Discovery of Clinically Approved Agents That Promote Suppression of Cystic Fibrosis Transmembrane Conductance Regulator Nonsense Mutations

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Abstract

Rationale: Premature termination codons (PTCs) in the cystic fibrosis transmembrane conductance regulator (CFTR) gene cause cystic fibrosis (CF). Several agents are known to suppress PTCs but are poorly efficacious or toxic.

Objectives: To determine whether there are clinically available agents that elicit translational readthrough and improve CFTR function sufficient to confer therapeutic benefit to patients with CF with PTCs.

Methods: Two independent screens, firefly luciferase and CFTR-mediated transepithelial chloride conductance assay, were performed on a library of 1,600 clinically approved compounds using fisher rat thyroid cells stably transfected with stop codons. Select agents were further evaluated using secondary screening assays including short circuit current analysis on primary cells from patients with CF. In addition, the effect of CFTR modulators (ivacaftor) was tested in combination with the most efficacious agents.

Measurements and Main Results: From the primary screen, 48 agents were selected as potentially active. Following confirmatory tests in the transepithelial chloride conductance assay and prioritizing agents based on favorable pharmacologic properties, eight agents were advanced for secondary screening. Ivacaftor significantly increased short circuit current following forskolin stimulation in cells treated with pyranoradine tetraphosphate, potassium p-aminobenzoate, and escin as compared with vehicle control. Escin, an herbal agent, consistently induced readthrough activity as demonstrated by enhanced CFTR expression and function *in vitro*.

Conclusions: Clinically approved drugs identified as potential readthrough agents, in combination with ivacaftor, may induce nonsense suppression to restore therapeutic levels of CFTR function. One or more agents may be suitable to advance to human testing.

Keywords: premature termination codon mutations; cystic fibrosis transmembrane conductance regulator; readthrough; clinical agents

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At a Glance Commentary

Scientific Knowledge on the

Subject: Currently available drugs that promote translational readthrough are either toxic or poorly efficacious in patients with cystic fibrosis (CF) with nonsense mutations.

What This Study Adds to the

Field: We identified agents that induce translational readthrough in the CF transmembrane conductance regulator gene, partially restoring CF transmembrane conductance regulator activity in an important preclinical model. Because the safety profile of these agents is already in place, they may be beneficial for patients with CF with nonsense mutations and other related genetic disorders.

Cystic fibrosis (CF) is an autosomal-recessive disorder caused by mutations in the gene encoding the CF transmembrane conductance regulator (*CFTR*), an anion channel primarily localized to the apical membranes of secretory epithelial cells lining the airways and multiple organs (1). Among the most common mutation class, premature termination codons (PTCs) in *CFTR* lead to translation termination caused by an in-frame nonsense mutation in the coding sequence, resulting in nonfunctional *CFTR* protein (2). PTCs are the proximate cause of approximately 7–11% of CF-causing alleles and many other genetic diseases (3).

Efforts to develop treatments for patients with CF with nonsense mutations have focused on strategies to promote termination suppression (also known as translational readthrough) of PTCs. Translational readthrough is accomplished when an amino acid carried by a near-cognate aminoacyl transfer RNA is inserted into a polypeptide chain at the erroneous stop codon, allowing translation to continue, and partially restoring full-length, functional protein (4). Several pharmacologic approaches to induce readthrough have been discovered, yet none has yielded an optimal combination of efficacy and safety. For instance, *in vitro* work has demonstrated that certain aminoglycosides can promote readthrough (5, 6) and have been tested in clinical trials

with mixed results (7–11), but are not well suited for long-term use. Synthetic aminoglycoside derivatives optimized for translation suppression of the eukaryotic ribosome have exhibited improved readthrough and reduced toxicity when compared *in vitro* (12). Ataluren (formerly PTC124) is an orally bioavailable small molecule that induces readthrough. In a subset analysis, ataluren demonstrated a modest treatment benefit in patients with CF not using chronic inhaled tobramycin, which interferes with its effect (13), a finding currently under prospective evaluation.

An attractive strategy for development of nontoxic, orally bioavailable, efficacious readthrough agents is to evaluate the utility of compounds already approved for clinical use, thus facilitating rapid evaluation of promising compounds in humans. The U.S. Food and Drug Administration–approved agent amlexanox was shown to inhibit nonsense-mediated mRNA decay, a pathway that targets aberrant mRNAs generated as a result of PTCs (14), and may also promote low-level PTC suppression. In the present study, we evaluated a library of 1,600 clinically approved compounds using high-throughput screening (HTS) to identify agents that suppress *CFTR*-PTC nonsense mutations. We conducted two primary assays in parallel (one mechanistic, the other phenotypic), and then confirmed readthrough activity of selected agents in a series of secondary assays specific to readthrough of *CFTR* nonsense mutations. Through this process, we identified several agents that induced readthrough. Among these was escin, a natural product that was efficacious and potent in a variety of primary human airway cells expressing *CFTR* premature termination alleles, indicating its potential as a clinical agent for nonsense-mediated CF and possibly other related genetic diseases. Some of the results of these studies have been previously reported in the form of abstracts (15, 16).

Methods

Detailed methods are available in the online supplement.

Cell Culture

Fischer rat thyroid (FRT) cells were stably transfected with a dual-luciferase

complementary DNA (cDNA) containing a UGAC or *CFTR*-G542X readthrough sequence (termed “G542X context”) as shown in Figure 1. For functional assays, cDNAs carrying *CFTR*-G542X, *CFTR*-W1282X, or *CFTR*-WT alleles were introduced into FRT cells using the Flp-In system (Invitrogen, Grand Island, NY). All primary human bronchial epithelial (HBE) cells were expanded using conditional reprogramming (17). The University of Alabama at Birmingham institutional review board approved use of HBE cells.

Luciferase HTS Reporter Assay

Luciferase assay was performed on the MicroSource-Pharmakon 1,600 library on FRT cells expressing either the dual-luciferase UGAC or *CFTR*-G542X context (12). Compounds were tested at 10 concentrations (100 nM to 30 μ M) in 0.3% dimethyl sulfoxide for 24 hours. In HTS, only firefly luciferase activity was measured, whereas the ratio between the readthrough product (firefly) and baseline expression (*Renilla*) was performed in the secondary evaluations.

Transepithelial Chloride Conductance Assay

Transepithelial chloride conductance (TECC) assay was performed on FRT cells carrying *CFTR*-G542X or W1282X cDNA (12). During initial evaluation of agents, cells were treated for 48 hours before transepithelial conductance (Gt) was assessed (18).

Confirmatory Dual-Luciferase Assay

The basic features of the dual-luciferase reporter system have been described previously (19). The UGA-containing readthrough cassette was inserted between the *Renilla* and firefly genes in the expression plasmid and stable FRT cell lines were made using the FlpIn system.

Ussing Chamber Studies in Primary Cell Monolayers

Short circuit current (Isc) was measured under voltage clamp conditions (18). Well-differentiated cells (G542X/F508del or W1282X/F508del) were treated with test compound or control for 48 hours.

Horseradish Peroxidase CFTR Expression Assay

To measure cell surface expression of *CFTR*, FRT cells expressing the fusion protein of

Figure 1. Enhanced readthrough activity in Fischer rat thyroid UGAC context and G542X context stable cell lines. (A) Dual-luciferase constructs showing the cystic fibrosis transmembrane conductance regulator (*CFTR*) premature termination codon context (G542X and UGAC) and wild type inserted between *Renilla* and firefly. In both UGAC context (B) and *CFTR* G542X context (C) cell lines, G418 showed high readthrough activity and was dose dependent, whereas it did not increase the reporter signal for *CFTR* wild type (D), an indicator of specificity. **** $P < 0.0001$. pA = poly(A) terminator signal; P_{CMV} = cytomegalovirus promoter.

CFTR-G542X cDNA and horseradish peroxidase (HRP) were treated with test compound for 48 hours followed by detection of HRP substrate using a microplate reader.

Real-Time Polymerase Chain Reaction

RNA was isolated using RNAeasy isolation kit (Qiagen, Germantown, MD) and real-time polymerase chain reaction was performed

using TaqMan One Step polymerase chain reaction master mix (Thermo Fisher Scientific, Waltham, MA) as described previously (20). The relative transcript levels were normalized to FRT wild-type (WT) mRNA expression.

Statistics

Thresholds for hit identification on HTS assays were greater than 95% of confidence interval for negative controls. For TECC, Isc, dual-luciferase, and HRP cell-based assays, descriptive statistics (mean, SD, and SEM) were compared using *t* tests or analysis of variance performed using Graphpad Prism software (La Jolla, CA) and Microsoft Excel (Seattle, WA), as appropriate. All statistical tests were two-sided and were performed at a 5% significance level.

Results

Development and Validation of Stable Cell Lines for Luciferase HTS Assay

We have recently shown in mammalian cells that the four most common CF-related nonsense mutations (G542X, R553X, R1162X, and W1282X) are all relatively resistant to readthrough induction by synthetic aminoglycosides optimized for readthrough (12). As such, we sought to identify compounds via HTS that would be efficient in promoting readthrough of PTCs. To measure readthrough activity, we first generated stable FRT cell lines using dual-luciferase reporter constructs comprised of *Renilla* and firefly genes surrounding a *CFTR* readthrough cassette with either G542X context or UGAC (Figure 1A). We selected G542X context because it is the most common *CFTR*-PTC nonsense mutation and we had previously established FRT *CFTR*-G542X for measurements of *CFTR* function (12, 18). UGAC was selected because it is the most responsive PTC context to positive control agents. Use of two distinct constructs minimized bias and maximized the potential of identifying effective drugs.

To validate these cell lines, we treated them with G418, a readthrough agent with established efficacy *in vitro* (4), at doses ranging from 0 to 500 $\mu\text{g}/\text{ml}$. G418 significantly increased firefly luciferase activity in a dose-dependent manner compared with dimethyl sulfoxide-treated vehicle ($P < 0.0001$) (Figures 1B and 1C), but had no significant effect in matched FRT WT *CFTR* control (Figure 1D), confirming that FRT cell lines with dual-luciferase G542X context and UGAC constructs would be informative models for use in the screens. For screening purposes, firefly luciferase levels were measured without assessment of *Renilla* expression,

an indication of total construct expression. Time dependence was established as described in the online supplement.

Initial HTS

As summarized in Figure 2, HTS of 1,600 agents was performed using two independent approaches in parallel: a *CFTR*-mediated TECC assay, which provides a readout of *CFTR* activity and thus ensures physiologic relevance; and a highly sensitive luciferase-based reporter assay (12), which enables simultaneous testing of multiple concentrations with much higher throughput.

In the TECC assay, FRT *CFTR*-G542X cells were treated with a single dose (10 μM) of each compound. Of the 1,600 compounds, 58 elicited an active forskolin-induced response, representing a hit rate of 3.6% (Figure 3A). Compounds that stimulated forskolin activity greater than or equal to 20% normalized to the G418 positive control were considered active. Agents that also responded to *CFTR* inhibitor (*CFTR*_{Inh})-172 (10 μM) after

forskolin were prioritized. Nonspecific compounds were excluded, as described in the online supplement.

In the firefly luciferase approach, FRT cells expressing UGAC and G542X context dual-luciferase reporters were treated with the same 1,600 compounds using a 10-point, serial twofold dilution concentration response assay (0.1–30 μM). In FRT UGAC cells, the initial screen yielded 115 active hits as defined by maximum firefly activation greater than or equal to 20% relative to the G418 positive control (Figure 3B). We excluded 90 agents with poor potency, a narrow dose response, unfavorable medicinal properties, or overlapping mechanisms (e.g., multiple corticosteroids were identified). Interestingly, none of the 1,600 agents reached the 20% threshold activity relative to the G418 control when tested in the FRT cell line with dual-luciferase *CFTR*-G542X context (data not shown), likely because this construct is relatively resistant to PTC suppression.

Together, the TECC and UGAC dual-luciferase approaches yielded a total of

Figure 2. Schematic representation of high-throughput screening and secondary evaluation. FRT = Fischer rat thyroid; HBE = human bronchial epithelial; HRP = horseradish peroxidase; Isc = short circuit current; TECC = transepithelial chloride conductance.

Figure 3. High-throughput screening. (A) Scatter plot representing the percent change in forskolin (FSK) (normalized to G418 control; 250 μ g/ml)-induced increase in transepithelial conductance (Gt) of Fischer rat thyroid cell monolayers ($n = 1$) expressing cystic fibrosis transmembrane conductance regulator G542X cDNA treated with a single dose (10 μ M) of compounds. (B) Scatter plot with active hits identified in Fischer rat thyroid UGAC cells from primary screen of high-throughput screening reporter assay indicating the maximum percent activation. The red dots are considered active agents with FSK-induced activation levels or the maximum percent activation levels $\geq 20\%$.

48 initial hits after accounting for specificity, medicinal properties, and other factors. Five agents were common hits for both assays at this stage.

Prioritization of Lead Agents

To prioritize efficacious agents from the 48 initial hits, we repeated treatment of FRT *CFTR*-G542X cells and FRT cells without *CFTR* for 48 hours with the selected 48 agents in triplicate. The change in Gt relative to dimethyl sulfoxide vehicle after forskolin activation was assessed, and agents that did not elicit *CFTR* activity were eliminated. As shown in Figure 4, a total of 13 agents consistently induced a forskolin-stimulated increase in Gt in FRT *CFTR*-G542X cells (0.1 ± 0.005). Five of these

13 agents were excluded for off-target effects (data not shown). The remaining eight agents were deemed lead agents; five had been originally identified through the TECC assay, and three through the dual-luciferase approach.

We next performed a series of functional assays to evaluate the efficacy of these lead agents in greater detail. Using FRT *CFTR*-G542X cells, we first assessed the optimal dose of each compound in augmenting Gt (Figure 5A) and mediating PTC suppression (Figure 5C) at concentrations ranging from 1 to 30 μ M and 0.01 to 10 μ M, respectively. Relative to vehicle control, cells exhibited significantly higher ($P < 0.05$) forskolin-stimulated *CFTR* activity (Figure 5A) when treated

with colchicine, cyclohexamide, escin, pyranoradine tetrphosphate (PT), prednicarbate, oxibendazole, and potassium paraamino benzoic acid (PABA) (Table 1). In general, readthrough efficacy was optimal across all eight agents at a 10- μ M dose, although it should be noted that, for escin, readthrough activity at approximately 2 μ M was similar to that at 10 μ M, and for doxorubicin, activity peaked at approximately 1 μ M.

These eight lead agents were also tested in FRT cells expressing *CFTR*-W1282X mutation, where colchicine (1 μ M), PT, prednicarbate, and escin (each at 10 μ M) was shown to significantly enhance *CFTR* activity compared with vehicle (see Figure E3A in the online supplement). To examine the off-target effects on *CFTR* processing, the lead agents was tested in FRT *CFTR*-F508del cells in comparison with the corrector molecule VX-809. None of the agents were active except for VX-809, which significantly enhanced *CFTR* activity, indicating that the readthrough agents are primarily acting to promote translational readthrough and not *CFTR* folding itself (see Figure E3B). To obtain an additional readout of *CFTR* function, lead agents were tested using the HRP *CFTR* cell surface expression assay (Figure 5B). The HRP activity was significantly higher in FRT *CFTR*-G542X cells treated with 10 μ M colchicine, cyclohexamide, oxibendazole, escin, and doxorubicin compared with vehicle control, validating functional measures with a biochemical assay of *CFTR* expression. *CFTR* expression was also positively correlated with firefly luciferase activity ($P = 0.02$) (Figure 5E) and forskolin-stimulated increase in Gt (Figure 5F). A moderate correlation between dual-luciferase activity and forskolin-stimulated increase in Gt also was observed (Figure 5G). We also tested amlexanox (see online supplement). Overall, these results suggested that restoration of readthrough activity was accompanied by restoration of *CFTR* function, further indicating the potential efficacy of these eight lead agents.

Evaluation of Lead Agents in Primary CF HBE Cells

Based on previous studies that demonstrated primary HBE cells have been a valuable preclinical tool for predicting CF clinical trial results (21, 22), we performed functional assays with HBE cells derived

Figure 4. Readthrough of compounds in Fischer rat thyroid cystic fibrosis transmembrane conductance regulator (*CFTR*)-G542X cells. Between the two assays, 48 compounds were selected for confirmatory testing in the transepithelial chloride conductance assay. Forskolin induced *CFTR* activity in Fischer rat thyroid *CFTR*-G542X cells ($n = 3$) treated with 48 compounds (10 μ M) and positive control G418 (250 μ g/ml).

from a G542X/F508del-*CFTR* heterozygous patient and WT HBE cells. The mean forskolin stimulated *I*_{sc} observed with a panel of non-CF WT HBE cells was $29.1 \pm 0.6 \mu\text{A}/\text{cm}^2$ (see Figures E5A and E5B). Previously we have shown that ivacaftor (formerly, VX-770), a *CFTR* potentiator that acts by increasing the open probability of *CFTR* at the cell surface, augments the ability of synthetic readthrough drugs in restoring *CFTR* function (12). Here, we assessed the effect of ivacaftor in combination with the optimal dose of each lead agent as determined in Figure 5A. Our results indicate that ivacaftor significantly increased *I*_{sc} following forskolin stimulation in cells treated with PT ($6.2 \pm 0.3 \mu\text{A}/\text{cm}^2$), prednicarbate ($4.9 \pm 1.2 \mu\text{A}/\text{cm}^2$), PABA ($6.6 \pm 1.2 \mu\text{A}/\text{cm}^2$), and escin ($7.1 \pm 0.3 \mu\text{A}/\text{cm}^2$) as compared with vehicle control (Figures 5A and 5B). In addition VX-770 significantly enhanced forskolin-induced *I*_{sc} in VX-809 (3 μ M)-treated HBE G542X/F508del cells (see Figures E5C and E5D). Together, these results are consistent with prior work suggesting the promise of therapeutic strategies combining PTC suppression compounds with ivacaftor, and provide

additional data supporting the potential utility of the agents identified in the screen.

Detailed Evaluation of Escin

Escin emerged as a particularly compelling compound among our eight leads. Escin elicited the highest levels of readthrough (Figure 5D), cell surface *CFTR* expression (Figure 5B), and forskolin-dependent *I*_{sc} alone and in combination with ivacaftor (Figures 6A and 6B). Importantly, escin, which has been used for its antiinflammatory, antiedematous, and vasoprotective properties (23), is a readily available herbal agent with no known side effects.

In an independent evaluation of escin, we first observed that FRT *CFTR*-G542X cells treated for 24 hours exhibited a significant increase in forskolin-stimulated *G*_t (Figure 7A) that was dose-dependent (Figure 7B). The effect of escin on *CFTR* function was confirmed by the complete attenuation of *G*_t levels on subsequent addition of *CFTR*_{Inh}-172 (Figure 7A). Although we observed efficacy at both 3 μ M and 10 μ M dose levels, we tested escin at a broader dose range (0.001–20 μ M)

using the HRP assay to further assess the effect of low doses (Figure 7C); EC_{50} was 2.3 μ M, indicating reasonable potency for human use. In primary CF HBE from a G542X/F508del donor, escin induced a significant increase in forskolin-dependent *I*_{sc} that was inhibited after administration of *CFTR*_{Inh}-172 (Figure 7D). *CFTR* activity on stimulation with forskolin alone ($11.9 \pm 2.5 \mu\text{A}/\text{cm}^2$) was approximately 46% of WT *CFTR* activity (Figure 7E), which was further augmented by ivacaftor. In addition, escin also enhanced *CFTR* mRNA expression levels by approximately twofold (Figure 7F). This finding corroborated the *I*_{sc} results and is consistent with previous findings that some compounds can both induce readthrough and inhibit nonsense-mediated mRNA decay (14).

To explore the potential benefit of escin for patients with CF with other common nonsense mutations, we tested the compound on cells with the W1282X PTC. Escin significantly enhanced *CFTR* function in FRT expressing W1282X *CFTR* and primary HBE derived from a W1282X/F508del donor (Figures 7G and 7H); ivacaftor increased these currents further, particularly in the heterologous

Figure 5. Cystic fibrosis transmembrane conductance regulator (CFTR) activity of potential readthrough compounds in Fischer rat thyroid (FRT) cells. (A) Forskolin-induced CFTR activity in FRT *CFTR*-G542X cells treated with different doses (1, 3, 10, and 30 μ M) of potential readthrough agents. (B) Readthrough compounds (10 μ M) identified by horseradish peroxidase (HRP) assay on HRP-tagged FRT *CFTR*-G542X cells with active agents showing significant HRP activity compared with dimethyl sulfoxide (DMSO) vehicle. (C) Dual-luciferase activity (ratio of firefly/*Renilla*) of lead agents in FRT UGAC cells at various doses (0.01–10 μ M). (D) Readthrough compounds identified by dual-luciferase assay represented as fold increase in firefly/*Renilla* ratio of highest efficacy. (E) Correlation between HRP assay (relative light units [RLU]) and dual-luciferase assay. (F) Correlation between Δ Gt and HRP. (G) Correlation between Δ Gt and dual-luciferase assay. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. PABA = paraamino benzoic acid; PT = pyranoradine tetraphosphate.

Table 1. Efficacy of Lead Agents across Model Systems

Agent	FRT G542X TECC Gt (mS/cm ²) (Mean ± SE)	FRT UGAC Dual-Luciferase Firefly/Renilla (Mean ± SD)	FRT G542X HRP RLU (Mean ± SE)	HBE G542X/ΔF508 HBE Ussing Chamber Isc (μA/cm ²) (Mean ± SE)
Colchicine	0.21 ± 0.0 ^{*†}	1.28 ± 0.0008	36000 ± 1554 [*]	1.2 ± 0.2 [†]
Cyclohexamide	0.13 ± 0.06 ^{‡§}	1.81 ± 0.01	38171 ± 1618 [*]	3.4 ± 0.07 [§]
Oxibendazole	0.16 ± 0.005 [*]	1.34 ± 0.01	28150 ± 908.6 [¶]	1.36 ± 0.04
Pyronaridine tetraphosphate	0.13 ± 0.08 [#]	1.1 ± 0.005	1091 ± 98.8	6.2 ± 0.3 [‡]
Prednicarbate	0.14 ± 0.02 [¶]	0.89 ± 0.0034	10515 ± 870.2	4.9 ± 1.2 [#]
Escin	0.20 ± 0.001 [*]	2.04 ± 0.02	57402 ± 2836 [*]	7.1 ± 0.3 [*]
Potassium paraamino benzoic acid	0.14 ± 0 [‡]	1.44 ± 0.02	10811 ± 701.1	6.6 ± 1.2 [‡]
Doxorubicin	0.11 ± 0.01	1.99 ± 0.007	26269 ± 1426 [¶]	2.38 ± 0.14

Definition of abbreviations: FRT = Fischer rat thyroid; HBE = human bronchial epithelial; HRP = horseradish peroxidase; Isc = short circuit current; RLU = relative light units; TECC = transepithelial chloride conductance.

Assays included TECC (Gt; mean values ± SE; mS/cm²), dual-luciferase (Firefly/Renilla ratio; mean ± SD), and HRP-detected protein expression (RLU; mean ± SE), each using FRT cells; and Ussing chamber analysis using primary HBE cells (Isc; mean ± SE; μA/cm²). Unless indicated, these compounds were treated at 10 μM. Concentration = 10 μM unless otherwise indicated.

^{*}P < 0.0001.

[†]1 μM.

[‡]P < 0.01.

[§]3 μM.

^{||}30 μM.

[¶]P < 0.001.

[#]P < 0.05.

expression system (Figure 7G). As expected, escin had no effect in primary HBE cells derived from a patient homozygous for F508del mutation that had a robust response to the corrector molecule lumacaftor (VX-809), an efficacious agent shown to partially restore CFTR function in patients with this type of mutation (24), indicating the effects of escin are caused by induction of readthrough (Figure 7I).

Discussion

We screened 1,600 clinically approved compounds to determine whether drugs with an established safety profile exist that promote nonsense suppression. Our goal was to identify active readthrough compounds that would be available for expedited testing in humans for this new indication.

We approached this using a two-pronged discovery strategy that relied on complementary assays that collectively assessed molecular readthrough and CFTR functional rescue. Our combined screening strategy led us to prioritize eight lead agents that were functional in both assay types (Figure 2). These agents also induced readthrough in a series of secondary evaluations, including Isc studies in

primary cells from patients with nonsense mutations (Figures 6A and 6B).

The agents that suppressed PTCs were a mixture of different medicinal classes (see Table E1). Categories that were represented by more than one agent were translational inhibitors (cycloheximide), antiinflammatory drugs (colchicine, escin), antihelminthic drug (oxibendazole), and corticosteroids (prednicarbate, several related agents). The identification of translational inhibitors in our screen, which efficiently enhanced PTC suppression in both CFTR expression and functional assays (Figure 5), suggests that their ability to reduce translational fidelity occurs at doses before complete translation inhibition (25). We suspect this may not be a viable strategy for long-term treatment of genetic disease without further optimization of readthrough specificity (vs. nonspecific translation inhibition).

Previous reports have shown the use of colchicine treatment for patients with CF for its antiinflammatory and antiproliferative properties (26), including those with renal amyloidosis (27), although it has potential for long-term toxicity. On the whole, the readthrough activity of many agents from our screen was modest, but may provide a fertile ground for future optimization. One such agent, PABA, an antifibrotic agent, was shown to be efficacious in HBE

G542X/F508del cells with an unexpectedly large degree of activity as compared with evaluation in cell lines and will be explored in future studies. The strength of our work is that we used two complementary assays coupled with a robust series of secondary testing focused on CFTR expression and function. In doing so, we avoided problems encountered with luciferase stabilization, which has been reported to enhance firefly luciferase signal independent of readthrough activity of PTCs (28, 29).

In our secondary screen, we used a dual-luciferase readthrough assay to validate firefly data obtained in the HTS and allow internal normalization to *Renilla* luciferase to eliminate variability in mRNA abundance or translation initiation. Although we were surprised with the overlap of only few agents from the initial screens, our subsequent secondary evaluation of agents revealed that the concordance between assays was generally high (Figures 5E–5G). The relatively weaker correlation between readthrough (dual-luciferase) and function (TECC) (Figure 5G) might be caused by the differences in how cell growth and translation affects the readouts detected in the assays. For example, in the TECC assay, drugs are added after the formation of monolayers when cell growth and

Figure 6. Restoration of cystic fibrosis transmembrane conductance regulator (CFTR) function in CF primary human bronchial epithelial (HBE) cells derived from a G542X/F508del donor. (A) Forskolin (FSK)-stimulated short circuit current (Isc) was significantly higher in HBE G542X/F508del cells (passage 4) treated (48 h) with prednicarbate, escin, pyranoradine tetraphosphate (PT), and paraamino benzoic acid (PABA). Ivacaftor (VX-770) further enhanced CFTR activity following FSK stimulation in cells treated with escin, PT, and PABA as compared with vehicle control. (B) Ussing chamber Isc recordings of HBE G542X/F508del monolayers treated with PT (10 μM), prednicarbate (10 μM), escin (10 μM), and PABA (30 μM) for 48 hours. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. CFTR_{inh} = CFTR inhibitor; WT = wild type.

translation is minimal. In contrast, in the dual-luciferase assay, drugs are added during cell seeding while growth and translation are both rapidly occurring. In

addition, there are multiple intervening steps (i.e., ER retention, cAMP dependent-gating) between translation and CFTR-dependent ion transport that are likely to

impact a precise correlation. We found that TECC was the most stringent assay, probably because readthrough, cell processing, and function are all required to observe activity, whereas HRP assay requires readthrough and processing and dual-luciferase requires only readthrough. Nevertheless, the relatively balanced number of compounds arising from each assay suggests that either approach could work as a primary screen when paired with an appropriate secondary evaluation strategy.

Another interesting aspect was the relatively strong effect of ivacaftor following readthrough with a variety of agents. The effect of ivacaftor was relatively large as opposed to activation seen in WT CFTR-expressing cells after high concentrations of forskolin (20); this may be caused by less limitation of transepithelial chloride transport by the basolateral membrane given the relatively small CFTR currents seen following effective readthrough.

Among the eight leads, escin exhibits an interesting profile that responded robustly to addition of CFTR_{inh}-172 (Figure 7A). Interestingly, functional CFTR activity in both FRT and HBE cells following escin treatment was relatively strong compared with its readthrough efficacy (Figures 6 and 7); this may be caused by stabilization of mRNA transcripts (Figure 7F), which would be expected to synergistically improve function in the context of PTC suppression. Another possibility is its dual role as a CFTR agonist. Escin improves outcomes in patients with chronic venous insufficiency (30, 31) because of its effects on nitric oxide metabolism (32). Nitric oxide is also involved in activation of CFTR channels (33) and chloride currents in human lung epithelial cells (34), which may be a potential advantage of the agent. Our studies that included evaluation of escin in CFTR-independent readthrough constructs, CFTR-deficient parental cells, and F508del homozygous HBE cells clearly indicate that the effect of escin is not caused by stimulation of CFTR activity outside of readthrough.

There is prior experience with herbal agents in the treatment of CF. For example, curcumin has been shown to activate CFTR channels *in vitro* (35, 36) and may also have species-specific corrector activity. Two resveratrol oligomers derived from a Chinese medicinal plant were identified as

Figure 7. Readthrough with medicinal agent escin. (A) Representative conductance (Gt) tracings of Fischer rat thyroid (FRT) cell monolayers expressing cystic fibrosis transmembrane conductance regulator (CFTR)-G542X cDNA treated with different doses (3 and 10 μ M) of escin. (B) FRT G542X cells showing significantly higher forskolin (FSK)-induced CFTR activity at 3 and 10 μ M compared with vehicle. (C) Horseradish peroxidase (HRP) activity of escin in FRT CFTR-G542X cells with half-maximal effective concentration (EC₅₀) at 2.7 μ M. (D) Representative tracings of the short circuit current (Isc) in human bronchial epithelial (HBE) G542X/F508del cells (Passage 2). Activity with escin was 46% of wild-type CFTR activity on stimulation with FSK alone. (E) Escin significantly increased FSK-induced CFTR activity and also (F) enhanced CFTR mRNA expression levels by $\sim$ 1.5-fold compared with vehicle. In addition, escin significantly increased readthrough in (G) FRT CFTR-W1282X cells and (H) HBE W1282X/F508del cells. The specificity of escin for readthrough is indicated by (I) no change in FSK-induced CFTR activity in HBE F508del/F508del cells compared with vehicle but higher levels of FSK activity with positive control VX-809 (Corrector). * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001. RLU = relative light units; WT = wild type.

CFTR inhibitors (37); others have been isolated from herbal plant *Rhodiola kirilowii* (38). In addition, escin has shown to induce extracellular Ca^{2+} in rat tissue (39) and because Ca^{2+} signaling has been shown to promote readthrough (40) the role of escin in mediating calcium signaling is not known and needs further exploration. This experience provides reason for optimism regarding the potential for escin to improve CF outcomes. Nevertheless, pharmacokinetic studies of escin followed

by escin treatment of G542X CFTR transgenic mice could be useful to guide the conduct of clinical trials in patients with CF. Escin is highly suitable for human administration and has been previously used effectively for chronic venous insufficiency, exhibiting reasonable safety and pharmacokinetic properties (41). Unfortunately, the complex pentacyclic structure (see Figure E6) makes it less suitable for chemical modifications. Despite this, if escin proves to be efficacious, we will

have accomplished our goal of achieving agents to mediate nonsense suppression for patients with CF and possibly other related genetic diseases. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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