Correctors and Potentiators Rescue Function of the Truncated W1282X-Cystic Fibrosis Transmembrane Regulator (CFTR) Translation Product**

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W1282X is the fifth most common cystic fibrosis transmembrane regulator (CFTR) mutation that causes cystic fibrosis. Here, we investigated the utility of a small molecule corrector/potentiator strategy, as used for ΔF508-CFTR, to produce functional rescue of the truncated translation product of the W1282X mutation, CFTR1281, without the need for read-through. In transfected cell systems, certain correctors and correctors, including VX-809 and VX-770, increased CFTR1281 activity. To identify novel correctors and potentiators with potentially greater efficacy on CFTR1281 functional screens were done of ~30,000 synthetic small molecules and drugs/nutraceuticals in CFTR1281-transfected cells. Corrector scaffolds of 1-arylpyrazole-4-arylsulfonyl-piperazine and spiro-piperidine-quinoxalinone classes were identified with up to ~5-fold greater efficacy than VX-809, some of which were selective for CFTR1281, whereas others also corrected ΔF508-CFTR. Several novel potentiator scaffolds were identified with efficacy comparable with VX-770; remarkably, a phenylsulfonamide-pyrrolopyridine acted synergistically with VX-770 to increase CFTR1281 function ~8-fold over that of VX-770 alone, normalizing CFTR1281 channel activity to that of wild type CFTR. Corrector and potentiator combinations were tested in primary cultures and conditionally reprogrammed cells generated from nasal brushings from one W1282X homozygous subject. Although robust chloride conductance was seen with correctors and potentiators in homozygous ΔF508 cells, increased chloride conductance was not found in W1282X cells despite the presence of adequate transcript levels. Notwithstanding the negative data in W1282X cells from one human subject, we speculate that corrector and potentiator combinations may have therapeutic efficacy in cystic fibrosis caused by the W1282X mutation, although additional studies are needed on human cells from W1282X subjects.

Premature termination codons (PTCs, or nonsense mutations) in the cystic fibrosis transmembrane conductance regulator (CFTR) gene are found in nearly 7% of patients with CF (1). PTCs result in synthesis of truncated protein, generally without normal function, as well as reduced transcript levels caused by nonsense-mediated mRNA decay (NMD) (2, 3). NMD suppressors that restore gene function by promoting incorporation of the correct or a near-cognate aminoacyl tRNA into nascent polypeptides represent attractive drug development candidates (2, 4, 5). The read-through candidate drug Ataluren (PTC124) is under evaluation for CF caused by PTCs, although initial phase III clinical trial data did not show efficacy (6, 7), and some laboratories were unable to demonstrate Ataluren-mediated translation read-through in various systems (8). One limitation of therapeutics causing NMD suppression is insertion of incorrect amino acids at the PTC site.

The W1282X mutation, which truncates CFTR to remove ~60% of nucleotide binding domain 2 (NBD2; Fig. 1A, top), is the fifth most common CF-causing mutation worldwide with a prevalence of ~50% in Ashkenazi Jewish subjects with CF (1). Motivated by the fact that the W1282X mutation is predicted to generate a truncated protein (CFTR1281) constituting most of the full-length wild type CFTR (1480 amino acids), and postulating that CFTR1281 has defective cellular processing and gat-

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5 The abbreviations used are: PTC, premature termination codon; CFTR, cystic fibrosis transmembrane regulator; endo H, endoglycosidase H; PNGase F, peptide-N-glycosidase F; fsk, forskolin; ANOVA, analysis of variance; 3HA, triplet hemagglutinin epitope tag; CF, cystic fibrosis; CRC, conditionally reprogrammed cell; NMD, nonsense-mediated mRNA decay.
ing, we investigated whether functional rescue of CFTR<sub>1281</sub> is possible using correctors and/or potentiators, a strategy used for CF caused by the ΔF508 mutation as well as G551D and other CFTR missense mutations (9–11).

Several lines of evidence suggest multiple defects in W1282X-CFTR and support corrector, potentiator, and read-through strategies (12). Transcript encoding W1282X-CFTR was found in nasal epithelial cells of homozygous W1282X CF subjects with 30–80% abundance compared with that of wild type CFTR in non-CF subjects (13). In bronchial epithelial cells virally infected to express W1282X-CFTR cDNA, butyrate-induced up-regulation of truncated CFTR expression produced cAMP/genistein-stimulated chloride conductance (14), and in the same cell model, VX-770 produced an ~4-fold increase in CFTR activity despite the apparent absence of full-length protein (15). Patch clamp experiments showed that VX-770 in combination with curcumin produced significant activation of W1282X-CFTR despite evidence of impaired ATP-dependent NBD1-NBD2 dimerization (16). Biochemical and functional approaches using heterologous expression systems have demonstrated that NBD2 is not required for efficient cell-surface presentation or CFTR activity, albeit with reduced open probability (17, 18). Partial deletion of NBD2 reduces channel biosynthetic processing and post-Golgi stability (18–21), probably by interfering with NBD2 folding and cooperative CFTR domain assembly (18).

In this study, we demonstrate the activity of available correctors and potentiators in transfected cells expressing the truncated protein product of the W1282X CF gene (CFTR<sub>1281</sub>), identify improved correctors and potentiators by high-throughput screening, and test compounds in human nasal epithelial cell cultures from a W1282X-homozygous CF subject. These studies were presented in part in abstract form at the 29th North American CF Conference in October, 2015 (22).

**Results**

**CFTR Correctors Stabilize W1282X-CFTR Translational Products in Respiratory Epithelial Cells**—To test whether the W1282X-CFTR gene product is susceptible to pharmacological modulation, initial biochemical assessment was done in a CF bronchial epithelial cell line (CFBE410—the designated as CFBE) lacking endogenous CFTR expression (23). Lentiviral vectors were generated to express W1282X-CFTR translational products stably under the control of the tetracycline transactivator (Tet) format (25).

Immunoblot analysis in combination with endo H and PNGase F digestion showed the expression of core-glycosylated (band B, endo H-sensitive; **black arrowhead**) and complex-glycosylated (band C, PNGase F-sensitive; **white arrowhead**) forms of both W1282X-CFTR and CFTR<sub>1281</sub> (Fig. 1B). The apparent molecular mass of the core- and complex-glycosylated forms of CFTR<sub>1281</sub> and W1282X-CFTR was reduced compared with wild type CFTR. Assuming comparable transcript levels, these observations suggest that the truncated CFTR<sub>1281</sub> has inefficient biosynthetic processing and/or impaired stability.

Supporting inefficient processing, the known ΔF508-CFTR correctors VX-809 and corr4a (C4) increased the complex-glycosylated forms of CFTR<sub>1281</sub> and W1282X-CFTR, as well as their plasma membrane expression as assessed by immunoblot analysis and live-cell ELISA (Fig. 1, C and D). As evidence for improved CFTR<sub>1281</sub> processing in the presence of VX-809, metabolic pulse-chase labeling with [35S] methionine and [35S] cysteine in polarized CFBE cell cultures showed ~75% increased incorporation of radiolabel into complex-glycosylated CFTR<sub>1281</sub>, with VX-809 (Fig. 1E).

**High Throughput Assay Development for W1282X-CFTR Correctors and Potentiators**—Motivated by the observations above, we developed a high throughput screen to identify correctors and potentiators of W1282X-CFTR. FRT cell lines were generated that stably express YFP-H148Q/I552L/F46L (YFP) and CFTR containing either the W1282X mutation (FRT-YFP-W1282X-CFTR) or CFTR<sub>1281</sub> (FRT-YFP-CFTR<sub>1281</sub>); FRT cells were used because of their low intrinsic permeability to iodide and other CFTR-permeable ions and prior experience with these cells in various CFTR modulator screens (26, 27). The corrector assay was done by 24-h incubation of cells with test compounds followed by brief (10 min) incubation with the cAMP agonist forskolin (fsk) and a potentiator prior to assay (Fig. 2A, **left**). For potentiator assays, cells were incubated with a corrector to increase cell-surface expression of W1282X-CFTR and then briefly incubated (10 min) with test compounds and forskolin prior to assay (Fig. 2A, **right**). CFTR activity was deduced from the initial rate of YFP fluorescence quenching in response to iodide addition to the extracellular solution.

**Known Correctors and Potentiators Rescue W1282X-CFTR Function in FRT Cells**—Incubation of FRT-YFP-W1282X-CFTR cells with forskolin and genistein (50 μM) produced limited fluorescence quenching in response to an iodide gradient, although 24-h culture with sodium butyrate, a pharmacological chaperone (28), increased this response, as found previously (Fig. 2B) (14). A similar pattern of CFTR activation was observed in cells incubated with VX-809, and combined VX-809 plus butyrate produced an additive response. In cells incubated with VX-809 alone or VX-809 plus butyrate, VX-770 (5 μM) had greater efficacy than genistein (Fig. 2, B and C). CFTR activation required both cAMP elevation and a potentiator (VX-770 or genistein; Fig. 2C and data not shown).

Fig. 2C (**left**) summarizes the experiments above and additional measurements for a panel of known CFTR modulators. Correctors VX-661, corr4a (C4), C3, and C18 each increased forskolin/VX-770-stimulated CFTR activity by ~2–3-fold over that in uncorrected cells, whereas CoPo-22 and C7 had minimal activity. Potentiators PG01 (P2) (29) and A04 (27) showed activity in VX-809-corrected/forskolin-stimulated cells, whereas P3, P5, P8, P9, P12, and CoPo-22 were inactive (Fig. 2C, **right**).

Further experiments were done in FRT-YFP-CFTR<sub>1281</sub> cells to investigate whether CFTR modulators that correct or poten-
tiate CFTR activity in W1282X-CFTR-expressing cells act on the truncated protein product produced by the W1282X mutation. Correctors (VX-809, VX-661, corr4a, C3, and C18) and potentiators (VX-770, PG01, and A04) that were active in FRT-YFP-W1282X-CFTR cells were comparably active in FRT-YFP-CFTR_{1281} cells. Arrowheads represent core-glycosylated (black arrowheads) and complex-glycosylated (open arrowheads) CFTR.

**FIGURE 1.** Biochemical rescue of CFTR_{1281} by correctors in CFBE cells. A, schematic of CFTR showing site of W1282X premature termination codon in nucleotide binding domain 2 (top). Wild type, mutated (W1282X-CFTR), and truncated (CFTR_{1281}) expression constructs were used in this study (bottom). B, immunoblot of wild type CFTR (WT, one-fifth the amount of protein loaded), W1282X-CFTR, and CFTR_{1281} in CFBE cells. Arrowheads represent core-glycosylated (black arrowheads) and complex-glycosylated (open arrowheads) CFTR. C, immunoblot of wild type CFTR, W1282X-CFTR, and CFTR_{1281} in CFBE cells in response to ΔF508-CFTR correctors (3 μM VX-809 and 10 μM C4) and putative modulators of read-through (200 μg/ml G418, 200 μg/ml gentamycin, and 10 μM PTC124). Arrowheads represent core-glycosylated (black) and complex-glycosylated (open) CFTR. D, surface presentation of W1282X-CFTR and CFTR_{1281} in response to CFTR modulators and PTC124 measured by live cell ELISA (mean ± S.E., ANOVA with Dunnett’s post hoc test compared with control (DMSO-treated) cells, *, p < 0.01). E, metabolic pulse-chase analysis of CFTR_{1281} maturation in response to VX-809. Right, quantification of VX-809 effect on CFTR_{1281} maturation (mean ± S.E., t test, *, p < 0.01). Data shown in B–E are representative of at least triplicate experiments. p, pulse; ch, chase.

**Pharmacological Correction of W1282X-CFTR**

**FIGURE 1.** Biochemical rescue of CFTR_{1281} by correctors in CFBE cells. A, schematic of CFTR showing site of W1282X premature termination codon in nucleotide binding domain 2 (top). Wild type, mutated (W1282X-CFTR), and truncated (CFTR_{1281}) expression constructs were used in this study (bottom). B, immunoblot of wild type CFTR (WT, one-fifth the amount of protein loaded), W1282X-CFTR, and CFTR_{1281} in CFBE cells. Arrowheads represent core-glycosylated (black arrowheads) and complex-glycosylated (open arrowheads) CFTR. C, immunoblot of wild type CFTR, W1282X-CFTR, and CFTR_{1281} in CFBE cells in response to ΔF508-CFTR correctors (3 μM VX-809 and 10 μM C4) and putative modulators of read-through (200 μg/ml G418, 200 μg/ml gentamycin, and 10 μM PTC124). Arrowheads represent core-glycosylated (black) and complex-glycosylated (open) CFTR. D, surface presentation of W1282X-CFTR and CFTR_{1281} in response to CFTR modulators and PTC124 measured by live cell ELISA (mean ± S.E., ANOVA with Dunnett’s post hoc test compared with control (DMSO-treated) cells, *, p < 0.01). E, metabolic pulse-chase analysis of CFTR_{1281} maturation in response to VX-809. Right, quantification of VX-809 effect on CFTR_{1281} maturation (mean ± S.E., t test, *, p < 0.01). Data shown in B–E are representative of at least triplicate experiments. p, pulse; ch, chase.

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Novel Small Molecule CFTR_{1281} Correctors Identified by High Throughput Screening—Screening was done to identify novel CFTR_{1281} correctors with improved efficacy over known ΔF508-CFTR correctors. Screening of ~30,000 synthetic small molecules identified six compound classes that at 25 μM nor-
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malized forskolin/VX-770-stimulated CFTR<sub>1281</sub> activity to >50% of that produced by 3 μM VX-809. Fig. 3A shows chemical structures of the two most active compounds, W1282X<sub>corr-A23</sub> and W1282X<sub>corr-B09</sub>, which are chemically distinct from VX-809. In YFP quenching assays, these compounds increased CFTR<sub>1281</sub> activity in a concentration-dependent manner producing ~5-fold greater maximal activity than VX-809 (Fig. 3B).

To quantify cell-surface expression, an FRT cell line expressing W1282X-CFTR containing a 3HA epitope tag in the fourth extracellular loop was generated. Cell-surface expression, as assayed in a live cell ELISA format, showed that the correctors VX-809, W1282X<sub>corr-A23</sub>, and W1282X<sub>corr-B09</sub> (at 3 μM) increased surface presentation of W1282X-CFTR in FRT cells with efficacy similar to that seen in the functional assays (Fig. 3C, top). To investigate whether CFTR<sub>1281</sub> or full-length (produced by read-through) CFTR was present at the cell surface in W1282X-CFTR-3HA-expressing cells, a luciferase-based W1282X read-through reporter was generated and expressed in FRT cells. As a positive control, the read-through compound geneticin (G418) produced a luciferase-dependent response, whereas W1282X<sub>corr-A23</sub>, W1282X<sub>corr-B09</sub>, and VX-809 did not (Fig. 3C, bottom). Together, these studies indicate that correctors identified by the screen, as well as VX-809, increase...
cell-surface CFTR$^{1281}$ in FRT cells rather than promoting read-through.

Further studies were done with the most potent corrector identified, W1282Xcorr-A23, a 1-arylpyrazole-4-arylsulfonyl-piperazine. Structure-activity studies of 100 commercially available analogs of W1282Xcorr-A23 revealed that pyrazoles substituted with benzene and 4-pyridine (R$^1$) gave good activity, whereas 2- and 3-pyridine reduced activity (Fig. 3D). Changing the five-member pyrazole (R$^2$) ring to 6-member pyrimidine or pyridazine rings reduced activity. The substituent (R$^3$) on the arylsulfonyl group also affected activity, with 3,5- and 3,4-disubstitution giving the best activity, whereas unsubstituted and 2-substitution reduced activity. Methyl and halide substituents were well tolerated.

Short-circuit current ($I_{sc}$) measurements using CFTR$^{1281}$-expressing cells and forskolin/VX-770 stimulation confirmed the concentration-dependent correction efficacy of W1282Xcorr-A23; CFTR$^{1281}$ correction by W1282Xcorr-A23 produced ~4-fold more current than VX-809, similar to values determined by YFP quenching assays, and current was fully blocked by CFTRinh-172 (Fig. 3E).

The correctors identified in the CFTR$^{1281}$ screen were also tested for corrector activity on CFTR$^{F508}$-CFTR using FRT-YFP-CFTR$^{F508}$ cells. Fig. 3F shows YFP fluorescence quenching in CFTR$^{F508}$-CFTR-expressing FRT cells treated with W1282Xcorr-A23 or VX-809 (3 μM). G, relative efficacy of correctors from classes A, B, and C (at 3 μM, 24 h) in CFTR$^{1281}$ and ΔF508-CFTR-expressing FRT cells (mean ± S.E., n = 4–12). Data were normalized to VX-809 efficacy. Compound structure provided in Table 1. In B, E, F, and G cells were stimulated with forskolin (10 μM) and VX-770 (5 μM). CFTRinh-172 was used at 10 μM in all studies.
Pharmacological Correction of W1282X-CFTR

TABLE 1
Chemical structures and corrector activities of selected Class A, B and C compounds

| Class | Structure | Name | R¹ | R² | R³ | %Correction |
|-------|-----------|------|----|----|----|-------------|
|       |           |      |    |    |    | W1282X | ΔF508 |
| A     |           |      |    |    |    |         |         |
| 1     | phenyl    | 3-F,4-Me | 51.4±4.8 | 21.2±0.6 |
| 2     | 4-Me-phenyl | 3,4-Me | 8.9±0.5 | 10.1±0.4 |
| 3     | 4-F-phenyl | 4-Et | 28.3±2.8 | 7.6±0.2 |
| B     |           |      |    |    |    |         |         |
| 4     | 3,5-Me    | H | 2-Cl | 46.0±3.8 | 7.3±1.0 |
| 5     | 3,5-Me    | Me | 4-Cl | 31.6±3.4 | 8.5±0.8 |
| 6     | 5-Me      | Me | 4-Cl | 18.2±1.4 | 6.7±0.4 |
| C     |           |      |    |    |    |         |         |
| 7     | 4-Me      | 4-Et | 210±9 | 49.4±1.2 |
| 8     | 4-Me      | 4-MeO | 137±23 | 39.3±0.8 |
| 9     | 4-Me      | 3-Me,4-F | 134±6 | 28.8±1.1 |
| 10    | -         | 3-Cl,4-Me | 122±4 | 13.4±0.9 |
| 11    | 4-Me      | 3-Me,4-Me | 110±11 | 15.1±1.4 |
| 12    | 4-F       | 4-Et | 103±5 | 13.1±0.8 |
| 13    | -         | 3-Me,4-F | 48.4±2.1 | 13.6±0.9 |
| 14    | -         | 4-EtO | 91.5±2.6 | 10.8±1.1 |
| 15    | -         | 4-MeO | 120±6 | 7.7±0.7 |
| 16    | -         | 2-F | 84.1±9.4 | 3.9±0.2 |
| 17    | -         | 2-F,5-F | 75.2±4.8 | 5.1±0.4 |

W1282Xcorr-A23, with near maximal activity at \( \sim 3 \mu M \). To investigate specificity of CFTR\(_{1281}\) correctors, active analogs from three compound classes were compared at 3 \( \mu M \) in CFTR\(_{1281}\) and ΔF508-CFTR-expressing cells. Whereas most known ΔF508-CFTR correctors (VX-809, VX-661, C18, and Corr4a) were comparably active on both CFTR mutants, many of the CFTR\(_{1281}\) correctors identified in the screen here were substantially more effective in CFTR\(_{1281}\)-expressing cells (Fig. 3G and Table 1). Although correctors with improved efficacy over VX-809 were identified by screening, subsequent assays were done with VX-809 as it is an approved drug.

Novel Small Molecule CFTR\(_{1281}\) Potentiators Identified by High Throughput Screening—Initial experiments were done to characterize VX-770 as a potentiator of VX-809-
corrected CFTR<sub>1281</sub>. Interestingly, increasing VX-770 increased CFTR<sub>1281</sub> activity without saturation at up to 200 μM, as seen in both plate reader and short-circuit current assays (Fig. 4A, left, and data not shown). In contrast, ΔF508-CFTR activity in VX-809-corrected cells was saturable, as expected (Fig. 4A, right). CFTR<sub>1281</sub> and ΔF508-CFTR activities were fully blocked by CFTRinh-172. These data suggest that CFTR<sub>1281</sub> has very low open probably at clinically achievable concentrations of VX-770 but has the potential to be greatly activated.

A small molecule screen was done to identify novel CFTR<sub>1281</sub> potentiators as diagrammed in Fig. 2A (right), in which cells were incubated with VX-809 to increase cell-surface CFTR<sub>1281</sub> expression, and test compounds with forskolin were added 10 min prior to assay. Fig. 4B shows structures of five active compounds emerging from the screen, each of which is chemically distinct from VX-770. Concentration dependence measurements in VX-809-corrected CFTR<sub>1281</sub>-expressing cells showed that the new CFTR<sub>1281</sub> potentiators had similar or lower activity than VX-770 (Fig. 4C). Control experiments confirmed that the potentiators did not activate CFTR<sub>1281</sub> in the absence of forskolin and that YFP quenching did not occur in cells without CFTR<sub>1281</sub>- CFTR<sub>1281</sub> activity was confirmed by short-circuit current measurements in VX-809-treated cells, with data shown for W1282X<sub>pot</sub>-A15 and W1282X<sub>pot</sub>-C01 (Fig. 4D).

**Potentiator Combinations Restore CFTR<sub>1281</sub> Activity to Wild Type Levels**—Because of the relatively low activity of CFTR<sub>1281</sub> in response to individual potentiators, we postulated that combinations of potentiators might show additive or perhaps synergistic effects. Initial experiments were done with VX-770 and the potentiators that were identified by screening. Remarkably, combination of VX-770 with W1282X<sub>pot</sub>-A15, -C01, -D01, or -E01 significantly increased CFTR<sub>1281</sub> activity, whereas W1282X<sub>pot</sub>-B01 did not increase the VX-770 response (Fig. 5A, top). In cells expressing ΔF508-CFTR, each of the potentiators (except W1282X<sub>pot</sub>-E01) increased channel activity; however, none of the potentiators further increased channel activity in the presence of VX-770 (Fig. 5A, bottom). Short-circuit current measurements showed W1282X<sub>pot</sub>-A15 activation of VX-809-corrected CFTR<sub>1281</sub> with EC<sub>50</sub> ~5 μM (Fig. 5B, left), which was independent of the order of addition (Fig. 5B, right). Activation by VX-770 and W1282X<sub>pot</sub>-A15 (100 ± 8 μA/cm<sup>2</sup>) (p < 0.0001 by ANOVA) was remarkably greater than by forskolin alone (2 ± 1 μA/cm<sup>2</sup>) or forskolin in combination with W1282X<sub>pot</sub>-A15 (4 ± 1 μA/cm<sup>2</sup>) or with VX-770 (9 ± 2 μA/cm<sup>2</sup>) (mean ± S.E., n = 3–10), indicating a synergistic response.

To estimate the CFTR<sub>1281</sub> channel activity in response to potentiators, cell-surface expression and channel activity were measured in FRT cells expressing epitope (3HA)-tagged wild type or W1282X-CFTR. Incubation of cells expressing CFTR-
3HA with forskolin produced robust current that was inhibited by CFTRinh-172 (Fig. 5C, top left). W1282X-CFTR-3HA-expressing cells corrected with W1282Xcorr-A23 produced CFTR channel activity in response to forskolin, VX-770, and W1282Xpot-A15 that was inhibited by CFTRinh-172 (Fig. 5C, bottom left). Following normalization by cell-surface expression, deduced per-channel activity for CFTR1281 relative to wild type CFTR (normalized to 1) is shown (Fig. 5C, right). Remarkably, the potentiator combination increased CFTR1281 channel activity to that of wild type CFTR.

Structure-activity studies were done on 120 commercially available analogs of W1282Xpot-A15 (Fig. 5D). Class A potentiators are composed of pyrrolo[2,3-b]pyridine with a phenylsulfonamide linked at the 3-position. Several alkyl groups (R1) on the pyrrolo[2,3-b]pyridine were studied, and n-butyl and isobutyl groups gave active potentiators showing synergy with VX-770. Shorter alkyl group such as propyl and ethyl reduced activity. The R2 substituent on the phenylsulfonamide ring also affected activity, with electron-neutral or donating groups such as ethyl, methyl, and methoxy giving the best activity, although halides such as fluoro and chloro reduced activity. The position of the substituent also affected activity, with para and ortho being more active than meta substitution, suggesting that electronic properties of the phenylsulfonamide ring affect activity.
Short-circuit current measurements were also done in VX-809-treated CFBE cells to confirm results from FRT cells in a human airway epithelial cell model (Fig. 5E). Little activation of CFTR1281 was seen with VX-770 or W1282Xpot-A15 alone; however, in combination these potentiators strongly increased channel activity (Fig. 5E).

Interestingly, we found that some of the new potentiators showed activity alone but limited synergy with VX-770, although others were relatively inactive alone but showed synergy with VX-770. Fig. 5F shows examples of each, with W1282Xpot-B01 in the former category and W1282Xpot-E01 in the latter.

**Screen of Approved and Investigational Drugs for Synergy with VX-770**—Motivated by the marked synergy of VX-770 with some potentiators, we carried out a “synergy screen,” reasoning that repurposing of an existing drug or bioactive molecule for use in combination with VX-809 and VX-770 might accelerate drug therapy for CF caused by the W1282X mutation. The screen might also identify bioactive compounds that are effective as potentiators when used alone. Screening was done using a collection of ~2600 bioactive compounds, natural products, and approved drugs that were added acutely with forskolin and VX-770 to VX-809-corrected CFTR1281-expressing cells (Fig. 6A). Screening identified several compounds that increased CFTR1281 activity above that produced by VX-770 and forskolin, including the flavones apigenin, genistein, and kaempferol, and the flavone-like anti-inflammatory compound isoliquiritigenin (Fig. 6B). Fig. 6C shows original YFP fluorescence quenching data and concentration-activity curves. Compounds did not increase CFTR1281 function when used alone (Fig. 6D). Apigenin and genistein had limited potentiator activity with forskolin, whereas isoliquiritigenin and kaempferol were inactive as potentiators.

**FIGURE 6. Synergy with VX-770 of bioactive small molecules identified in a synergy screen.** A, schematic of screen used to identify bioactive molecules that act in synergy with VX-770. CFTR1281-expressing FRT cells were corrected with VX-809, and test compounds were added with forskolin and VX-770. B, structures of bioactive compounds that act in synergy with VX-770. C, left, YFP fluorescence quenching in CFTR1281-expressing FRT cells in response to VX-770 alone (top trace) and with indicated compounds. Right, concentration dependence of CFTR1281 activity in response to VX-770 and indicated compounds (mean ± S.E., n = 3–6). gen, genistein; api, apigenin; kam, kaempferol; iso, isoliquiritigenin. D, compounds do not activate CFTR1281 in FRT cells alone and produce limited activity in the presence of forskolin (mean ± S.E., n = 3–6). Statistical analysis was by ANOVA with Dunnett’s post hoc test. For treatment of cells with compounds alone, or with forskolin and compounds, data are compared with forskolin-alone treatment; for cells treated with forskolin/VX-770 and compound, data are compared with cells treated with forskolin/VX-770 (*, p < 0.05; **, p < 0.0001). E, short-circuit current in FRT cells expressing CFTR1281 in response to VX-770 (5 μM) and apigenin. F, dose-response of CFTR1281 activation in FRT cells by VX-770 and apigenin. G, left, CFTR1281 activation in CFBE cells by apigenin and VX-770 is independent of addition order and (right) is more efficacious than genistein. Statistical analysis was by ANOVA with Dunnett’s post hoc test (*, p < 0.05; **, p < 0.001). In C–G cells were corrected with VX-809 (3 μM, 24 h) and stimulated with forskolin (10 μM). Data in E–G are representative of 3–5 experiments.
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Nasal epithelial cells were obtained by brushings from a single W1282X-CFTR homozygous CF subject. In primary cells, VX-770 and W1282Xpot-A15 produced no significant CFTR current without or with VX-809 incubation (Fig. 7C). The amiloride and ATP responses were similar in these cells to those in non-CF and ΔF508-CFTR P0 cultures and CRCs. No CFTR current was observed under the same experimental conditions using CRCs generated from nasal epithelial cells from the same CF subject (data not shown). Further experiments tested the activity of read-through compounds in CRCs generated from the W1282X-CFTR P0 cultures. As with P0 cultures, CRCs without and with VX-809 showed no response to CFTR modulators (Fig. 7D). VX-809-corrected W1282X-CFTR CRCs treated with the read-through agents PTC124/ataluren (6), amlexanox (30), and geneticin (14) produced no significant CFTR current in response to correctors and potentiators but showed robust amiloride and ATP responses.

Quantitative PCR was done to determine CFTR transcript levels in the human airway epithelial cell models (Fig. 7E). In nasal brushings, CFTR transcript was reduced only by ~20% in CF cells as compared with a non-CF subject (Fig. 7E, top left). In P0 cultures, non-CF nasal and bronchial airway epithelial cells had similar CFTR transcript levels (data not shown), and cells from the W1282X-CFTR subject had ~50% less CFTR transcript (Fig. 7E, top right). CRCs from non-CF and W1282X-CFTR subjects had similar levels of CFTR transcript (Fig. 7E, bottom left). Finally, in P0 cultures and CRCs from non-CF or W1282X-CFTR subjects, CFTR transcript levels were similar, suggesting that reprogramming does not greatly reduce CFTR transcript. First passage CRC cultures were morphologically

\[ I_{sc} \text{ data for apigenin, the most potent compound identified in the synergy screen, revealed limited CFTR}_{1281} \text{ activity when used alone but robust CFTR}_{1281} \text{ activity in the presence of VX-770 (Fig. 6E). CFTR}_{1281} \text{ activation by apigenin in the presence of VX-770 had an EC}_{50} \text{ of } \sim 20 \mu M \text{ (Fig. 6F), } \sim 4\text{-fold less potent than W1282Xpot-A15. As found for W1282Xpot-A15, activation of CFTR}_{1281} \text{ by VX-770 and apigenin was independent of the order of addition (data not shown). Comparing with cells incubated with W1282Xpot-A15, the per-channel activity of CFTR}_{1281} \text{ relative to wild type CFTR in cells incubated with VX-770/apigenin is } \sim 0.6 \text{ and with VX-770/genistin was } \sim 0.2. \text{ Experiments in VX-809-treated CFBE cells confirmed synergy of VX-770 with apigenin (Fig. 6G).}

Corrector and Potentiator Testing in W1282X Homozygous Human Airway Epithelial Cells—Compounds were tested in airway epithelial cells from a homozygous W1282X-CFTR subject. Initial experiments were done with airway epithelial cells from non-CF and homozygous ΔF508-CFTR subjects to confirm the utility of conditionally reprogrammed cells (CRCs) for these studies. Primary and CRCs from a non-CF subject showed similar responses when treated with amiloride (ENaC channel blocker), forskolin, CFTR\_1281\_pot, and ATP (activator of calcium-activated chloride channels) (Fig. 7A). Also, primary and conditionally reprogrammed human bronchial epithelial cells from a homozygous ΔF508-CFTR CF subject showed similar responses following correction with VX-809, with no activity in the absence of corrector (Fig. 7B). These studies support the use of CRCs to study pharmacology of CFTR modulators, which allows considerable expansion of cell cultures derived from rare patient specimens, as needed for the W1282X studies here.
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indistinguishable from P0 cultures with both showing pseudostratified and ciliated differentiation (data not shown).

Discussion

Therapeutic approaches for CF caused by the W1282X mutation and other PTC mutations such as G542X have focused on promoting read-through to generate full-length CFTR protein (5). The limited efficiency of read-through and function of the translated full-length proteins, which generally contain non-native amino acids at the PTC, are major challenges in this approach. We demonstrate here that in the absence of read-through the truncated protein product generated by the W1282X mutation (CFTR1281) has the potential to be efficiently processed and gated using combinations of small molecules. The major novel findings of this work include the application of correctors to facilitate processing of CFTR1281, the identification of correctors and potentiatotors with much greater efficacy than existing compounds, and the discovery of potentiatotor combinations that restore CFTR1281 channel conductance to that of wild type CFTR. However, whether the findings from cell cultures models will translate to native human airway epithelial cells remains uncertain at this time, as corrector and potentiator combinations did not show efficacy in airway epithelial cell cultures from a single homozygous W1282X CF subject.

The results here suggest that pharmacological activation of CFTR1281 may require triple drug combination therapy with a corrector and two potentiatotors acting in synergy. Of note, therapies for ΔF508-CFTR in development include a single potentiatotor and two correctors, supporting the viability of triple drug CFTR modulator therapy for CF (25, 31–33). High throughput screening in this study produced novel chemical scaffolds that corrected CFTR1281 trafficking with greater efficacy than existing ΔF508-CFTR correctors. Perhaps second and third generation correctors under development for ΔF508-CFTR may be active as CFTR1281 correctors; and VX-770 together with newer corrector and potentiator combinations did not show efficacy in airway epithelial cell cultures from other W1282X CF patients. It is well recognized that the efficacy of ΔF508-CFTR correctors can be cell type-dependent (37, 38), as correctors identified in transfected cells often are not effective in patient-derived human airway epithelial cells. However, potentiatotors tend to be cell context-independent as they likely target mutant CFTRs at the plasma membrane directly.

There are limited prior reports on the biological properties of the 1-arylprazole-4-arylsulfonyl-piperazine corrector scaffold identified in this study. Some naphthalene arylsulfonyl-pipera- zines were identified as inhibitors of activated coagulation factor X (39) and human 11β-hydroxysteroid dehydrogenase type 1 (40). Limited biological data have been reported for the phenylsulfonamide-pyrolyprolyridine class of W1282X potentiatotors. Some phenylsulfonamide-pyrolyprolyridines were found as cannabinoïd agonists for therapy for pain and nausea (41). Virtual screening also identified phenylsulfonamide-pyrolyprolyridines as potential inhibitors of heat shock protein Hsp90 (42). The arylpyrazole-arylsulfonyl-piperazine and phenylsulfonamide-pyrolyprolyridine scaffolds have favorable drug-like properties, including the presence of multiple hydrogen bond acceptors, molecular masses of 396 and 357 Da, aLogP values of 3.2 and 3.8, and topological polar surface areas of 79.4 and 61.8 Å², respectively (43, 44). In addition, these scaffolds do not belong to promiscuous binder families known as pan-assay interfer- ence compounds (45). Further medicinal chemistry to generate targeted analogs may give compounds with improved potency.

In summary, the studies here support the potential utility of small molecule correctors and potentiatotors as a therapeutic approach for CF caused by the W1282X mutation and perhaps
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for other CF-causing mutations in the carboxyl-terminal region of CFTR. Although compounds did not produce measurable current in nasal epithelial cells from a single homozygous W1282X CF subject, further testing of CFTR1281 correctors and potentiators in cells from additional W1282X homozygous subjects may show benefit, as might testing in subjects with appropriate compound mutations such as W1282X/G542X.

Experimental Procedures

Chemicals—VX-809, VX-661, VX-770, and CFTRinh-172 were purchased from Selleck Chemicals (Boston, MA). The previously described potentiators and correctors (preixed with P and C, respectively) P3, P8, P9, C3, C7, and C18 were obtained from the CFTR Compound Program, The Cystic Fibrosis Foundation Therapeutics Inc., which is administered at Rosalind Franklin University of Medicine and Science; Corr4a (C4), CoPo-22, PG01, A04, and P12 were obtained from an in-house repository of CFTR modulators. Genetin (G418) was purchased from Gibco-Life Technologies, Inc. Screening was done using a collection of ~30,000 drug-like synthetic small molecules (ChemDiv, San Diego) and collections of approved and investigational drugs (~2500 molecules; Microsource Spectrum Library, Gaylordsville, CT; the National Institutes of Health Clinical Collection, Biofocus, South San Francisco, CA). All other chemicals were purchased from Sigma unless otherwise stated.

Complementary DNA Constructs—Expression cassettes containing CFTR cDNA were generated in pcDNA3.1/Zeo (+) (Invitrogen) that contains a cytomegalovirus intermediate-early (CMV) promoter. Constructs were generated in two steps. The 5’-region of CFTR (~3.8 kb) was excised as a KpnI-BstXI fragment from a plasmid encoding enhanced GFP-CFTR (46). Subsequently, gBlock gene fragments (Integrated DNA Technology, Coralville, IA) were synthesized to regenerate the 3’-region of wild type and mutant CFTR cDNAs (Fig. 1A, bottom). Gene fragments were designed to contain an endogenous BstXI site and an engineered XhoI site for cloning, together with the 5′-region of wild type and mutant CFTR cDNAs (Fig. 1A, bottom). Gene fragments were designed to contain an endogenous BstXI site and an engineered XhoI site for cloning, together with the 5′-region of wild type and mutant CFTR cDNAs (Fig. 1A, bottom). Gene fragments were designed to contain an endogenous BstXI site and an engineered XhoI site for cloning, together with the 5′-region of wild type and mutant CFTR cDNAs (Fig. 1A, bottom). Gene fragments were designed to contain an endogenous BstXI site and an engineered XhoI site for cloning, together with the 5′-region of wild type and mutant CFTR cDNAs (Fig. 1A, bottom).

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removing cell clumps. For some experiments, isolated nasal epithelial cells were plated on permeable tissue culture inserts (12 mm diameter, 0.4-μm polyester membrane; Corning Costar Transwell) for polarized growth (termed P0 cultures) as described (25). Conditional reprogramming of nasal epitelial cells was performed using a modification of reported methods (51, 52). Briefly, 3T3-L1 fibroblasts (obtained from the UCSF Cell Culture Facility) were suspended in F-medium (3:1 Ham’s F-12/DMEM, supplemented with 5% FBS, 5 μg/ml bovine insulin, 8.4 ng/ml cholera toxin, 10 ng/ml recombinant human EGF (Atlanta Biologicals, Norcross, GA), 25 ng/ml hydrocortisone, 1 μM Y27632-ROCK inhibitor (Enzo Life Sciences, Farmingdale, NY), penicillin/streptomycin (as above), 250 μg/ml amphotericin B, and 10 μg/ml gentamicin) and irradiated in a Gammacell 3000 Elan (Best Theratronics, Springfield, VA) at 30 gray. Irradiated cells were then plated (3 × 10^6 per 10-cm tissue culture dish) and allowed to attach for 2 h. Nasal cells (1.5 × 10^6) were added to each dish and allowed to proliferate for 4–7 days and were subsequently harvested by differential trypsinization when they reached ~80% confluence. Conditionally reprogrammed cells were subsequently plated (5 × 10^6 cells/cm^2) on tissue culture inserts coated with human placental collagen (20 μg/cm^2) in DF12 containing 5% FBS and antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml) to generate differentiated nasal epithelial cell sheets (termed P1 CRCs). After 24 h, the inserts were rinsed with PBS containing antibiotics and cultured with UNC-ALI media as described (25). CFTR transcript levels in human cells were measured by TaqMan quantitative RT-PCR using the HS00357011_1_m1 probe set (Thermo Fisher Scientific, Waltham, MA) with data normalized to GAPDH transcript levels. Experiments were done at the UCSF Helen Diller Cancer Center Genome Analysis Core.

**Screening Procedures**—High throughput screening used a semi-automated screening platform (Beckman, Fullerton, CA) configured as described (53). FRT cells were plated in 96-well black-walled, clear-bottom tissue culture plates (Corning) at a density of 20,000 cells/well and cultured to confluence over 48 h prior to assays. For the corrector screen (Fig. 2A, left), cells expressing W1282X-CFTR and YFP were treated with test compounds in culture medium for 1 day, then washed twice with PBS, and incubated for 10 min in 100 μl of PBS containing forskolin (10 μM) and VX-770 (5 μM) prior to assay of CFTR activity. All plates in the corrector screen contained wells with positive (3 μM VX-809, 5 μM VX-770, and 10 μM forskolin) and negative (5 μM VX-770 and 10 μM forskolin) controls. For the potentiator screen (Fig. 2A, right), cells expressing CFTR_1281 were cultured in medium containing VX-809 (3 μM) for 1 day, washed twice with PBS, and then incubated for 10 min in 100 μl of PBS containing forskolin (10 μM) and test compounds prior to assay. All plates in the potentiator screen contained positive (3 μM VX-809, 5 μM VX-770, and 10 μM forskolin) and negative (3 μM VX-809 and 10 μM forskolin) controls. A “synergy” screen to investigate combined effects of VX-770 with bioactive molecules and approved drugs was done using cells expressing CFTR_1281 in a similar manner as the potentiator screen, with the exception that test wells contained VX-770 (5 μM). Assays were performed using a BMG Labtech FLUOstar OMEGA (Cary, NC) over 12 s with initial fluorescence intensity recorded for 1 s prior to additional of 100 μl of NaI-substituted PBS (137 mM NaCl replaced with NaI). Initial I_+ influx was computed from fluorescence intensity by single exponential regression (26). Control experiments were done using FRT cells expressing EYFP-H14Q/I152L/F46L alone.

**I_+ Measurements**—Measurements of I_+ were done with cells cultured on permeable supports as described (27). For FRT cells, the basolateral membrane was permeabilized with 250 μg/ml amphotericin B, and experiments were done using a HCO_3^-buffered system (in mM: 120 NaCl, 5 KCl, 1 MgCl_2, 1 CaCl_2, 5 Hepes, 25 NaHCO_3, pH 7.4) with a basolateral to apical chloride gradient (generated by replacing 60 mM NaCl with sodium gluconate in the apical solution). For nasal epithelial cells, experiments were done using symmetrical HCO_3^-buffered solutions (containing 120 mM NaCl). Modulators of ion conductance were added to both apical and basolateral bathing solutions, and cells were equilibrated with 95% O_2, 5% CO_2 and maintained at 37 °C.

**CFTR Cell Surface Assays**—FRT cells expressing 3HA-tagged CFTR constructs were washed in PBS (supplemented with 0.5% BSA) and incubated at 4 °C for 10 min prior to incubation with anti-hemagglutinin antibody (2 μg/ml, 1 h, 4 °C; clone 16B12, Biolegend, San Diego, CA), HRP-conjugated secondary antibody (0.16 μg/ml, 1 h, 4 °C; goat anti-mouse IgG antibody, Thermo Scientific, Waltham, MA), and Western Bright Sirius luminescence detection reagent (Advanta Inc., Menlo Park, CA). Luminescence representing surface CFTR was detected using a Tecan Infinite F500 plate reader (San Jose, CA). CFTR activity was measured by I_+ for computation of relative channel activity. A similar approach was used for CFBE cells but with Amplex-Red (Molecular Probes, Waltham, MA) HRP substrate to measure CFTR cell-surface density (50).

**Author Contributions**—P. M. H. conceived the study, designed experiments, generated cDNA constructs and cell models used in the study, conducted read-through and surface presentation assays, analyzed results, and wrote the paper. P. W. P. conducted structure-activity studies and electrophysiological experiments using FRT and reprogrammed cell models, analyzed results, and wrote aspects of the paper. J. A. T. conducted HTS and structure-activity studies, and analyzed data. H. X. participated in generation of lentiviral expression constructs and performed biochemical analysis. R. G. A. participated in generation of lentiviral expression constructs, performed electrophysiological studies on CFBE cultures, and was responsible for the initial observation of combined VX-770/genistein potentiation. D. P. performed pulse-chase studies. L. Z. cultured primary and conditionally reprogrammed human cell cultures. D. W. N. acquired patient samples. W. E. F. conceived certain studies, designed experiments, and wrote aspects of the paper. G. L. L. and A. S. V. conceived the study, designed experiments, and wrote aspects of the paper. J. A. T. conducted HTS and structure-activity studies, and wrote the paper.

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