK293 than in BHK cells, presumably because of differences in the chaperone-folding environment (Wang et al., 2008).

Glafenine caused a modest 2 to 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 to 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 nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit cyclooxygenases (Wolfe et al., 1999). Medicinal chemistry or altered formulation or route of administration could be useful for minimizing these problems. Alternatively, hybrid molecules might be developed that combine a nonselective 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 ΔPhe508-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 ΔPhe508-CFTR trafficking by acting on inflammatory signaling pathways. It is noteworthy that clinical trials have shown that the anti-inflammatory drug ibuprofen slows the progression of CF lung disease when taken for 2 to 4 years (Konstan et al., 2007). Its beneficial effect was presumed to be due to the management of inflammation rather than improved trafficking of ΔPhe508-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 and 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 analog 7-chloro-4-[4-[4-chlorophenyl)sulfonyl]-1-piperazinyl]quinoline (KM11060), corrector 4a, miglustat, and VRT-

325 (Rubenstein et al., 1997; Dormer et al., 2001, 2005; Egan et al., 2004; 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 the large body of data that has already been collected about it, which may shorten the time to clinical trials and provide hints regarding its mode of action.

The relative potency of glafenine compared with other reported small-molecule correctors remains to be established; however, glafenine increased ΔPhe508-CFTR surface expression in BHK cells to ~40% of that observed for wild-type CFTR, which is comparable with 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 ΔPhe508-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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