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Design and Synthesis of a Hybrid Potentiator-Corrector Agonist of the Cystic Fibrosis Mutant Protein Δ F508-CFTR

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Abstract

A developing therapy of Δ F508-CFTR, the most common CFTR mutation causing cystic fibrosis, employs correction of defective CFTR chloride channel gating by a ‘potentiator’ and of defective CFTR protein folding by a ‘corrector’. Based on SAR data for phenylglycine-type potentiators and bithiazole correctors, we designed a hybrid molecule incorporating an enzymatic hydrolysable linker to deliver the potentiator (**PG01**) fragment **2** and the corrector (**Corr-4a**) fragment **13**. The hybrid molecule **14** contained PG01-OH and Corr-4a—linker—CO₂H moieties, linked with an ethylene glycol spacer through an ester bond. The potentiator **2** and corrector fragments **13** (after cleavage) had low micromolar potency for restoration of Δ F508-CFTR channel gating and cellular processing, respectively. Cleavage of hybrid molecule **14** by intestinal enzymes under physiological conditions produced the active potentiator (**2**) and corrector fragments (**13**), providing proof-of-concept for small-molecule potentiator-corrector hybrids as a single drug therapy for CF caused by the Δ F508 mutation.

Keywords

Cystic fibrosis transmembrane conductance regulator protein (CFTR); Δ F508-CFTR; potentiators; correctors; hybrids; multi-ligand drug

Introduction

The genetic disease cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (CFTR), which encodes a cAMP-regulated chloride channel.^{1–3} Mutations in Δ F508-CFTR lead to distinct defects in channel gating and cellular processing.¹ Cystic fibrosis results in chronic lung infection, deterioration of lung function, and death. Δ F508-CFTR is misfolded, retained at the

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Supplementary data Supplementary data associated with this article can be found, in the online version, at doi: #####

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endoplasmic reticulum (ER), and rapidly degraded.⁴ Small-molecule therapy will likely require compounds that correct the two major underlying problems in CF: a) CFTR misfolding and ER retention, and b) defective channel gating.⁵⁻⁷ This cleavable conjugate approach requires significant SAR data to determine tolerant sites for individual ligand conjugation and is a key first step in the multiple ligand approach.⁸ Herein, we report proof-of-principle data toward the design, synthesis, and component conjugation-site tolerance of a potentiator–corrector linked hybrid.

Previously, we identified several chemical classes of small-molecules that correct each of these two defects.⁵⁻⁷

One class of activators, phenylglycines (Figure 1, **PG01**), are believed to bind to Δ F508-CFTR at the cell surface and increase chloride channel gating; these activators are referred to as ‘potentiators’. Another class of activators, bithiazoles (Figure 1, **Corr-4a**), are believed to bind to Δ F508-CFTR at the endoplasmic reticulum and facilitate its folding and plasma membrane targeting; these activators are referred to as ‘correctors’. These compounds (phenylglycines and bithiazoles) were identified from screening a diverse, small-molecule collection of 150,000 compounds.^{6,7}

Because both a potentiator and corrector are likely required to treat cystic fibrosis⁹ caused by the Δ F508 mutation, cystic fibrosis is an attractive target for the development of a multi-ligand drug. This approach to the treatment of complex diseases with single compounds containing multiple drug ligands is an emerging paradigm in drug discovery.⁹ Multi-ligand ‘hybrid’ drugs have advantages and disadvantages. One advantage is that the synthetic chemistry development takes place in the less expensive, early stages of drug development. Another advantage is that the multi-ligand approach can avoid complex pharmacokinetic/pharmacodynamic relationships, which require extensive clinical studies to clarify drug-drug interactions in cocktail/multicomponent drugs.⁸ Disadvantages with this strategy involve issues in variable potencies of the individual moieties as well as differences in their bioavailability and in vivo processing.¹⁰

Results and Discussion

To synthesize a molecule containing potentiator and corrector fragments, possible linker connection strategies were considered (Figure 1). A review of recent work and SAR data for bithiazole correctors⁹ suggested that connection of the linker through the 2-methoxy group may be a suitable position for installation of the linker to the corrector. Reviewing SAR data for the phenylglycine potentiators pointed toward incorporation of a benzyl alcohol functionality on the aniline ring in place of the 4-isopropyl group.

Chemistry

The synthesis of the hybrid complex began with the synthesis of the potentiator fragment **2** (Scheme 1). Starting from commercially available 4-aminobenzyl alcohol and coupling with *N*-methyl-Boc-phenylglycine under EDC coupling conditions gave the Boc protected 2° amine **1**. TFA mediated Boc deprotection and EDC coupling of the resulting 2° amine with 3-indole acetic acid gave the potentiator fragment **2** in 75% yield together with a small amount of ester **3** (11%).

The synthesis of the corrector fragment began with the synthesis of the linker (Scheme 2). Diethylene glycol was reacted with *t*-butylbromoacetate to give ethylene glycol *t*-butyl ester **4**. Mitsunobu coupling¹¹ with 2-nitro-4-chlorophenol gave **5** in excellent yield. Reduction of the nitro group using stannous chloride followed by treatment with thiophosgene gave isothiocyanate **7**.

Subsequent treatment of isothiocyanate **7** with ammonia gas in DCM afforded the thiourea **8**. Commercially available benzoylisothiocyanate was reacted with ammonia gas to afford thiourea **9**, which was reacted with 3-chloro-2,4-pentanedione to yield thiazole **10**. Bromination of **10** with pyridinium tribromide in HBr gave α -bromoketone **11** in excellent yield. Thiazole cyclization of thiourea **8** and α -bromoketone **11** gave Corr-linker-*t*-butylester **12**, which was deprotected with TFA affording Corr-4a-CO₂H **13**. The hybrid molecule **14** (Scheme 3) was constructed via an EDC coupling reaction of PG01-OH fragment **2** and Corr-4a-CO₂H fragment **13** which gave the hybrid in 11% yield.

Activity measurements

The Δ F508-CFTR potentiator and corrector activities of fragment **2**, fragment **13**, and hybrid **14**, were measured, and compared with reference compounds **PG01** and **Corr-4a**. Activities were assayed by established methodology utilizing FRT epithelial cells stably coexpressing human Δ F508-CFTR and the high-sensitivity halide-sensing green fluorescent protein YFP-H148Q/I152L as described previously.⁵⁻⁷ For measurement of potentiator activity, cells were grown at 27 °C for 24 h to allow Δ F508-CFTR trafficking to the cell plasma membrane. Δ F508-CFTR channel function was measured from the kinetics of halide (iodide) influx after incubation with test compound for 10 min in the presence of the cAMP agonist forskolin. For measurement of corrector activity, cells were incubated at 37 °C for 20 h in the presence of test compound, washed with PBS, and assayed for iodide influx in the presence of forskolin and the reference potentiator genistein (50 μ M). Measurements were made on a fluorescence plate reader with an automated syringe pump, incorporating appropriate positive and negative controls in all measurements, each of which was made in quadruplicate. The first graph (Figure 2) summarizes concentration-activity data (iodide influx rates) for potentiator **PG01**, PG01-OH **2**, and hybrid **14** in the potentiator assay. In the second panel of Figure 2, **Corr-4a** is compared with corrector fragment **13** and potentiator—corrector hybrid **14** in the corrector assay. Both the potentiator (**2**) and corrector (**13**) fragments exhibit strong activity in their corresponding assays. This retained activity validates that the structural modifications necessary to synthesize hybrid **14**. The lack of activity of hybrid **14** is likely due to its low penetration into the cell interior.

LCMS

An LC method to identify the potentiator, corrector, and hybrid peaks was developed using a Waters 2695 LC and a Waters PDA 996 detector coupled to an Alliance mass spectrometer, ionization mode; electrospray (+), mass range 200-1200 Da, 23-V cone voltage, column; XTerra MS C₁₈ (Waters, 2.1 mm $\times$ 50 mm $\times$ 3.5 μ m). Reversed-phase HPLC separations were carried out using a C₁₈ column connected to a solvent delivery system (model 2690; Waters, Milford, MA). The solvent system consisted of a linear gradient from 0% CH₃CN/100% H₂O, to 100% CH₃CN/0% H₂O, over 28 min, followed by 4 min at 100% CH₃CN/0% H₂O, and 3 min at 0% CH₃CN/100% H₂O to equilibrate the column for the next run (0.2 ml/min flow rate). The retention times for PG01-OH (**2**), Corr-4a-CO₂H (**13**), and hybrid **14** were: 17 min, 20 min, and 23 min, respectively, detected at 256 nm. Mass spectra were used to confirm compound identity. Mass spectrometer specifications are as follows: Waters Alliance (HT 2790 + ZQ) mass spectrometer utilizing positive ion detection mode, scanning from 200 to 1200 Da.

Hydrolysis of the Hybrid

The hybrid was first treated with carbonic anhydrase, a major enzyme present on the intestinal mucosa, incubating for 10 min at rt (Figure 3).¹² Methanol was added to the solution, cooled in an ice bath at 0 °C, and centrifuged at 16,000 $\times$ g for 10 min. The supernate was analyzed by LC/MS. The hybrid was completely hydrolyzed to the

corresponding potentiator and corrector fragments as identified by LC/MS (Figure 3A). Hydrolysis of the hybrid **14** was performed in parallel by incubation with the intestinal contents from mice at 37°C (90% humidity, 5% CO₂) for 4h. Methanol was added to the solution, cooled to 0 °C, and centrifuged at 16,000 × g for 10 min. The supernate was analyzed by LC/MS. The hybrid was completely hydrolyzed to the corresponding potentiator and corrector fragments identified by LC/MS (Figure 3B). Peaks were correlated to spectra of purified compounds run under identical conditions. Disappearance of the hybrid **14** peak (retention time, 23 min) in both HPLC experiments (A and B) and the appearance of the two corresponding active fragments PG-OH **2** (retention time, 17 min), Corr-CO₂H **13** (retention time, 20 min) illustrate that the hybrid is being hydrolyzed to the active fragments.

Conclusion

This work provides proof-of-concept for the design, synthesis, and component conjugation-site tolerance of an ester-linked Δ F508-CFTR potentiator–corrector hybrid molecule. Further, this study provides insight into the structural requirements/tolerances of a multiple ligand approach for CF treatment. The resultant potentiator and corrector fragments following hydrolysis, as would occur in the gastrointestinal tract, were active in the correction of defective Δ F508-CFTR cellular processing and chloride channel gating, respectively. Thus, a single compound can be engineered to confer the distinct activities required to restore chloride channel function in cystic fibrosis caused by the Δ F508 mutation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
A) $\Delta F508$ -CFTR corrector **Corr-4a** and potentiator **PG01**. B) Strategy for a potentiator—corrector hybrid molecule.

Figure 2. Dose-response data for potentiator assay of compounds **PG01**, **2**, and **14** (top panel SEM; n = 4) and for corrector assay of compounds **13** and **Corr-4a**, **14** (bottom panel, SEM; n = 4).

Figure 3.

A) HPLC spectra of the supernate from the reaction of hybrid **14** and carbonic anhydrase. B) HPLC spectra of the supernate from the reaction of hybrid **14** and mouse intestinal contents.

Scheme 1.
Synthesis of PG01-OH fragment **2**.

Scheme 2.
Synthesis of Corr-CO₂H fragment **13**.

Scheme 3.

Synthesis of hybrid **14** (arrow A) and subsequent hydrolysis by carbonic anhydrase or intestinal contents of this hybrid (arrow B) to the active potentiator PG-OH **2** and corrector Corr4a-CO₂H **13** fragments.