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Recovery of ΔF508-CFTR Function by Analogs of Hyaluronan Disaccharide

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ABSTRACT

We recently discovered that hyaluronan was exported from fibroblasts by MRP5 and from epithelial cells by cystic fibrosis (CF) transmembrane conductance regulator (CFTR) that was known as a chloride channel. On this basis we developed membrane permeable analogs of hyaluronan disaccharide as new class of compounds to modify their efflux. We found substances that activated hyaluronan export from human breast cancer cells. The most active compound 2-(2-acetamido-3,5-dihydroxyphenoxy)-5-aminobenzoic acid (Hylout4) was tested for its influence on the activity of epithelial cells. It activated the ion efflux by normal and defective ΔF508-CFTR. It also enhanced the plasma membrane concentration of the ΔF508-CFTR protein and reduced the transepithelial resistance of epithelial cells. In human trials of healthy persons, it caused an opening of CFTR in the nasal epithelium. Thus compound Hylout4 is a corrector that recovered ΔF508-CFTR from intracellular degradation and activated its export function. J. Cell. Biochem. 113: 156–164, 2012.

KEY WORDS: CYSTIC FIBROSIS; CFTR; ION TRANSPORT; HYALURONAN

Cystic fibrosis (CF) is one of the most common inherited diseases, afflicting 1 in approximately 2,500 white individuals [Bobadilla et al., 2002]. The primary cause of morbidity and mortality in CF is chronic lung infection and deterioration of lung function. CF is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, which encodes a cAMP-regulated chloride channel expressed at the apical membrane of epithelial cells in the airways, pancreas, testis, and other tissues [Pilewski and Frizzell, 1999; Sheppard and Welsh, 1999]. The most common CFTR mutation producing CF is deletion of phenylalanine at residue 508 (ΔF508) in its amino acid sequence, which is present in at least 1 allele in approximately 90% of CF subjects [Bobadilla et al., 2002]. The ΔF508-CFTR protein is misfolded and retained at the endoplasmic reticulum (ER), where it is degraded rapidly [Denning et al., 1992; Lukacs et al., 1994; Du et al., 2005]. The misfolding of ΔF508-CFTR is thought to be mild, because it can be "rescued" in cell culture models by incubation for 18 h or more at reduced (<30°C) temperature or with chemical chaperones such as glycerol [Sato et al., 1996] or phenylbutyrate [Rubenstein et al., 1997], which results in partial restoration of ΔF508-CFTR plasma membrane expression. However, channel gating of the plasma membrane-rescued ΔF508-CFTR protein remains defective such that its open probability after cAMP stimulation is reduced by more than threefold compared with that of wild-type CFTR [Dalemans et al., 1991; Haws et al., 1996]. Small-molecule correctors of defective ΔF508-CFTR folding/cellular processing ("correctors") and channel gating ("potentiators") may provide a strategy for therapy of CF that corrects the underlying defect. A potential advantage of pharmacotherapy for defective ΔF508-CFTR processing and gating is that it minimizes concerns about treating the wrong cells or losing physiological CFTR regulation, as might occur with gene therapy or activation of alternative chloride channels. A number of small-molecule ΔF508-CFTR potentiators [Drumm et al., 1991; Hwang et al., 1997; Al-Nakkash and Hwang, 1999] and correctors [Yang et al., 2003; Pedemonte et al., 2005; Carlile et al., 2007; Wang et al., 2007; Loo et al., 2008; Yoo et al., 2008] have been identified. These potentiators and correctors were mostly discovered by high-throughput screening for activation of the chloride channel.

Recently, we discovered that hyaluronan is exported from fibroblasts by MRP5 [Schulz et al., 2007] and from epithelial cells by CFTR [Schulz et al., 2010]. These studies added hyaluronan as another important natural substrate to CFTR in addition to chloride.

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They also suggested a compromise in the controversy of the CF pathology between the hydration and the salt hypothesis [Guggino, 1999], because hyaluronan fulfills both properties being an extremely hydrated salt. Hyaluronan is not only exported from bronchial epithelia by CFTR upon inflammation, but also from human breast carcinoma cells, because they have an epithelial origin. Hyaluronan export by CFTR can best be analyzed on breast carcinoma cell lines, because bronchial epithelial cell lines lose the hyaluronan synthesizing capacity.

Therefore, we intended to chemically synthesized compounds that modulate hyaluronan production. These compounds should be useful as drugs, be membrane permeable and mimic important structural features of the terminal ends of the hyaluronan chain. These prerequisites guided us to the synthesis of diaryls substituted with hydroxyl-, acetamido-, and carboxyl groups in positions as in hyaluronan disaccharides. Here we describe a new class of compounds that activate chloride export from bronchial epithelial cells and hyaluronan export from breast carcinomal cells.

MATERIALS AND METHODS

MATERIALS

Mouse-anti-CFTR-IgM was from Acris Antibodies, Hiddenhausen, Germany. Other chemical were from Sigma–Aldrich Chemical Corporation.

CELL LINES

The epithelial breast cancer cells HMT3552 [Jojovic et al., 2002] have been described. Human epithelial cells containing wild-type CFTR (16HBE14o$^-$) and the mutant cell line containing ΔF508-CFTR (CFBE41o$^-$) were kindly provided by Dr. D.C. Gruenert [Kunzelmann et al., 1993]. The cell line 16HBE14o$^-$ is an SV40 large T-antigen transformed epithelial cell line derived from human bronchial epithelium which retains differentiated epithelial morphology and functions. The cell line CFBE41o$^-$ are transformed human airway epithelial cells carrying a homozygous mutation for DeltaF508. The cells were grown in Dulbecco's medium supplemented with streptomycin/penicillin (100 units of each/ml) and 10% foetal calf serum.

CYTOTOXICITY ASSAYS

The cytotoxicity was measured by the Alamar blue® assay which determined the cell viability by reduction of resazurin with NADH [Nakayama et al., 1997].

WESTERN BLOTTING OF CFTR

The amounts of CFTR in membranes of CFBE41o$^-$ cells were analyzed by Western blotting. Briefly, equal amounts of cells were incubated with increasing concentrations of 2-(2-acetamido-3,5-dihydroxyphenoxy)-5-aminobenzoic acid (Hylout4) for 24 h, washed washed twice with ice-cold phosphate-buffered saline, scrapped off with 1 ml of ice-cold phosphate-buffered saline and sedimented for 5 min at 1,000*g*. The cell pellets were suspended in 200 μl of homogenizing buffer containing 0.1 M sodium acetate, 0.2 M NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin pH 6.0. The suspensions were homogenized by ultrasonication three times for 10 s and undissolved materials were removed by centrifugation for 50 min at 48,000*g*. The membranes containing pellets were solubilized in 200 μl of 0.5% Triton X-100, 50 mM imidazol, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin pH 7.0. Undissolved proteins were removed by centrifugation for 50 min at 48,000*g*. Equal aliquots were loaded onto a 10% SDS polyacrylamide gel and analyzed by Western blotting with mouse-anti-CFTR-IgM. The blots were probed using an ECL light-based immunodetection system (Amersham Biosciences, Inc.).

CHEMICAL SYNTHESIS OF Hylout4

Nitrophloroglucinol (1 g, 6.5 mM) was dissolved in 10 ml of methanol and hydrogenated in a hydrogen atmosphere in the presence of 0.1 g of 10% Pd/C overnight at room temperature. The catalyst was removed by filtration and the solvent was evaporated to obtain aminophloroglucinol. It was dissolved in a 1:1 mixture of water and ethanol and an equimolar amount of acetic anhydride was added at 60°C. After 1 h at 60°C the solution was concentrated and *N*-acetylaminophloroglucinol was obtained. *N*-Acetylamino-phloro-glucinol (6 mM) was dissolved in 12 ml of dimethylformamide, mixed with 2-chloro-5-nitrobenzoic acid (1.2 g; 6 mM), 1.7 g of K_2CO_3, 0.18 g of copper powder, and 0.18 g of CuCl and refluxed for 3 h. After cooling to room temperature, the solution was filtered through celite. Water (120 ml) was added and the solution was acidified with concentrated HCl to pH 2, and the product was extracted with 120 ml of ethylacetate. The organic phase was dried over Na_2SO_4 and evaporated. The product was dissolved in 12 ml of methanol, 0.1 g of palladium (10% on charcoal) was added and hydrogenated in a hydrogen atmosphere overnight at room temperature. The catalyst was removed by centrifugation, and the solvent was evaporated to obtain Hylout4. It was further purified by chromatography on silica gel with ethylacetate as solvent.

TRANSEPITHELIAL RESISTANCE (TER)

Human bronchial epithelium cells (16HBE14o$^-$ and CFBE41o$^-$) were seeded on a 12-well plate ThinCertTM cell culture inserts (area 1.13 cm^2, pore size 0.4 μm, pore density 10^8/cm^2, Greiner Bio-One, Germany) and cultured for 7–10 days at 37°C, 5% CO_2. We developed a system which allows measuring the TER of 8 culture inserts simultaneously at time intervals of 20 s or 5 min. Each well was equipped with six titanium electrodes, three to inject current and three to measure voltage. Electrodes were arranged in a way that resulted in a fairly homogenous electrical field. Current was injected at the given time interval for 1 s and voltage was measured. Data acquisition and processing was done by 2-channel-PowerLab system (26 Series, ADInstruments GmbH, Germany). 8cpt-cAMP (8-(4-chlorophenylthio)-adenosine-3′,5′-cyclic monophosphate) or Hylout4 were added simultaneously to both compartments (final concentration 100 μM). TER was measured inside of the incubator at 37°C and 5% CO_2. TER data are expressed as relative values to allow appropriate comparisons between the different series of experiments. The value measured directly before adding any substances was set as 100% (reference value).

DETERMINATION OF HYALURONAN EXPORT

The breast cancer cells HMT3552 were incubated for 24 h at 37°C, the media were replaced with fresh media and after additional 24 h aliquots (5 and 20 µl) of the culture medium were used for measurement of the hyaluronan concentration in the cell culture medium by an ELISA. The wells of a 96 well Covalink-NH-microtiter plate (NUNC) were coated with 100 µl of a mixture of 100 mg/ml of hyaluronan (Healon®), 9.2 µg/ml of *N*-hydroxysuccin-imide-3-sulfonic acid and 615 µl/ml of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide for 2 h at room temperature and overnight at 4°C. The wells were washed three times with 2 M NaCl, 41 mM $MgSO_4$, 0.05% Tween-20 in 50 mM phosphate buffered saline pH 7.2 (buffer A) and once with 2 M NaCl, 41 mM $MgSO_4$, in phosphate buffered saline pH 7.2. Additional binding sites were blocked by incubation with 300 µl of 0.5% bovine serum albumin in phosphate buffered saline for 30 min at 37°C. Calibration of the assay was performed with standard concentrations of hyaluronan ranging from 15 ng/ml to 6,000 ng/ml in equal volumes of culture medium as used for measurement of the cellular supernatants. A solution (50 µl) of the biotinylated hyaluronan binding fragment of aggrecan (Applied Bioligands Corporation, Winnipeg, Canada) in 1.5 M NaCl, 0.3 M guanidinium hydrochloride, 0.08% bovine serum albumin 0.02% NaN_3, 25 mM phosphate buffer pH 7.0 was preincubated with 50 µl of the standard hyaluronan solutions or cellular supernatants for 1 h at 37°C. The mixtures were transferred to the hyaluronan-coated test plate and incubated for 1 h at 37°C. The microtiter plate was washed three times with buffer A and incubated with 100 µl/well of a solution of streptavidin-horseradish-peroxidase (Amersham) at a dilution of 1:100 in phosphate buffered saline, 0.1% Tween-20 for 30 min at room temperature. The plate was washed five times with buffer A and the color was developed by incubation with a 100 µl/well of a solution of 5 mg *o*-phenylenediamine and 5 µl 30% H_2O_2 in 10 ml of 0.1 M citrate-phosphate buffer pH 5.3 for 25 min at room temperature. The adsorption was read at 490 nm. The concentrations in the samples were calculated from a logarithmic regression curve of the hyaluronan standard solutions.

IODIDE EFFLUX

Iodide efflux experiments were performed as described [Lansdell et al., 1998]. Briefly, 16HBE14o$^-$ or CFBEo$^-$ cells (80–90% confluent) were incubated for 1 h in a loading buffer containing 136 mM NaI, 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 11 mM glucose, and 20 mM Hepes, adjusted to pH 7.4 with NaOH. To remove extracellular iodide, cells were thoroughly washed with efflux buffer (136 mM $NaNO_3$ replacing 136 mM NaI in the loading buffer) and then equilibrated in 2.5 ml efflux buffer for 1 min. The efflux buffer was changed at 1 min intervals over the duration of the experiment. Four minutes after anion substitution, cells were exposed to Hylout4. The amount of iodide in each 2.5 ml sample of efflux buffer was determined using an iodide-selective electrode (HNU Systems Ltd, Warrington, UK). In a voltage clamp experiment, the cells were incubated in the presence of 100 mM KCl and 10 µM valinomycin. Cells were loaded and experiments performed at room temperature.

DETERMINATION OF THE NASAL TRANSEPITHELIAL POTENTIAL DIFFERENCE

The effect of compound Hylout4 on the transepithelial nasal potential difference (NPD) was measured with healthy human volunteers by a modified method of Schüler et al. [Schuler et al., 2004]. The nasal mucosa was perfused at a rate of 5 ml/min. Baseline PD was measured after perfusion of the nasal epithelium with isotonic NaCl solution (147 mM NaCl, 2 mM $CaCl_2$). PD changes were recorded during these perfusion protocols:

(1) Isotonic NaCl (147 mM NaCl, 2 mM $CaCl_2$).
(2) No chloride solution (147 mM Na-gluconate, 2 mM $CaCl_2$).
(3) No chloride solution + 100 µM Hyalout4.
(4) No chloride solution + 10 µM isoprenaline.
(5) Isotonic NaCl.

In order to test, if the different effects of Hyalout4 and isoprenaline on nasal transepithelial PD, step 3 and 4 of this perfusion protocol were interchanged for the second experiment. Solutions were changed as soon as a steady voltage tracing was achieved for at least 30 s, and the differences in PD values were measured between the plateaus of the corresponding solutions.

RESULTS

HYALURONAN DISACCHARIDE ANALOGS

In attempts to develop specific membrane permeable inhibitors of hyaluronan export, we synthesized the diaryl analogs of the two possible hyaluronan dissacharides 1 and 2 with the non-reducing GlcNac terminus and a non-reducing GlcA terminus, respectively (Fig. 1) that were designed as membrane permeable analogs: 2-(2-acetamido-3-hydroxyphenoxy)benzoic acid (Hylout2) and 2-(2-acetamidophenoxy)-6-hydroxybenzoic acid (Hylout3). The structures differ only in the position of one hydroxyl group being in *o*-position of the acetylamido group in Hylout2 or in *o*-position of the carboxyl group in Hylout3. Thus these compounds resemble the non-reducing end of a hyaluronan chain with a terminal *N*-acetylamino group for Hylout2 and with a terminal glucuronic acid for Hylout3. These compounds were tested for their effect on hyaluronan export. Much to our surprise and contrary to our expectation, compound Hylout2 was activating at low micromolar concentrations, whereas Hylout3 was inactive. Then we modified the lead compound Hylout2 by introducing additional hydroxyl, amino, or hydrophobic groups. All of these compounds also activated and the most active one was Hylout4 (Fig. 2). The toxicity of compound Hylout4, in concentrations up to of 400 µM, was measured by the Alamar blue assay and it was found to be not toxic (data not shown).

IODIDE EFFLUX

CFTR is known as a channel that does not only export chloride and hyaluronan, but also other ions such as iodide. Iodide is a convenient ion to determine the functional activity of CFTR in living cells, because its concentration can be determined by an iodide selective electrode. We used the iodide efflux technique to assess the effect of Hylout4 on epithelial cell lines 16HBE14o$^-$ and

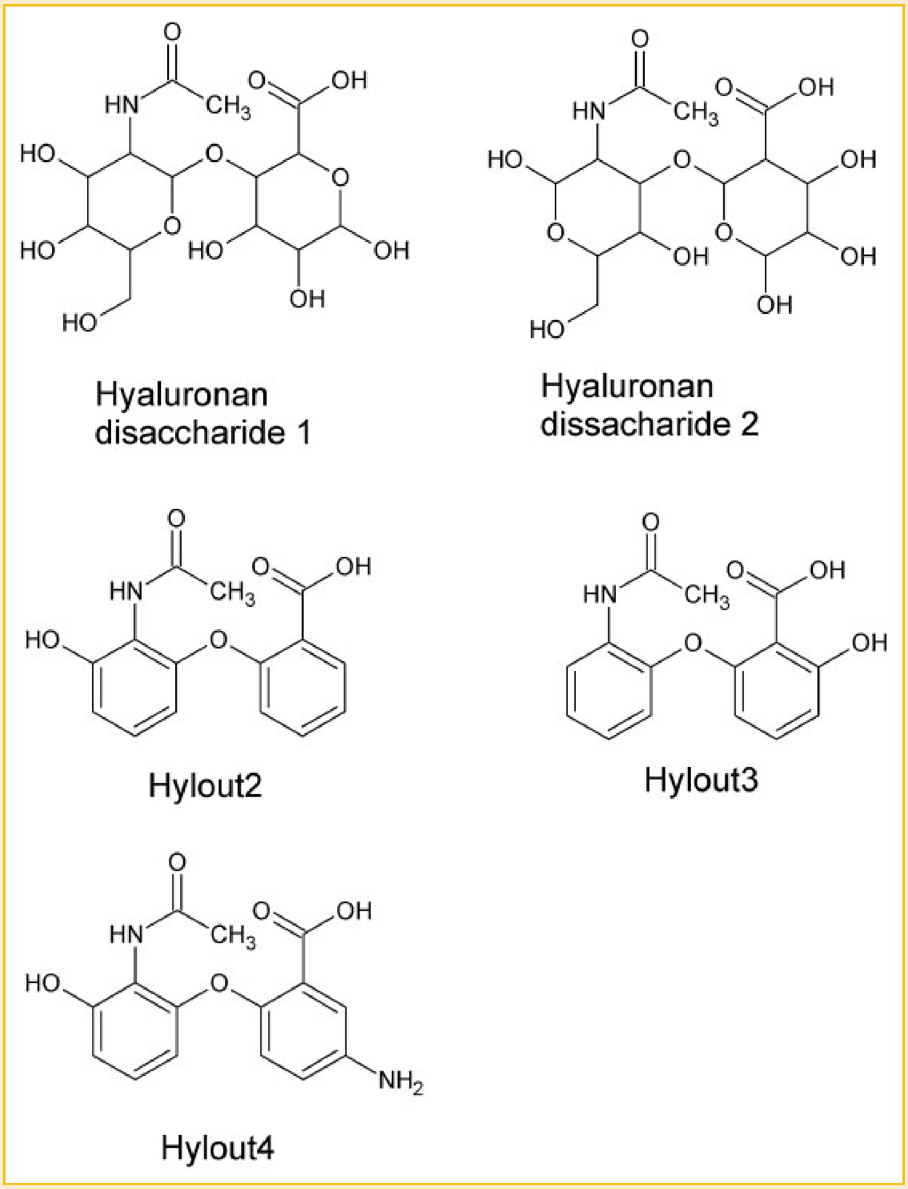

Fig. 1. Structures of hyaluronan disaccharide and analogs.

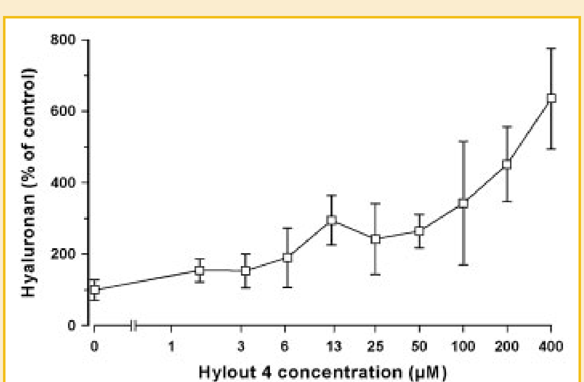

Fig. 2.

CFBE41o^{-} cells which express the intact and ΔF508-defective CFTR channel, respectively. Iodide efflux was measured at a concentration of 100 μM of Hylout4, because this concentration was also effectively activating hyaluronan export from breast carcinoma cells in the previous experiment. Figure 3 shows that Hylout4 stimulated a sudden burst of iodide efflux from 16HBE14o^{-} as well as from CFBE41o^{-} cells. The immediate opening of the channels indicates that compound Hylout4 functions as an potentiator. The

Fig. 2. Activation of hyaluronan export by Hylout4. Human breast carcinoma cells HMT3552 were grown to 50% confluence and incubated for 2 days with increasing concentrations of Hylout4, and the hyaluronan concentrations were determined in the culture supernatant. The error bars indicate the sd of nine determinations.

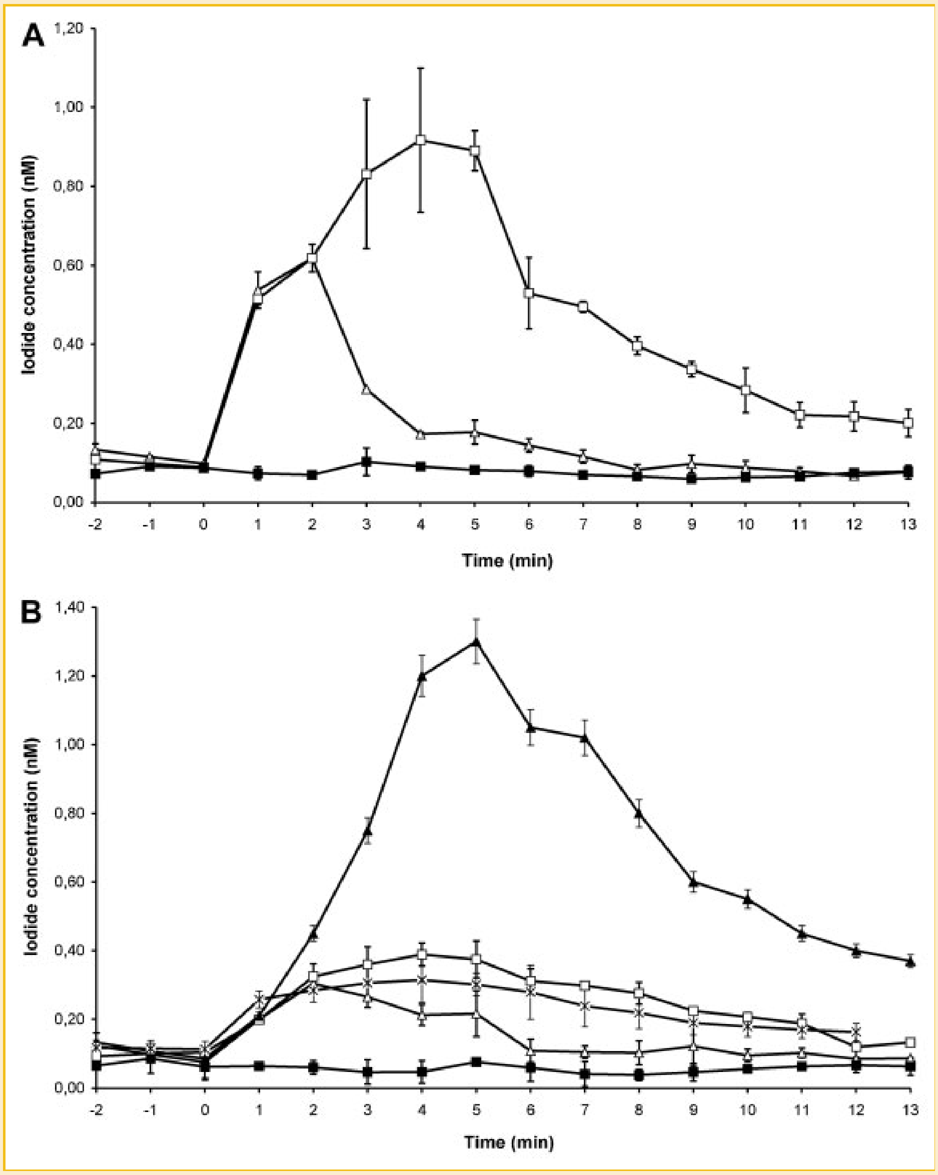

Fig. 3. Compound Hylout4 stimulates iodide efflux. CFTR can also export iodide instead of chloride. We made use of this property to measure the kinetics of export with an iodide sensitive electrode. 16HBE14o⁻ (A) or CFBE41o⁻ (B) were loaded with iodide and the iodide concentration was measured by an iodide sensitive electrode in 1 min intervals (■). Parallel cultures were exposed to 100 μM of Hylout4 (□) at time point 0 for 4 min. After 2 min of Hylout4 addition, the CFTR-specific inhibitor $CFTR_{inh}$-172 was added to CFBE14o⁻ cells (△). Preincubation for 24 h with Hylout4 and additional exposure at time point 0 for 4 min further activated the iodide efflux of CFBE41o⁻ cells (▲). In a control experiment, the cells were voltage clamped by 100 M KCl and 10 μM valinocin (×). The error bar indicates the sd of three determinations.

activation of iodide export by Hylout4 was blocked by the specific $CFTR_{inh}$-172 inhibitor. When 16HBE14o⁻ cells were preincubated for 24 h with Hylout4, the iodide efflux from CFBE41o⁻ cells was even more activated. To eliminated the possibility of an influence of Hylout4 on the membrane potential rather than opening of CFTR, a control experiment was performed with voltage clamped cells in the presence of 100 mM KCl and 10 μM valinomycin. This treatment led to a similar iodide efflux in CFBE41o⁻ cells.

TRANSEPITHELIAL RESISTANCE (TER)

The transport activity of epithelial cells can conveniently be measured by the TER. We measured the TER of wild-type CFTR expressing 16HBE14o⁻ and ΔF508-CFTR expressing CFBE41o⁻ cell monolayers in the presence of 100 μM Hylout4 or the membrane permeable cAMP analog 8cpt-cAMP that is known to activate CFTR [Moran, 2010]. Figure 4 shows that Hyalout4 exerts the same short term effect on 16HBE14o⁻ cells as 8cpt-cAMP. In both cases TER dropped immediately after application. While TER remains low with 8cpt-cAMP even 20 h after application, TER of Hyalout4 treated 16HBE14o⁻ cells recovered to values of untreated cells within 20 h. In CFBE41o⁻ cells 8cpt-cAMP and compound Hylout4 caused a transient increase of TER followed by a slow decrease of the resistance over a period of 20 h. In a separate experiment, the decrease of resistance in 16HBE14o⁻ cells was determined for

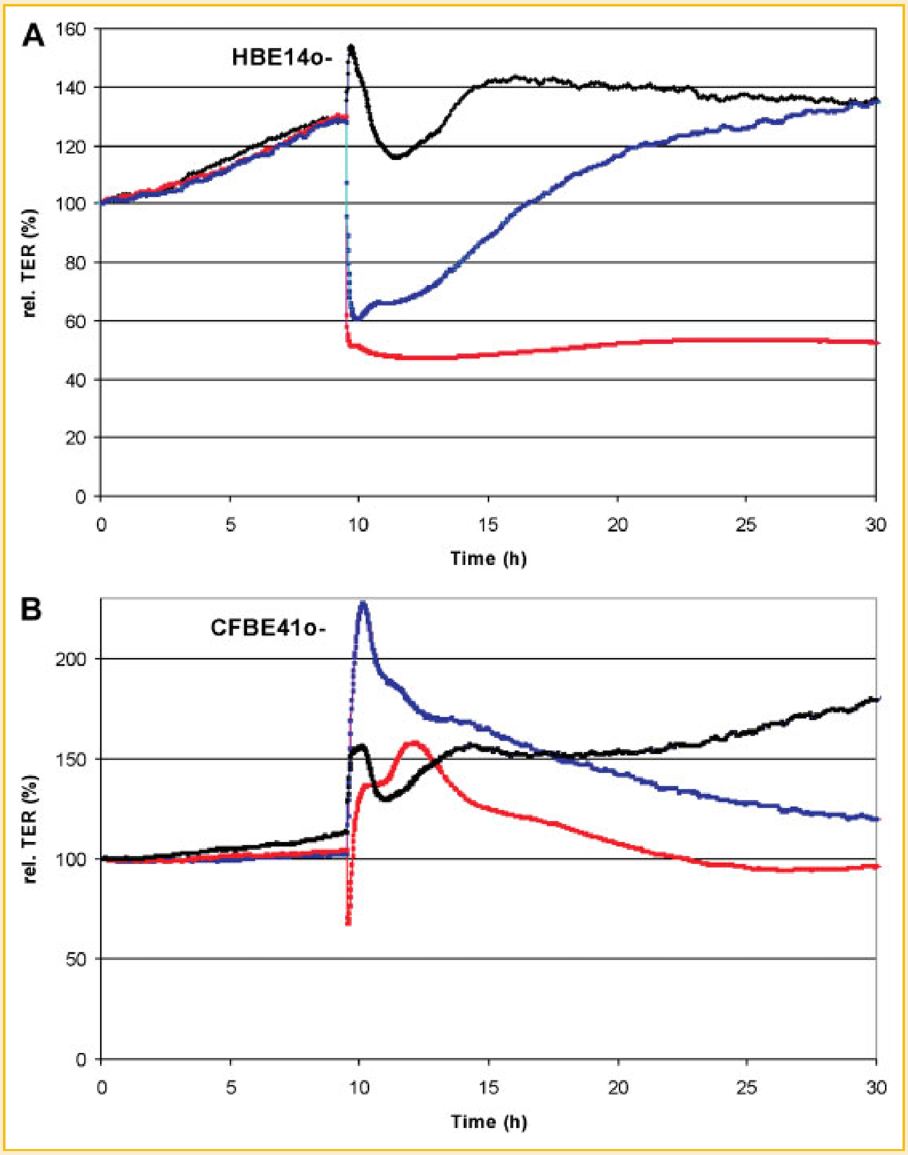

Fig. 4. Transepithelial resistance. Long time observation of relative TER in 16HBE14o$^-$ (A) and CFBE41o$^-$ cells (B) in the absence (black) or presence of 100 μM of the membrane permeable CFTR activator 8cpt-cAMP (red) or 100 μM of Hylout4 (blue). Substances were added after 9 h, the value measured directly before adding any substances was set as 100% (reference value). The traces were calculated from the mean of three wells and the standard errors were below 5%.

Hylout4 at concentrations of 10, 50, 100, and 200 μM after 20 h and found to be 94%, 89%, 67%, and 65%, respectively. The initial absolute resistances were $407 \pm 41\ \Omega \times cm^2$ and $440 \pm 48\ \Omega \times cm^2$ for 16HBE14o$^-$ and CFBE41o$^-$ cells, respectively. These results suggested a long term recovery of functionally active CFTR.

ACTION OF Hylout4 ON ΔF508-CFTR EXPRESSION

The ΔF508-CFTR mutation impairs maturation and transport competence at the ER and destabilizes ΔF508-CFTR in post-Golgi compartments. Correctors may facilitate post-translational folding of newly synthesized ΔF508-CFTR and/or enhance the stability of mature ΔF508-CFTR. Therefore, we analyzed the amount of ΔF508-CFTR in the cells in the presence of increasing Hylout4 concentrations in CFBE41o$^-$ cells. Correction of this primary defect is evident as the appearance of complex glycosylation (i.e., an increase in the ratio of the C-band to the B-band form of the protein). Western blotting with anti-CFTR antibodies showed that the amounts of intact ΔF508-CFTR increased with Hylout4 (Fig. 5). In a control

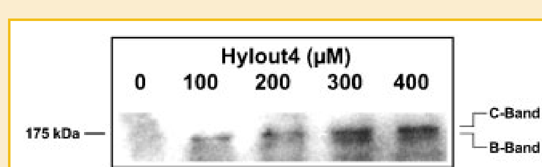

Fig. 5. Expression of ΔF508-CFTR. CFBE41o$^-$ cells were incubated with increasing concentrations of Hylout4 for 24 h and the amounts of ΔF508-CFTR was analyzed by Western blotting as described in the methods section.

experiment, we evaluated the level of ΔF508-CFTR mRNA and found that it was not altered by Hyalout4. This result indicated that compound Hylout4 enhanced cellular processing of ΔF508-CFTR.

NASAL TRANSEPITHELIAL POTENTIAL DIFFERENCE

There is no proper animal model for testing potential drugs that could correct the defective ΔF508-CFTR function [Kukavica-Ibrulj and Levesque, 2008]. Therefore we preliminarily tested Hylout4 on healthy human volunteers with the approval of the local ethical committee (Fig. 6). The NPD is used to assess ion conductance in the upper respiratory epithelium. It is used for diagnosis of CF and to monitoring the effects of pharmacological agents to correct the abnormalities of ion transport in CF [Schuler et al., 2004]. During perfusion with isotonic NaCl, both individuals showed a PD of −5 mV. Changing the perfusion to no-chloride caused a decrease of PD by −15 mV. This reflects a depolarization of the apical membrane of epithelial cells caused by chloride efflux which was induced by an extracellular directed gradient for chloride. Application of no-chloride solution together with 100 μM Hyalout caused a further decrease of PD by −14 mV with a slope smaller than for no-chloride alone (Fig. 6A). It is likely that Hyalout4 induced a recruitment of CFTR molecules from submembraneous stores into the plasma membrane, allowing a further chloride efflux and subsequently a further depolarization of the apical membrane. The following perfusion with no-chloride and 10 μM isoprenaline caused a further PD decrease by −5 mV by a chloride efflux through the activation of freshly recruited CFTR channels. In order to test the reliability of the potential measurement, isotonic NaCl was perfused. This resulted in a sharp increase of the PD reaching the basal NPD withing a few minutes (Fig. 6). The change of PD upon maximal activation of chloride conductance was about −34 mV, more than 40% caused by Hyalout4. In a second approach, isoprenaline was perfused before Hyalout4 (Fig. 6B). The decrease of PD was about −9 mV for isoprenaline in no-chloride solution. After the maximal response to isoprenaline (plateau) Hyalout4 in no-chloride solution caused a decrease of PD by maximum −10 mV. The pharmacological induced decrease of PD in both experimental setting (Hyalout4 + isoprenaline) was about −20 mV. The effect of Hyalout4 was more pronounced when perfused before isoprenaline. This indicates that Hyalout4 (1) activated CFTR chloride conductance and (2) induced the insertion of submembraneous stored CFTR molecules into the plasma membrane. In the case of "isoprenaline before Hyalout4," the activation of CFTR molecules already present in the plasma membrane, was done by isoprenaline. Therefore, the portion of Hyalout4 at the pharmacological induced decrease of PD is limited to the recruitment and activation of submembraneous stored CFTR.

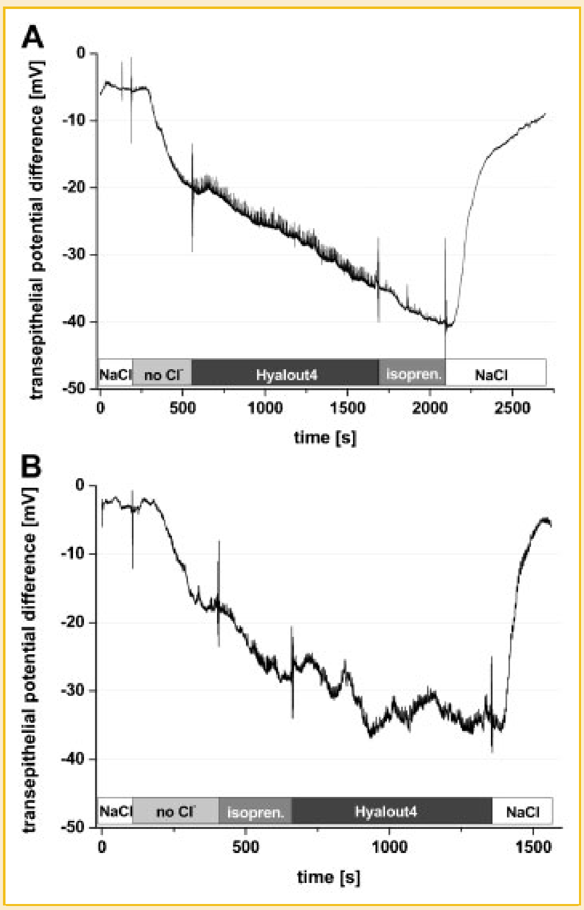

Fig. 6. Effect of compound Hylout4 on the nasal transepithelial potential difference. The effect of Hylout4 was analyzed by the nasal potential difference (NPD) of healthy individuals (A and B). Basal PD (isotonic NaCl) was −5 mV for both individuals. No-chloride causes a decrease of PD by −15 mV. Perfusion of Hyalout4 before isoprenaline causes a further decrease of PD by −14 mV with a small slope. Isoprenaline causes a further PD decrease by −5 mV (A). In the case of "isoprenaline before Hyalout4" (B), the effect of Hyalout4 was smaller (−10 mV) than in the "Hyalout4 before isoprenaline" configuration. Perfusion with isotonic NaCl at the end of the experiment ensures the reliability of the potential measurement because of a PD increase back to the basal PD within a few minutes.

DISCUSSION

Recently, we discovered that CFTR can export hyaluronan in addition to chloride or other halides such as iodide [Schulz et al., 2010] and that hyaluronan export is defective in epithelial cells expressing the mutated ΔF508-CFTR. This defective export could be responsible for the highly viscous mucous of aggregated hyaluronan protein mixtures in patients with CF, because hyaluronan is an extremely hydrated polyanion that is the major water binding component. In an attempt to modify hyaluronan export, we synthesized two disaccharide analogs that differed only in the position of one hydroxyl group mimicking either the non-reducing terminus GlcNac or GlcA. These compounds were tested on human breast carcinoma cells for their influence on hyaluronan exporter CFTR. Surprisingly, the disaccharide with the non-reducing terminus GlcNac was activating, whereas the non-reducing terminus GlcA was inactive. Thus the activating property resided in the position of the hydroxyl group indicating a stringent specificity. Further modifying the chemical structure of the activating disaccharide led to the hitherto most activating compound Hylout4.

In order to assess whether Hylout4 is a general activator of CFTR in epithelial cells, we analyzed the effect of compound Hylout4 on iodide transport as a convenient surrogate of chloride export. It activated iodide export immediately in wild-type and ΔF508-CFTR mutated epithelial cells, and it was CFTR specific, because activation was reduced by the inhibitor $CFTR_{inh}$-172. In addition, Hylout4 corrected ΔF508-CFTR cellular misprocessing and restored expression and halide permeability. When ΔF508-CFTR cells were preincubated with Hylout4 for 24 h to allow full expression and cellular processing, the iodide outflow increased about 2.6-fold. This suggested that more functional ΔF508-CFTR channels were present in the plasma membranes.

We verified correction by electrophysiological and biochemical measurements. In wild-type cells it opens CFTR channels immediately and intracellular chloride is exported reducing the resistance. In contrast, in ΔF508-CFTR cells it caused a transient increase in the TER. This phenomenon was recently described [Lesimple et al., 2010] and explained by a regulatory effect of CFTR on paracellular permeability increasing the barrier function of tight junctions. Only after new ΔF508-CFTR channels were synthesized, rescued from intracellular degradation and integrated into the plasma membranes in active form, the TER enduringly dropped. The different kinetic responses of Hylout4 and 8cpt-cAMP could be due to the different mechanisms of action. cAMP is known to activate CFTR by activation of CFTR phosphorylation [Moran, 2010], whereas Hylout4 probably activated ΔF508-CFTR by altering its conformation and rescued it from intracellular degradation.

Transepithelial nasal difference measurements were used to evaluate Hylout4 efficacy on healthy individuals. It turns out that Hyalout activates chloride conductance (CFTR) and that this pharmacological activation is quantitatively beyond the effect of isoprenaline. The slope of PD decrease induced by Hyalout4 indicates a recruitment of CFTR molecules from submembraneous stores into the plasma membrane.

The identification of small-molecule ΔF508-CFTR correctors presents a greater conceptual difficulty than that of ΔF508-CFTR potentiators or CFTR activators/inhibitors, because correction of cellular misprocessing could involve multiple targets, whereas the primary target for potentiators, activators, and inhibitors is CFTR itself. CFTR cellular processing involves translation, folding at the ER, Golgi transport, post-translational glycosylation, and apical plasma membrane targeting [Kopito, 1999]. Plasma membrane CFTR is internalized by endocytosis and then recycled to the plasma membrane or targeted for lysosomal degradation [Gentzsch et al., 2004]. ΔF508-CFTR folding is inefficient, with 99.5% of newly synthesized ΔF508-CFTR in BHK cells targeted for degradation without reaching the Golgi apparatus. Our results indicated that Hylout4 is an activator of ΔF508-CFTR function as well as a corrector of ΔF508-CFTR cellular misprocessing.

One of the main problems in the development of small molecule correctors is their high log *P* value and low solubility which effects the bioavailabilty. Recently, correctors have been synthesized which were very hydrophobic and have a log *P* of 4.1 and an EC50 of 1 μM [Ye et al., 2010]. In contrast, the log *P* and solubility for Hylout4 under physiological conditions were calculated to be −0.06 and 57 mM (http://www.vcclab.org/lab/alogps/start.html), respectively. Hylout4 thus appeared superior by orders of magnitude. Another challenge for therapy discovery has been an inhibition of halide flux by small molecule correctors such as VRT-325 [Kim Chiaw et al., 2010]. Hylout4 met this challenge.

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REFERENCES

Al-Nakkash L, Hwang TC. 1999. Activation of wild-type and deltaF508-CFTR by phosphodiesterase inhibitors through cAMP-dependent and -independent mechanisms. Pflugers Arch 437:553–561.

Bobadilla JL, Macek M, Jr., Fine JP, Farrell PM. 2002. Cystic fibrosis: A worldwide analysis of CFTR mutations—Correlation with incidence data and application to screening. Hum Mutat 19:575–606.

Carlile GW, Robert R, Zhang D, Teske KA, Luo Y, Hanrahan JW, Thomas DY. 2007. Correctors of protein trafficking defects identified by a novel high-throughput screening assay. Chem Biochem 8:1012–1020.

Dalemans W, Barbry P, Champigny G, Jallat S, Dott K, Dreyer D, Crystal RG, Pavirani A, Lecocq JP, Lazdunski M. 1991. Altered chloride ion channel kinetics associated with the delta F508 cystic fibrosis mutation. Nature 354: 526–528.

Denning GM, Anderson MP, Amara JF, Marshall J, Smith AE, Welsh MJ. 1992. Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. Nature 358:761–764.

Drumm ML, Wilkinson DJ, Smit LS, Worrell RT, Strong TV, Frizzell RA, Dawson DC, Collins FS. 1991. Chloride conductance expressed by delta F508 and other mutant CFTRs in *Xenopus* oocytes. Science 254:1797–1799.

Du K, Sharma M, Lukacs GL. 2005. The DeltaF508 cystic fibrosis mutation impairs domain–domain interactions and arrests post-translational folding of CFTR. Nat Struct Mol Biol 12:17–25.

Gentzsch M, Chang XB, Cui L, Wu Y, Ozols VV, Choudhury A, Pagano RE, Riordan JR. 2004. Endocytic trafficking routes of wild type and DeltaF508 cystic fibrosis transmembrane conductance regulator. Mol Biol Cell 15:2684–2696.

Guggino WB. 1999. Cystic fibrosis and the salt controversy. Cell 96:607–610.

Haws CM, Nepomuceno IB, Krouse ME, Wakelee H, Law T, Xia Y, Nguyen H, Wine JJ. 1996. Delta F508-CFTR channels: Kinetics, activation by forskolin, and potentiation by xanthines. Am J Physiol 270:C1544–C1555.

Hwang TC, Wang F, Yang IC, Reenstra WW. 1997. Genistein potentiates wild-type and delta F508-CFTR channel activity. Am J Physiol 273:C988–C998.

Jojovic M, Delpech B, Prehm P, Schumacher U. 2002. Expression of hyaluronate and hyaluronate synthase in human primary tumours and their metastases in scid mice. Cancer Lett 188:181–189.

Kim Chiaw P, Wellhauser L, Huan LJ, Ramjeesingh M, Bear C. 2010. A chemical corrector modifies the channel function of F508del-CFTR. Mol Pharmacol 78:411–418.

Kopito RR. 1999. Biosynthesis and degradation of CFTR. Physiol Rev 79: S167–S173.

Kukavica-Ibrulj I, Levesque RC. 2008. Animal models of chronic lung infection with *Pseudomonas aeruginosa*: Useful tools for cystic fibrosis studies. Lab Anim 42:389–412.

Kunzelmann K, Schwiebert EM, Zeitlin PL, Kuo WL, Stanton BA, Gruenert DC. 1993. An immortalized cystic fibrosis tracheal epithelial cell line homozygous for the delta F508 CFTR mutation. Am J Respir Cell Mol Biol 8:522–529.

Lansdell KA, Kidd JF, Delaney SJ, Wainwright BJ, Sheppard DN. 1998. Regulation of murine cystic fibrosis transmembrane conductance regulator Cl^{-} channels expressed in Chinese hamster ovary cells. J Physiol 512:751–764.

Lesimple P, Liao J, Robert R, Gruenert DC, Hanrahan JW. 2010. Cystic fibrosis transmembrane conductance regulator trafficking modulates the barrier function of airway epithelial cell monolayers. J Physiol 588:1195–1209.

Loo TW, Bartlett MC, Clarke DM. 2008. Correctors promote folding of CFTR in the endoplasmic reticulum. Biochem J 413:29–36.

Lukacs GL, Mohamed A, Kartner N, Chang XB, Riordan JR, Grinstein S. 1994. Conformational maturation of CFTR but not its mutant counterpart (delta F508) occurs in the endoplasmic reticulum and requires ATP. EMBO J 13:6076–6086.

Moran O. 2010. Model of the cAMP activation of chloride transport by CFTR channel and the mechanism of potentiators. J Theor Biol 262: 73–79.

Nakayama GR, Caton MC, Nova MP, Parandoosh Z. 1997. Assessment of the Alamar Blue assay for cellular growth and viability in vitro. J Immunol Methods 204:205–208.

Pedemonte N, Lukacs GL, Du K, Caci E, Zegarra-Moran O, Galietta LJ, Verkman AS. 2005. Small-molecule correctors of defective DeltaF508-CFTR cellular processing identified by high-throughput screening. J Clin Invest 115:2564–2571.

Pilewski JM, Frizzell RA. 1999. Role of CFTR in airway disease. Physiol Rev 79:S215–S255.

Rubenstein RC, Egan ME, Zeitlin PL. 1997. In vitro pharmacologic restoration of CFTR-mediated chloride transport with sodium 4-phenylbutyrate in cystic fibrosis epithelial cells containing delta F508-CFTR. J Clin Invest 100:2457–2465.

Sato S, Ward CL, Krouse ME, Wine JJ, Kopito RR. 1996. Glycerol reverses the misfolding phenotype of the most common cystic fibrosis mutation. J Biol Chem 271:635–638.

Schuler D, Sermet-Gaudelus I, Wilschanski M, Ballmann M, Dechaux M, Edelman A, Hug M, Leal T, Lebacq J, Lebecque P, Lenoir G, Stanke F, Wallemacq P, Tummler B, Knowles MR. 2004. Basic protocol for transepithelial nasal potential difference measurements. J Cyst Fibros 3(Suppl 2): 151–155.

Schulz T, Schumacher U, Prehm P. 2007. Hyaluronan export by the ABC-transporter MRP5 and its modulation by intracellular cGMP. J Biol Chem 282:20999–21004.

Schulz T, Schumacher U, Prante C, Sextro W, Prehm P. 2010. The cystic fibrosis transmembrane conductance regulator (CFTR) can export hyaluronan. Pathobiology 77:200–209.

Sheppard DN, Welsh MJ. 1999. Structure and function of the CFTR chloride channel. Physiol Rev 79:S23–S45.

Wang Y, Loo TW, Bartlett MC, Clarke DM. 2007. Correctors promote maturation of cystic fibrosis transmembrane conductance regulator (CFTR)-processing mutants by binding to the protein. J Biol Chem 282: 33247–33251.

Yang H, Shelat AA, Guy RK, Gopinath VS, Ma T, Du K, Lukacs GL, Taddei A, Folli C, Pedemonte N, Galietta LJ, Verkman AS. 2003. Nanomolar affinity small molecule correctors of defective Delta F508-CFTR chloride channel gating. J Biol Chem 278:35079–35085.

Ye L, Knapp JM, Sangwung P, Fettinger JC, Verkman AS, Kurth MJ. 2010. Pyrazolylthiazole as DeltaF508-cystic fibrosis transmembrane conductance regulator correctors with improved hydrophilicity compared to bithiazoles. J Med Chem 53:3772–3781.

Yoo CL, Yu GJ, Yang B, Robins LI, Verkman AS, Kurth MJ. 2008. 4′-Methyl-4,5′-bithiazole-based correctors of defective DeltaF508-CFTR cellular processing. Bioorg Med Chem Lett 18:2610–2614.