Discovery of a picomolar potency pharmacological corrector of the mutant CFTR chloride channel

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F508del, the most frequent mutation causing cystic fibrosis (CF), results in mistrafficking and premature degradation of the CFTR chloride channel. Small molecules named correctors may rescue F508del-CFTR and therefore represent promising drugs to target the basic defect in CF. We screened a carefully designed chemical library to find F508del-CFTR correctors. The initial active compound resulting from the primary screening underwent extensive chemical optimization. The final compound, ARN23765, showed an extremely high potency in bronchial epithelial cells from F508del homozygous patients, with an EC50 of 38 picomolar, which is more than 5000-fold lower compared to presently available corrector drugs. ARN23765 also showed high efficacy, synergy with other types of correctors, and compatibility with chronic VX-770 potentiator. Besides being a promising drug, particularly suited for drug combinations, ARN23765 represents a high-affinity probe for CFTR structure-function studies.

INTRODUCTION

The F508del mutation is the most frequent among patients with cystic fibrosis (CF) (1). F508del severely impairs the folding and stability of the CF transmembrane conductance regulator (CFTR) protein, a chloride channel expressed in the apical membrane of various epithelia in the respiratory, gastrointestinal, and reproductive systems (1–2). As a consequence, F508del-CFTR protein trafficking is stopped at the endoplasmic reticulum where it is largely degraded through the ubiquitin-proteasome pathway (3, 4). Peripheral quality control systems are also involved in F508del-CFTR degradation if the protein reaches the plasma membrane (5). F508del also causes a defect in CFTR channel gating that is evident as a reduced time spent in the open (ion-conducting) state (6). F508del mutation, in the homozygous condition or in combination with other types of severe mutations, is associated with a classical form of CF, characterized by progressive loss of respiratory function, pancreatic insufficiency, and infertility (1). These problems are due to defective epithelial secretion of chloride and bicarbonate, which results in the production of dense mucus secretions that clog the airways and exocrine gland ducts. An additional characteristic of patients with severe CF mutations is the high NaCl concentration (>100 mM) in sweat, a defect caused by impairment of chloride reabsorption by the sweat gland duct (1).

The trafficking defect caused by F508del can be rescued by small molecules called correctors (7). Various high-throughput screening campaigns have led to the identification of correctors that rescue F508del-CFTR activity (8–12). The mechanism of action of these compounds has not yet been defined, but they may act by directly binding to F508del-CFTR or by modulating other proteins involved in the cell quality control system or the protein trafficking machinery. Only a few molecules have progressed to clinical trial stage. Most of the other correctors lack adequate efficacy, specificity, and consistent activity in different cell contexts. Two drugs have been approved so far for the rescue of F508del-CFTR, i.e., Orkambi and Symdeko, both developed by Vertex Pharmaceuticals. Orkambi consists of a combination of the corrector VX-809 (10) and the potentiator VX-770 (13). The potentiator is included to address the gating defect and, hence, to maximize the rescue of F508del-CFTR function. However, Orkambi treatment is associated with a relatively modest clinical benefit in patients homozygous for F508del (14) and no effect at all, at least on pulmonary function, on individuals with a single copy of the mutation (15). These results contrast with the high clinical benefit obtained by the potentiator VX-770 alone on patients having mutations (e.g., G551D) that only cause a gating defect (16). The insufficient efficacy of corrector therapy in vivo has been explained with the particular resistance of F508del trafficking defect to treatment with a single agent since the mutation not only causes multiple defects to protein stability and folding (17–20) but also may be due to specific liabilities of VX-809 when administered in vivo. Symdeko was recently approved by the U.S. Food and Drug Administration and consists of the combination of the corrector VX-661 (tezacaftor) and the potentiator VX-770. As for Orkambi, the efficacy of Symdeko in F508del homozygous patients was smaller than that of VX-770 in patients with the G551D mutation (21). Another possible reason for the limited efficacy of corrector-potentiator combinations tested so far is the “anti-corrector” effect that VX-770 seems to cause when administered chronically in vitro (22, 23). More recently, better clinical results have been obtained with triple combinations including the potentiator VX-770 and two correctors having complementary mechanisms of action, i.e., VX-661 together with VX-445 or VX-659 (24, 25). The triple combinations were designed to include VX-661 and not VX-809 since the latter one was reported to have a relatively high frequency of adverse events (26).

In this study, we report the discovery and biological characterization of novel and potent F508del correctors. Starting from a hit identified by high-throughput screening of a carefully selected compound library, an iterative process of chemical synthesis and functional/biochemical
evaluation of hit analogs in multiple cell systems, including bronchial epithelial cells from patients with CF, allowed us to obtain a lead compound that rescued F508del-CFTR at low nanomolar concentrations. Further elaboration of the chemical structure of the lead compound led to the discovery of ARN23765, a corrector with picomolar potency and high efficacy over a 3-log unit concentration range.

RESULTS
For the primary screening, we used a carefully selected collection of compounds, which was assembled starting from an initial set of ca. 300,000 commercially available molecules. A series of progressively more stringent filters were applied to discard compounds with suboptimal drug-like properties and with core chemical structures known to bind to multiple target proteins or that are extensively exploited in medicinal chemistry. A subsequent stepwise clustering protocol based on an unweighted pair group method with arithmetic mean hierarchical agglomerative algorithm (27) allowed us to select 11,334 maximally diverse compounds.

The whole chemical library was screened in parallel on two cell lines, FRT and CFBE41o−, both stably expressing F508del-CFTR (28, 29). Cells also expressed the halide-sensitive yellow fluorescent protein (HS-YFP), which allows evaluation of F508del-CFTR activity in the cell membrane by calculating the fluorescence quenching rate resulting from iodide influx (30). The search for correctors was done by treating the cells with each compound at 5 μM (CFBE41o−) or 10 μM (FRT) for 24 hours. The screening on each cell line was run in duplicate using two separate cell preparations. Each microplate also contained corr-4a (5 μM) and VX-809 (1 μM) as positive controls. The Z factor (31) was satisfactory for all screenings: 0.5 for FRT cells and 0.65 for CFBE41o− cells. Figure 1A summarizes the results obtained for both cell types. It can be easily appreciated that FRT cells showed a much larger number of putatively active compounds, a feature that is consistent with a more relaxed cell quality control system. We considered as hits all compounds whose activity, calculated from the two rounds of the screening, was higher than a threshold resulting from the average plus 4 SDs of activity measured in vehicle-treated cells. This threshold corresponds to 125% (FRT) or 135% (CFBE41o−) of vehicle-treated cells. In this way, we selected 117 hits in FRT and 11 hits in CFBE41o− cells. Two compounds appeared as hits in both cell types. All 126 compounds were retested on FRT and CFBE41o− at different concentrations to confirm activity and obtain information on potency and efficacy. A large fraction of correctors identified in the FRT screening was confirmed when tested in the same cells. However, in agreement with the primary screening results, most FRT hits were inactive in CFBE41o− cells. The two hits that were found in both FRT and CFBE41o− screenings were confirmed when retested with the HS-YFP assay, and three additional compounds, which were initially missed in the CFBE41o− screening, were found to be active in both cell types. Figure 1B shows dose-response relationships for the five active compounds, tested as correctors on FRT and CFBE41o− cells. Incubation with these compounds at micromolar concentrations modestly increased the function of F508del-CFTR. These compounds were subsequently evaluated on...
primary CF bronchial epithelial cells homozygous for the F508del mutation (Fig. 1C). Cells differentiated under air-liquid condition on porous membranes were treated by including correctors (5 to 10 µM) or vehicle alone in the basolateral medium for 24 hours. After treatment, the epithelia were mounted in a perfusion chamber to measure short-circuit current. After blocking the epithelial sodium channel ENaC with amiloride (10 µM), cells were stimulated with chlorophenylthio–adenosine 3′,5′-cyclic monophosphate (CPT-cAMP) (100 µM) plus VX-770 (1 µM) to maximally activate F508del-CFTR. The resulting current was then inhibited with the potent CFTR inhibitor-172 (inh-172; 10 µM). The drop in transepithelial current elicited by inh-172 was taken as the parameter reflecting F508del-CFTR function in the apical membrane. Only one compound, ARN5562, was effective, leading to a rescue of F508del-CFTR equivalent to nearly 60% of that achieved with VX-809 (Fig. 1C). ARN5562 was also evaluated in Western blot experiments. In these experiments, the electrophoretic mobility of F508del-CFTR reports the extent of maturation and trafficking. Mutant CFTR migrates as “band B” (at approximately 150 kDa) that corresponds to the core-glycosylated immature form of the protein. Correctors improve the trafficking of the protein from the endoplasmic reticulum to the Golgi, thus allowing full glycosylation and appearance of “band C” (at approximately 170 kDa), the prevalent form in cells expressing wild-type CFTR. Figure 1D shows that ARN5562 is effective in improving F508del-CFTR maturation, although less than VX-809.

The promising results obtained with ARN5562 prompted us to start a medicinal chemistry campaign with the goal to improve potency and efficacy. Changes were introduced into the structure of ARN5562 to explore the structure–activity relationships (SARs) in the rescue of F508del-CFTR activity for this class of compounds. At each round of synthesis, all compounds were first tested at multiple concentrations on FRT and CFBE41o− with the HS-YFP assay. Then, the active compounds were evaluated with the transepithelial electrical conductance (TEEC) assay on FRT cells. Last, the best compounds resulting from HS-YFP and TEEC assays were tested on CF bronchial epithelial cells and in Western blot experiments.

Figure 2A shows the structure of hit ARN5562 and of two key compounds that were discovered during the optimization process: the advanced hit ARN21586, and the lead compound ARN22081. The chemical evolution of hit ARN5562 focused primarily on its right end part, i.e., the benzodioxane ring, and left end part, i.e., the 3,5-dimethyl-4-chloropyrazol-1-yl moiety. The key compounds identified in the SAR study and the data obtained in the HS-YFP assay in F508del-FRT cells are reported in Table 1. The full SAR study will be reported elsewhere. Replacement of the benzodioxane with a benzodioxole ring on the right end part of ARN5562 and introduction of a trifluoromethyl group at position 3 of the pyrazolyl moiety led to ARN21586, an analog with improved efficacy and potency. The extensive exploration of substituents on the pyrazolyl ring resulted in the identification of the 4-methoxybenzoic acid at position 5 as a group imparting good potency and efficacy. Combining this group with a gem-difluoro-benzodioxole ring on the right end part of the ARN21586 scaffold yielded the lead compound ARN22081.

Figure 2B shows dose-response relationships obtained for the three key compounds with the HS-YFP assay. It can be seen that a marked...
improvement in efficacy and potency was achieved. While the initial hit ARN5562 was only modestly effective at micromolar concentrations, ARN22081 markedly rescued F508del-CFTR with single-digit nanomolar potency (Fig. 2B). This extent of activity was confirmed with the TEEC assay (Fig. 2C). Rescue of F508del-CFTR to the plasma membrane generates a pathway for transepithelial flux of chloride ions that results in an increase in CFTR-dependent TEEC, a parameter that can be measured with chopstick electrodes. A detailed description of this method is reported elsewhere (32). Figure 2C shows that ARN5562 has a limited ability to correct F508del-CFTR, compared to VX-809, which is only achieved at 5 \( \mu \text{M} \). A progressive improvement in potency and efficacy is observed with ARN21586 and ARN22081. With the latter compound, a.

### Table 1. Chemical structure, maximal efficacy \( (E_{\text{max}}) \), and potency data of ARN5562 and selected analogs in F508del-CFTR FRT cells (HS-YFP assay). The \( E_{\text{max}} \) values are normalized to the activity with DMSO (negative control).

| ID         | \( R^1 \) | \( R^2 \) | \( E_{\text{max}} \) | \( EC_{50} \) (\( \mu \text{M} \)) |
|------------|-----------|-----------|----------------------|-----------------------------|
| ARN5562   |           |           | 1.56                 | 1.04                        |
| ARN21586  |           |           | 2.29                 | 0.346                       |
| ARN22081  |           |           | 2.19                 | 0.0019                      |
| ARN22652* |           |           | 2.4                  | 0.001                       |
| ARN23765† |           |           | 2.28                 | 0.0004                      |
| ARN23766‡ |           |           | 2.18                 | 0.063                       |
| VX-809     |           |           | 2.53                 | 0.319                       |

*Racemate. †(S)-enantiomer of ARN22652. ‡(R)-enantiomer of ARN22652.
correction similar to that of VX-809 is obtained with submicromolar concentrations. These results were confirmed at the protein level with Western blot experiments (Fig. 2D) and at the functional level with short-circuit current experiments done on CF primary bronchial epithelial cells (Fig. 2E). In these experiments, it can be appreciated that ARN22081 rescues mutant CFTR maturation and function to an extent that is comparable to that obtained with VX-809.

Further medicinal chemistry efforts on ARN22081 led to the discovery of the tetrahydroindazole analog ARN22652, a racemate retaining the maximal efficacy and potency of the parent compound. Separation of ARN22652 components by chiral column chromatography yielded the two enantiomers, ARN23765 and ARN23766 (Fig. 3A). Notably, using the HS-YFP assay (Fig. 3B), the eutomer, ARN23765, showed efficacy comparable to ARN22652 but subnanomolar potency [median effective concentration (EC50) = 0.4 nM], whereas the dystomer, ARN23766, displayed a ca. 150-fold drop in potency (EC50 = 63 nM). The rescue of F508del-CFTR protein by ARN23765 at low concentrations was also observed using Western blot experiments (Fig. 3C). The high potency and efficacy of ARN23765, compared to ARN23766 and the racemate, was confirmed with the TEEC assay on FRT cells (Fig. 3D) and, particularly, with short-circuit current experiments on bronchial epithelial cells from a patient with F508del/F508del (Fig. 3E). Note the marked difference in potency of ARN23765 with respect to VX-809 in bronchial epithelial cells. While VX-809 had an EC50 of nearly 200 nM, ARN23765 showed a value of 38 pM, thus indicating a more than 5000-fold improvement (Fig. 3E). Rescue activity by ARN23765 decreased at 1 μM. However, a rescue close to maximal effect was maintained in the range of concentrations between 0.1 and 100 nM. Besides VX-809, we also tested other correctors including VX-661, FDL-169 (developed by Flatley Discovery Lab), and GLPG2222 (developed by Galapagos). ARN23765 was consistently more potent than the other correctors, with GLPG2222 (EC50 ~ 2 nM) being the one that had the closest properties to our compound (Fig. 3E). Unexpectedly, VX-661 appeared to have a modest corrector activity, 40% of VX-809, only observed at micromolar concentrations.

To confirm that ARN23765 activity is not affected by genetic background, we used cells from separate patients homozygous for F508del mutation. For this purpose, we modified the TEEC assay used for FRT cells. In this way, we had a better throughput compared to conventional short-circuit recordings. Besides acquiring TEEC values, we also measured the transepithelial electrical potential difference (PD) at each step. All values were taken at resting, after addition of apical amiloride, after maximal stimulation of F508del-CFTR activity (with forskolin plus genistein), and after block with PPQ102. From TEEC and PD, we calculated the equivalent short-circuit current.

![Fig. 3. Evaluation of ARN23765 as F508del-CFTR corrector.](image)

(A) Chemical structures of the two enantiomers ARN23765 and ARN23766. (B) Dose-response relationships for the two compounds (24 hours of treatment) determined with the HS-YFP assay in CFBE41o− cells. Each symbol is the mean ± SD of n = 3 experiments. (C) Western blot analysis of corrector effects at the indicated concentrations (in nM or μM; 24 hours of treatment). The image is representative of n = 3 similar experiments. (D) Evaluation of correctors (0.1 nM to 5 μM; 24 hours) in FRT cells by TEEC (n = 7 to 11). The dashed lines indicate the values for vehicle and VX-809 (1 μM; 24 hours). *P < 0.05 and **P < 0.01 versus vehicle (ANOVA with Dunnett’s post hoc test; n = 6 to 11). (E) Representative traces and summary of data obtained from short-circuit current recordings on F508del/F508del bronchial epithelial cells. The graphs report the means ± SD of amplitude of current drop elicited by inh-172 in epithelia treated with the indicated correctors (24 hours of treatment) and concentrations (n = 10 to 27).
(I_{eq})]. The results from a typical experiment are shown in Fig. 4A. Treatment with VX-809 (1 μM) or with ARN23765 (10 nM) elicited an increase in the response to the activating cocktail and, correspondingly, to the CFTR blocker with respect to cells treated with the vehicle alone. For each epithelium, we measured the difference in I_{eq} before and after block with PPQ102 (ΔI_{eq}). These values are reported in Fig. 4B for the different conditions. Tests on cells from four separate patients with F508del/F508del confirmed the results obtained with the conventional short-circuit current technique: Incubation with ARN23765 (10 nM) markedly increased F508del-CFTR function, with results comparable to those of VX-809 (1 μM). We also evaluated epithelia from two patients with a single copy of F508del and a premature stop codon (R553X and G542X) on the other allele (Fig. 4B). ARN23765 was also notably effective on these cells. As expected from the halved amount of F508del-CFTR produced, absolute values of ΔI_{eq} were smaller than those obtained for F508del/F508del epithelia. We also carried out TEEC/PD measurements on non-CF epithelia (Fig. 4B). In agreement with the expression of wild-type CFTR protein, ΔI_{eq} values in vehicle-treated cells were more than 10-fold larger compared to those measured in CF epithelia under the same conditions. Treatment of non-CF epithelia with ARN23765, but not with VX-809, elicited a significant increase of wild-type CFTR function (Fig. 4B).

It has been reported that long-term treatment of cells with corrector-potentiator combinations leads to a reduced rescue of F508del-CFTR (22, 23). This effect has been attributed to an impairment of F508del-CFTR stability caused by VX-770 and other potentiators. To verify whether this effect was observed also with our corrector, we treated F508del/F508del epithelia with ARN23765 (10 nM) plus VX-770 (0.5 μM) for 24 hours. Then, these compounds were removed, and cells were acutely stimulated with forskolin and genistein for the other TEEC/PD experiments. As shown in Fig. 4C, the function of F508del-CFTR (ΔI_{eq}) in cells rescued with ARN23765 alone was the same as that of cells also treated with VX-770 for 24 hours.

Next, we carried out short-circuit current recordings on F508del/F508del epithelia treated with VX-809 (1 μM) or ARN23765 (1 nM) for 72 hours (Fig. 4D). The extent of rescue was comparable to that of epithelia treated with these correctors for only 24 hours (compare with Fig. 3E), indicating that prolonged exposure to ARN23765 did not negatively affect the epithelia. In this set of experiments, we also removed the correctors in the last 24 hours of treatment. Epithelia that had been treated with ARN23765 showed a persistent level of F508del-CFTR function, while rescue was lost in epithelia previously treated with VX-809 (Fig. 4D and Fig. S1A). To further confirm these data, we repeated the experiments for a longer time, treating the cells for 120 hours with VX-809 (1 μM) or ARN23765 (1 nM). F508del-CFTR rescue was still maintained for both compounds compared to 72 hours (Fig. S1, A and B). In this set of experiments, correctors were also removed in the last 36 hours. F508del-CFTR function was partially maintained in cells treated with ARN23765 but returned to control levels in cells treated with VX-809 (Fig. S1B).

To further characterize the corrector activity of ARN23765 on mutant CFTR, we evaluated the stability of F508del-CFTR in CFBE41o- cells following 24 hours of treatment with ARN23765 or with VX-809, followed by addition of cycloheximide to the medium to block protein synthesis. We then lysed cells at different time points, and cell lysates were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblotting to evaluate CFTR expression. As shown in Fig. 5 (A and B), the expression of mutant CFTR (both bands B and C) decreases over time. However, treatment with ARN23765 (1 nM) significantly increased, by threefold, the half-life of mature CFTR (band C). Upon treatment with VX-809 (1 μM), the stability of band C was increased by twofold.

The conformational stability of mutant CFTR protein under resting conditions and following rescue with correctors was evaluated by determining the denaturation temperature, which induces conversion of detergent-solubilized CFTR into SDS-resistant aggregates (33). Accordingly, F508del-CFTR CFBE41o- cells were treated for 24 hours with ARN23765 or VX-809 and then lysed. In parallel, cell lysates were generated from CFBE41o- cells stably expressing
wild-type CFTR. Cell lysates were heat-denatured at 28°C to 70°C, and the aggregation-resistant CFTR protein was quantified by SDS-PAGE, followed by Western blotting (Fig. 5, C and D). Treatment with ARN23765 (1 nM) and, to a lower extent, with VX-809 (1 μM) significantly increased the resistance to thermoaggregation of mature CFTR, suggesting that ARN23765 promotes the accumulation of conformationally stabilized complex-glycosylated F508del-CFTR (Fig. 5, C and D).

Recently, Lukacs and collaborators (11) have reported the identification of novel correctors targeting distinct structural defects of CFTR, which synergistically rescue mutant expression and function at the plasma membrane. We were able to retrieve from a commercial source (Life Chemicals) one of the compounds, referred to as 4172, that was classified as a type 3 corrector, according to its mechanism of action (11, 17). Thus, we tested 4172 alone and in combination with ARN23765 or VX-809 on FRT and CFBE41o− cells using the
with correctors were also investigated to assess the extent of Ca\(^{2+}\) -
enhancement of recordings on F508del/F508del bronchial epithelia. Cells were treated
for 24 hours with 4172 (10 \(\mu\)M), VX-809 (1 \(\mu\)M), or ARN23765 (10 nM) alone and in combination (Fig. 6, C and D). The combinations between 4172 and ARN23765 or 4172 and VX-809 resulted in a similar extent of rescue, although the concentration used for ARN2765 was 100 times lower than the one used for VX-809 (Fig. 6, C and D). These results suggested that ARN2765 and VX-809 belong to the same class of correctors, i.e., class 1 (11). In agreement with this interpretation, we found no additive effect when the two compounds were combined together (Fig. 6E).

We evaluated the effect of single correctors and corrector combinations on the pH of the apical fluid of cultured CF bronchial epithelia as a relevant pathophysiological parameter. Both ARN23765 (10 nM) and 4172 (10 \(\mu\)M) elicited a significant increase in pH with respect to control-treated cells (Fig. 6F). The combination of the two correctors further increased pH to a level significantly higher than that measured with each corrector alone (Fig. 6F).

To further characterize ARN23765, we carried out a series of additional experiments. First, we asked whether ARN23765 improves the response of F508del cells to a receptor-mediated physiological stimulus. Therefore, we stimulated the cells with isoproterenol, a \(\beta\)-adrenergic agonist that, by increasing cytosolic cAMP, should lead to CFTR activation. The response to isoproterenol, as well as that resulting from subsequent addition of VX-770, was very small in control-treated cells (Fig. S2A). In contrast, cells treated with VX-809 (1 \(\mu\)M) or ARN23765 (10 nM) showed relatively large responses to both agents, thus indicating that correctors improve the amount of F508del-CFTR responding to the \(\beta\)-adrenergic stimulus and that the VX-770 potentiator further amplifies this type of response. In agreement with the involvement of CFTR, the addition of inh-172 at the end of recordings caused a current drop that was significantly larger in corrector-treated versus control-treated cells (Fig. S2A). Cells treated with correctors were also investigated to assess the extent of Ca\(^{2+}\) -

As a second type of experiment, we evaluated the possibility that ARN23765 has an additional activity as CFTR potentiator. Cells were treated for 24 hours at low temperature (32\(^\circ\)C) or with correctors, VX-809 or ARN23765, to correct the trafficking defect. Then, ARN23765 was acutely added to the cells at various concentrations. There was no increase in activity in contrast to real potentiators genistein and VX-770 (Fig. S2C).

We also tested ARN23765 on other types of mutations, N1303K and P574H. N1303K is characterized by severe trafficking and gating defects. In particular, in a previous study, it was found that rescue of N1303K-CFTR function requires the combination of a potentiator plus the co-potentiator ASP-11 (34). We cotransfected human embryonic kidney (HEK) 293 cells with N1303K-CFTR and HS-YFP plasmids. We found that N1303K-CFTR function was significantly stimulated by VX-770 and ASP-11 and that this effect was further amplified by previous incubation with correctors ARN23765 or VX-809 (Fig. S4, A and B). To study P574H, we used FRT cells with stable coexpression of this CFTR mutant and HS-YFP. The assay showed that ARN23765 improves the function of P574H-CFTR, particularly in combination with low temperature (Fig. S5).

Last, we used a panel of assays to assess the effect of ARN23765 on cell viability. In contrast to positive controls, i.e., celastrol or
cytochalasin D, ARN23765 did not impair cell proliferation nor caused cell toxicity (fig. S6, A to C). In addition, to detect potential off-target effects, we outsourced to contract research organizations (Eurofins Cerep and SOLVO Biotechnology) the test of ARN23765 on a panel of relevant biological targets including receptors, monoamine transporters, adenosine 5′-triphosphate–binding cassette (ABC) proteins, and hERG channel. There were no alerting effects of the compound on these targets at a concentration (10 µM) well above the EC$_{50}$ of ARN23765 as CFTR corrector. As for ABC transporters, at 10 µM, ARN23765 inhibited ABC-B1 (multidrug resistance mutation 1 or P-glycoprotein) and ABC-C2 (multidrug resistance-associated protein 2) by 13 and 27%, respectively.

**DISCUSSION**

Identification of novel pharmacological correctors of the misfolding caused by the F508del mutation is highly needed to develop effective treatments for a large number of patients with CF. These treatments require the combination of small molecules having a complementary mechanism of action since F508del causes multiple defects (11, 17). It is generally accepted that a single agent is not enough to rescue the mutant protein to high levels and to generate a significant clinical benefit.

Here, we used the screening of a maximally diverse chemical library to find novel correctors. We previously showed that corrector activity is often cell type–dependent (35), i.e., correctors identified by screening in a single cell line often fail to be confirmed in other cell lines. Instead, compounds with activity on more than one cell line have a higher probability to be also effective in native airway epithelial cells from patients with CF. For this reason, to maximize the probability of finding real correctors, we decided to perform a double screening in parallel on two cell lines expressing F508del-CFTR, i.e., FRT and CFBE410$. The small number of hits detected in the bronchial cell line may arise from a more stringent cell quality control system. By retesting all hits in the two cell lines, we found five compounds with activity on both cell systems. One of them, ARN5562, was active in native bronchial epithelial cells. Because of this very promising profile, we concentrated our efforts to improve the efficacy and potency of this molecule.

After several rounds of chemical synthesis and functional/biochemical evaluation with multiple assays, we obtained ARN23765, an extremely potent and highly effective corrector for the F508del mutant protein. Regarding potency, ARN23765 appears as the compound with the lowest EC$_{50}$ developed so far. Compared to correctors already used in patients with CF, namely, VX-809 and VX-661, it is effective at concentrations that are more than 5000 times lower. Another interesting property of ARN23765 is the lack of interference by VX-770, previously shown to be a critical negative aspect of the latter compound (22, 23). We found no decrease in activity rescue when the cells were chronically treated with both compounds together. Furthermore, ARN23765 elicits a long-term correction that can be observed up to 36 hours after removal of the compound. This behavior was not observed for VX-809 despite the use of a concentration that was 1000 times higher. At the moment, the mechanism explaining the lasting correction produced by ARN23765 is not clear. It may derive from a persistence of the compound in intracellular compartments or from a long-term effect of the compound on mutant CFTR. Future studies are required to elucidate this issue.

Regarding the mechanism of action, the lack of additivity with VX-809 suggests that ARN23765 is a class 1 corrector (17). Comparison of the chemical structures of ARN23765 and the Vertex corrector shows that the two compounds share a common moiety, i.e., the gem-difluorobenzodioxole residue. However, the connectivity of the latter group with the rest of the molecule in ARN23765 is different from that in VX-809. Overall, ARN23765 appears to have less conformational freedom than VX-809. We speculate that, if the two compounds share the same binding site, the higher potency of ARN23765 may derive from (i) a lower energy to achieve the productive binding conformation, (ii) more favorable interactions with critical amino acid side chains at the binding pocket, or a combination of the two. Further studies are required to explain the unexpectedly higher potency of ARN23765 compared with VX-809 and other correctors.

To assess the value of ARN23765 as a possible drug candidate in the CF field, we carried out a series of experiments to determine its effects on other epithelial functions, other CFTR mutants, and cell viability. Treatment of bronchial epithelial with ARN23765 caused an increase in apical fluid pH that was further enhanced when combined with 4172. It has been shown that the apical surface of CF airway epithelia is acidic because of defective CFTR-dependent bicarbonate secretion (36, 37). This abnormality may lead to defective mucociliary clearance and impaired antibacterial defense. Therefore, the change in pH elicited by correctors appears as an effect that may be beneficial on important epithelial functions. We also found that the response to isoproterenol, a stimulus that mimics physiological activation of CFTR, was significantly improved by ARN23765. We tested ARN23765 and VX-809 on Ca$^{2+}$-activated Cl$^-$/secretion and ENaC-dependent Na$^+$ absorption. While the former process was unaffected, the latter was partially inhibited by both compounds. In the original study on VX-809, it was found that the corrector enhances the amount of fluid on the apical surface, possibly as a result of enhanced fluid secretion and/or decreased ENaC-dependent Na$^+$ absorption (10). Future studies will need to address whether the decrease in ENaC function is a consequence of CFTR rescue. Furthermore, we evaluated the efficacy of ARN23765 on other CF mutations. We found a significant increase in CFTR-dependent function, particularly in combination with other agents. These results suggest that ARN23765 may be used as an adjuvant agent for mutations other than F508del. Last, we performed an initial assessment of ARN23765 safety in vitro. There was no significant effect on cell viability using different assays. Furthermore, no safety alert emerged from a screening on a panel of receptors, transporters, and ion channels. Despite the promising pharmacological profile of ARN23765 in vitro, it needs to be stressed that further studies are needed to rule out potential liabilities (systemic/organ toxicity and/or unsatisfactory pharmacokinetics and pharmacodynamics) that could hinder its use in vivo.

In conclusion, we discovered a small molecule that rescues mutant CFTR with very high potency. This characteristic may be crucial in the future development of therapies requiring combinations of up to three CFTR modulators. In this respect, the use of drugs that work at very low concentrations, thus decreasing the probability of unwanted side effects, would be highly desirable. In addition to potency, ARN23765 appears to have very good efficacy, markedly higher than that of VX-661, which is the class 1 corrector presently included in triple combinations.
The high potency of ARN23765 is also appealing for its use as a research tool. There is evidence suggesting that class 1 correctors directly bind to CFTR protein (11, 38, 39), possibly with the first transmembrane domain, but a definite proof is lacking. ARN23765 may represent a high-affinity probe to identify the corresponding binding site using the strategy that was recently used for potentiators (40). The high affinity of VX-770 and GLPG1837 was exploited to determine the cyto–electron microscopy structures of potentiator–CFTR complexes. Studies are ongoing to determine the binding site of ARN23765 to CFTR.

MATERIALS AND METHODS
Cell models and cell culture procedures
We generated CFBE41o− and FRT cells stably expressing either mutant F508del or wild-type CFTR and the HS-YFP YFP-H148Q/I152L, as previously described (28–30). CFBE41o− cells were cultured using minimum essential medium (MEM), while for FRT cells, we used the Coon’s modification of Ham’s F12 medium. Both media were supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). For HS-YFP assays of CFTR activity, CFBE41o− or FRT cells were plated (50,000 cells per well) on clear-bottom 96-well black microplates (Corning Life Sciences, Acton, MA). We also generated FRT cells with stable expression of P574H-CFTR and HS-YFP.

The isolation, culture, and differentiation methods of primary bronchial epithelial cells have previously been described in detail (41). Briefly, epithelial cells were obtained from mainstem bronchi of CF individuals undergoing lung transplant. For this study, cells were obtained from four patients with CF (homozygous for F508del mutation), from two patients with CF (compound heterozygous for F508del mutation), and from one non-CF patient. Bronchi were set overnight at 4°C in a solution containing protease XIV to detach cells. Epithelial cells were then cultured in a serum-free medium (LHC9 mixed with RPMI 1640, 1:1) supplemented with hormones and supplements that favor cell number amplification. For cells deriving from patients with CF, the culture medium contained a complex mixture of antibiotics (usually colistin, pipercillin, and tazobactam) to eradicate bacteria in the first days.

To obtain differentiated epithelia, cells were seeded at high density on porous membranes [500,000 cells for 1-cm² Snapwell inserts (Corning, code 3801), for Ussing chamber studies; 200,000 cells for 0.33-cm² mini-Transwell inserts (Corning, code 3379), for transepithelial electrical resistance (TEER)/PD measurements]. After 24 hours, the serum-free medium was replaced with Dulbecco’s modified Eagle’s medium/Ham’s F12 containing 2% fetal bovine serum plus hormones and supplements. Differentiation of cells into a tight epithelium was monitored by measuring TEER and PD with an epithelial voltohmmeter (EVOM1; World Precision Instruments). The medium was replaced daily on both sides of the permeable supports up to 8 to 10 days (liquid-liquid culture). Subsequently, the apical medium was totally removed, and the cells received nutrients only from the basolateral side [air–liquid culture (ALC)]. This condition favored a further differentiation of the epithelium. Cells were maintained under ALC for 2 to 3 weeks before experiments.

To test putative correctors, compounds were added to the basolateral medium 24 hours before the experiments to achieve the required concentration. Control epithelia were treated with vehicle alone [dimethyl sulfoxide (DMSO)].

HS-YFP assay
At the time of the assay, cells were washed with phosphate-buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 1 mM CaCl₂, and 0.5 mM MgCl₂. Cells were then incubated for 25 min with 60 μl of PBS plus forskolin (20 μM) and VX-770 (1 μM) to maximally stimulate F508del-CFTR. Cells were then transferred to microplate readers (FLUOstar OPTIMA; BMG LABTECH, Offenburg, Germany) for CFTR activity determination. The plate readers were equipped with high-quality excitation (HQ500/20x: 500 ± 10 nm) and emission (HQ535/30m: 535 ± 15 nm) filters for YFP (Chroma Technology). For the primary screening, the assay consisted of a continuous 12-s fluorescence reading, 1 s before and 11 s after injection of 165 μl of an iodide-containing solution (PBS with Cl⁻ replaced by I⁻; final I⁻ concentration, 100 mM). Data were normalized to the initial background-subtracted fluorescence. To determine I⁻ influx rate, the final 10 s of the data for each well were fitted with a linear function to extrapolate initial slope (df/dt).

For the secondary evaluation of compounds, the assay had a duration of 14 s, with continuous fluorescence reading, 2 s before and 12 s after the injection of the iodide-containing solution. I⁻ influx rate was determined by fitting the final 11 s of the data for each well with an exponential function. This assay was used for CFBE41o− cells expressing F508del-CFTR, FRT cells expressing F508del-CFTR or P574H-CFTR, and HEK-293 cells transiently transfected with N1303K-CFTR.

TEER and PD difference measurements
Both primary bronchial and FRT cells were treated with compounds included in the appropriate culture medium at the indicated concentrations for 24 hours at 37°C and 5% CO₂ before measuring the TEER and/or PD. In all experiments, TEER and PD were evaluated with an epithelial voltohmeter (EVOM1; World Precision Instruments).

For primary bronchial cells, the electrical measurements were done in Coon’s modified Ham’s F12 medium where NaHCO₃ was replaced with 20 mM Na-Hepes (pH 7.3). After equilibration for 1 hour, TEER and PD were measured in each well under basal conditions, after ENaC inhibition with apical amiloride (10 μM), after CFTR stimulation with forskolin (10 μM) and genistein (50 μM) on both sides, and after CFTR inhibition with apical PPQ102 (30 μM). After each treatment, we waited 10 min before recording electrical parameters. The TEER and PD values for each well were converted into short-circuit current equivalent by Ohm’s law.

For FRT cells, experiments were performed in a solution containing 130 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 1 mM CaCl₂, 0.5 mM MgCl₂, 10 mM Na-Hepes (pH 7.3), and 10 mM glucose. We measured TEER under basal conditions with the same solution on both sides of the permeable supports. Then, we added 20 μM forskolin and 50 μM genistein to activate CFTR channels. Last, we added 30 μM PPQ102 to block CFTR. As done for bronchial epithelial cells, we waited 10 min after each treatment before taking the measurement. The TEER values were converted into TEEC.

Short-circuit current recordings
Snapwell inserts carrying differentiated bronchial epithelia were mounted in a vertical diffusion chamber, resembling an Ussing chamber with internal fluid circulation. Both apical and basolateral hemichambers were filled with 5 ml of a solution containing 126 mM NaCl, 0.38 mM KH₂PO₄, 2.13 mM K₂HPO₄, 1 mM MgSO₄, 1 mM CaCl₂, 24 mM NaHCO₃, and 10 mM glucose. Both sides were continuously bubbled with a gas mixture containing 5% CO₂:95% air,
and the temperature of the solution was kept at 37°C. The transepithelial voltage was short-circuited with a voltage clamp (DVC-1000; World Precision Instruments) connected to the apical and basolateral chambers via Ag/AgCl electrodes and agar bridges (1 M KCl in 1% agar). The offset between voltage electrodes and the fluid electrical resistance were cancelled before each set of experiments. The short-circuit current was recorded with a PowerLab 4/25 (ADInstruments) analog-to-digital converter connected to a PC.

**Antibodies**

The following antibodies were used: mouse monoclonal anti-CFTR (570 and 596), provided by J. R. Riordan through a program of the Cystic Fibrosis Foundation (42); mouse monoclonal anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (clone 6C5, Santa Cruz Biotechnology); horseradish peroxidase (HRP)–conjugated anti-mouse immunoglobulin G (IgG) (Abcam); or HRP-conjugated anti-rabbit IgG (Dako).

**Western blot**

CFBE41o− cells treated with vehicle alone (DMSO), with VX-809, Western blot IgG (Dako).

**Immunoglobulin G (IgG) (Abcam); or HRP-conjugated anti-rabbit technology); horseradish peroxidase (HRP)–conjugated anti-mouse immunoglobulin G (IgG) (Abcam); or HRP-conjugated anti-rabbit IgG (Dako).

**Cell proliferation**

CFBE41o− cells stably expressing F508del-CFTR and the HS-YFP were plated at low density (5000 cell per well) on 96-well plates suitable for high-content imaging. After 24 hours, cells were treated with different concentrations of test compounds or vehicle alone (DMSO). Cell proliferation was monitored for 24 hours using the Opera Phenix (PerkinElmer) high-content screening system.

**MTT cell viability assay**

CFBE41o− cells were plated (50,000 cell per well) on 96-well plates. After 24 hours, cells were treated with different concentrations of test compounds or vehicle alone (DMSO). The following day, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) substrate was added to the medium at a final concentration of 0.5 mg/ml and incubated for 3 hours at 37°C. Formazan production (proportional to the number of viable cells) was then quantified by recording changes in absorbance at 560 nm using a plate reader equipped for absorbance measurements (VICTOR3; PerkinElmer).

**Cell death analysis**

CFBE41o− cells were plated at high density (50,000 cells per well) on 96-well plates suitable for high–content imaging. After 24 hours, cells were treated with test compounds or vehicle alone (DMSO). After 24 hours, cells were counterstained with Hoechst 33342 and propidium iodide to visualize total cells and dead/dying cells, respectively, and imaged using the Opera Phenix (PerkinElmer) high-content screening system. Wells were imaged using a ×20 air objective. Hoechst 33342 signal was laser-excited at 405 nm, and the emission was collected between 435 and 480 nm. The propidium iodide signal was laser-excited at 560 nm, and the emission was measured between 570 and 630 nm.

**Statistics**

Statistical significance of the effect of single treatments on CFTR activity or expression was tested by parametric one-way analysis of variance (ANOVA), followed by Dunnett’s multiple comparisons test (all groups against the control group) as post hoc test. In the case of combination of treatments, statistical significance was verified by ANOVA, followed by Tukey’s test (for multiple comparisons) as post hoc test. Differences were considered statistically significant when P was less than 0.05.
Study approval
The collection of bronchial epithelial cells and their study to investigate
the mechanisms of transepithelial ion transport and the response to
CFTR modulators were specifically approved by the Ethics Commi-
mittee of the Istituto Giannina Gaslini following the guidelines of the
Italian Ministry of Health (updated registration number: ANTECER,
042-09/07/2018). Each patient provided informed consent to the
study using a form that was also approved by the Ethics Committee.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/
content/full/6/6/eaay9669/DC1

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Competing interests: T.B., F. Berto., P.D.F., A.R.-G., F.S., F. Berti, E.C., L.F., N.P., and L.J.V.G. are inventors on a patent/patent application related to this work filed by Fondazione Istituto Italiano di Tecnologia, Istituto Giannina Gaslini, and Fondazione per la Ricerca sulla Fibrosi Cistica–Onlus (PCT international publication no. WO 2018167690 A1; international publication date, 20 September 2018). T.B., F. Berto., P.D.F., E.C., L.F., N.P., and L.J.V.G. are inventors on a patent/patent application related to this work filed by Fondazione Istituto Italiano di Tecnologia, Istituto Giannina Gaslini, and Fondazione per la Ricerca sulla Fibrosi Cistica–Onlus (PCT international publication no. WO 2018167695 A1; international publication date, 20 September 2018). The authors declare no other competing interests.

Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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