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Phenylhydrazones as Correctors of a Mutant Cystic Fibrosis Transmembrane Conductance Regulator

Erika Nieddu[1], Benedetta Pollarolo[1], Marco T. Mazzei[1], Maria Anzaldi[1], Silvia Schenone[1], Nicoletta Pedemonte[2], Luis J. V. Galietta[2], and Mauro Mazzei[1]

[1] Department of Pharmacy, University of Genova, Genova, Italy
[2] Laboratory of Molecular Genetics, Giannina Gaslini Institute, Genova, Italy

The phenylhydrazone RDR-1 is endowed with moderate activity as F508del-CFTR corrector; nevertheless, its simple structure enables stimulating developments in this class of correctors. Therefore, we synthesized a number of phenylhydrazones **3** by reacting phenylhydrazine derivatives **1** with furfural derivatives **2**. By the same reaction, also the pyridine derivatives **4**, the thiophene derivatives **5**, and the hydrazides **6** and **7** were prepared. All compounds were tested as F508del-CFTR correctors in the cystic fibrosis (CF) bronchial epithelial cell line CFBE41o-, using corr-4a and VX-809 as controls. Some of the tested compounds emerged as interesting F508del-CFTR correctors at 20 μM (**3c**) and 2 μM (**5d**). **3c** and **5d** administered together with VX-809 produced a satisfactory additivity of action. When the structure of **5d** was overlapped with RDR-1 and five other established correctors, a shared central design was clearly visible. This fact may be of interest in the search for new F508del-CFTR correctors.

Keywords: CFTR / Correctors / Cystic fibrosis / Ion channels / Phenylhydrazones

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Introduction

Cystic fibrosis (CF) is a genetic disease due to mutations affecting the gene coding for the protein CFTR (cystic fibrosis transmembrane conductance regulator). CFTR is a channel for anions (particularly, for chloride) located in the apical membrane of epithelial cells. When CFTR is missing or heavily damaged, electrolyte and fluid secretion from the epithelial cells to the lumen of many organs (lung, pancreas, intestine, etc.) are hampered. In addition, the mucus coating the lungs becomes more viscous and is transformed into a favorable place for the growth of bacterial infections [1, 2].

Although the CFTR gene is subjected to many mutations (more than 1900 mutations have been identified so far), the disease occurs in very severe form when the CFTR is lacking the phenylalanine in position 508. This mutation is present in ca. 70% of CF homozygote patients. In this case, the mutant CFTR (F508del-CFTR) remains entrapped in the endoplasmic reticulum (ER) where a prolonged association with the chaperone HSC70 conveys the F508del-CFTR toward the degradation via the ubiquitin/proteasome pathway. Therefore, only a marginal amount of the mutant CFTR reaches the plasma membrane and patients present a severe form of CF [3].

Since 1991, researchers know that F508del-CFTR may be rescued *in vitro* by culturing cells at low temperature (27–30°C) [4]. In fact, under these conditions, the cellular quality control system allows the trafficking of the F508del-CFTR from the ER to the plasma membrane [5]. Unfortunately, F508del-CFTR also presents a gating defect when it succeeds in getting to the plasma membrane [6]. Furthermore, a third issue arises from the fact that the F508del-CFTR in the membrane is subjected to a rapid recycle leading to a fast lysosomial degradation; therefore, the F508del-CFTR also presents a stability defect [7]. Although the F508del-CFTR shows multiple faults, pharmacological correction of trafficking defect, is feasible [8]. Since 1996, researchers have found that some

Correspondence: Prof. Mauro Mazzei, Department of Pharmacy, University of Genova, Viale Benedetto XV, 3, 16132 Genova, Italy.
E-mail: mauro.mazzei@unige.it
Fax: +39 010 3538358

chemicals may partially rescue the mutant protein obtaining a result similar to the use of low temperature [9, 10]. Later on, such chemicals were called correctors. By the time, the number of the correctors has been growing. At present, such class is constituted of several compounds recently reviewed [8, 11]. The therapeutic use of correctors has, until now, been hampered by their toxicity, as a high dosage is required to rescue the F508del-CFTR. Moreover, their mechanism of action is still unclear. For instance, some of them could bind to CFTR itself [12–15], while others could act favorably on the complex system of proteins (namely, molecular chaperones, phosphodiesterases, kinases, and PARPs), helping in the folding and trafficking of F508del-CFTR [16–19].

Clearly, the more interesting correctors are those binding the F508del-CFTR itself with respect to correctors favoring the partial inhibition of proteins essential for the cell-life, for example, chaperones. Among the more recent correctors, our interest was pointed at the phenylhydrazone RDR-1. RDR-1 is a medium level corrector and its mechanism of action is still not clear, especially if it binds or not NBD1 [14, 20]. Nevertheless, this molecule possesses a quite simple structure, and therefore it is easy to extend the synthesis to other phenylhydrazones and investigate their activity on F508del-CFTR rescue. Interestingly, the synthesis of RDR-1 derivatives also leads to investigate in more detail on a common molecular design we have already identified [21] for some different correctors, including RDR-1. This fact may be of interest in the search of new F508del-CFTR correctors.

Results and discussion

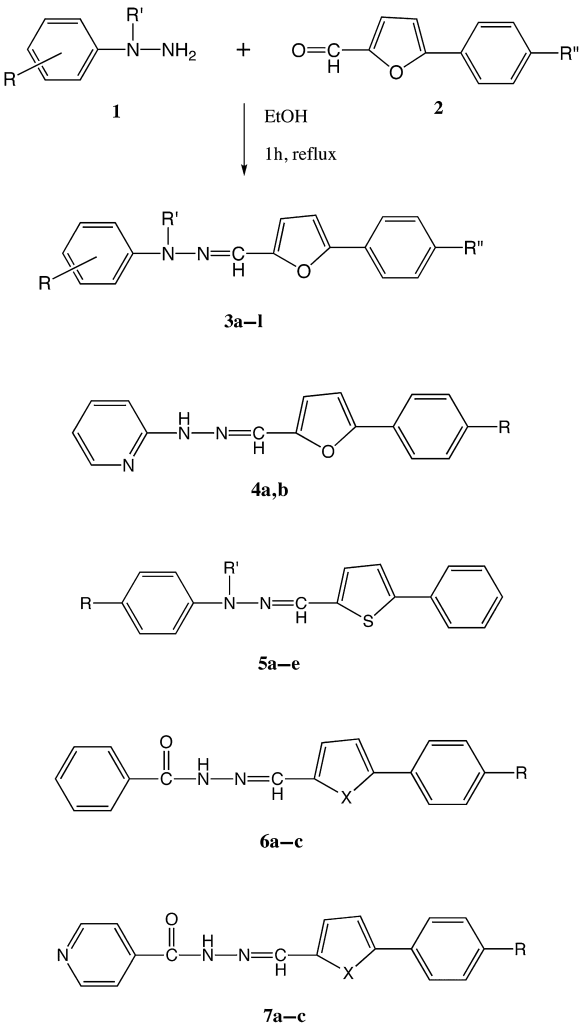
Chemistry

As depicted in Scheme 1, phenylhydrazones **3a–l** were easily prepared by reacting phenylhydrazines **1** with furfural derivatives **2** in ethanol. Then, by the same reaction, the pyridine derivatives (**4a** and **4b**), the thiophene derivatives (**5a–e**), the benzohydrazides (**6a–c**), and the isonicotinohydrazides (**7a–c**) were prepared. The formulas of the synthesized compounds are shown in Table 1.

All compounds are very colored crystals. The proposed structures were confirmed by spectral data and elemental analyses (see Experimental section).

CFTR corrector activity

Table 1 reports the effectiveness of compounds at both 20 and 2 μM. Activity was calculated as variation of fluorescence during time due to influx of iodide through CFTR and quenching of YFP fluorescence (fluorescence quenching rate, FQR). Two known correctors (corr-4a and VX-809) were used as reference compounds at 10 and 1 μM, respectively [22–24]. Although corr-4a has a moderate activity in CFBE41o- cells at 10 μM, it was not tested at higher concentration (i.e., at 20 μM) because of signs of cellular toxicity. After incubation with correctors, the functional assay evaluating F508del-CFTR in the membrane was done in the presence of genistein as a potentiator. Indeed, F508del-CFTR has also a partial gating defect as well that requires treatment with a potentiator to generate full anion transport.

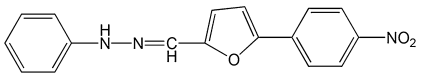
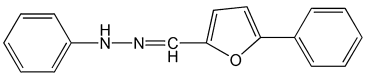
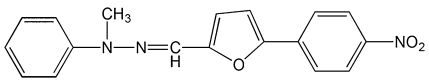
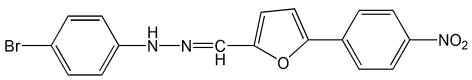
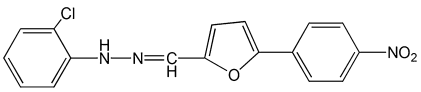
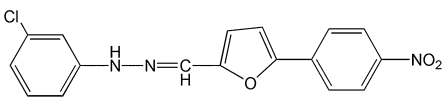
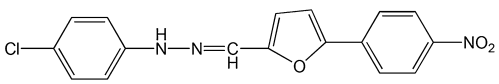
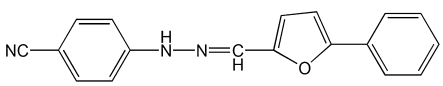
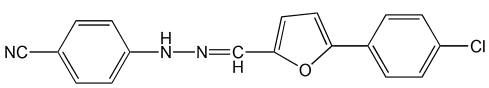
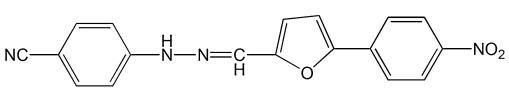
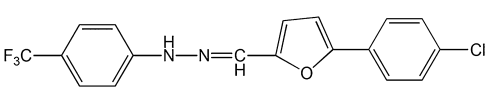


Scheme 1. Synthesis of phenylhydrazones **3**, **4**, **5**, **6**, and **7**.

Data were normalized with respect to control dimethyl sulfoxide (DMSO): compound activity in Table 1 shows how many times the compound is more efficient than DMSO. In parallel experiments, the selective blocker $CFTR_{inh}$-172 [25] was added to the solution containing forskolin plus genistein to demonstrate that the activity was CFTR-dependent.

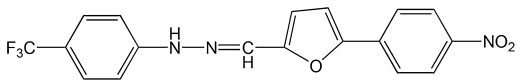
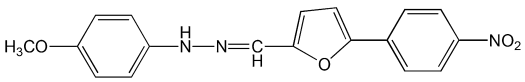
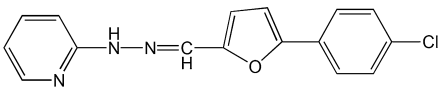
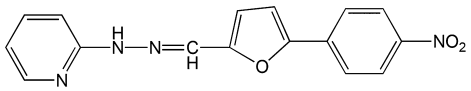
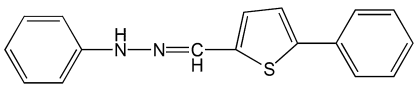
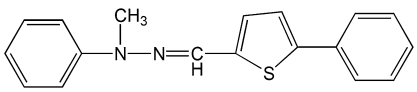
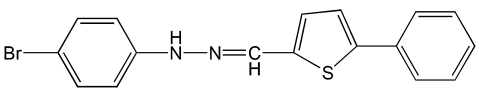
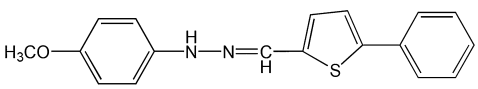
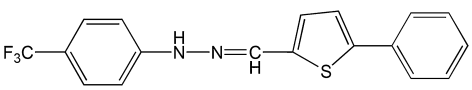
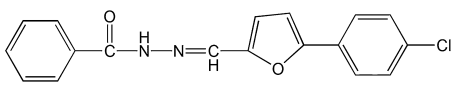
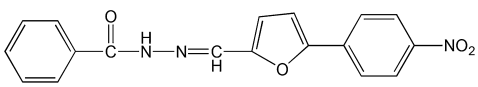
From the compounds presented in Table 1, it is possible to draw some interesting considerations. Looking at the general formula of Fig. 1, which encompasses only 15 derivatives in Table 1, the best results in the activity are found when the A substituent is Br or CH_3O. About the C substituent, we can conclude that the presence of the nitro group exerts indubitably a positive effect for the F508del-CFTR correction: in fact, some of the better correctors presented in Table 1 (**3c**, **3f**, **3l**, and RDR-1) benefit from such substitution.

Table 1. Evaluation of compounds on CFBE41o- F508del-CFTR cells.[a)]

Compound	Formula	Activity (FQR with respect to DMSO)	
		20 μM	2 μM
RDR-1[b)]		1.3 ± 0.1	1.3 ± 0.2
3a		0.7 ± 0.1	1.1 ± 0.1
3b		1.0 ± 0.2	1.0 ± 0.1
3c		1.8 ± 0.1	1.2 ± 0.2
3d		1.0 ± 0.1	0.9 ± 0.1
3e		1.0 ± 0.2	1.0 ± 0.1
3f		1.2 ± 0.1	1.0 ± 0.1
3g		1.0 ± 0.1	1.1 ± 0.2
3h		1.2 ± 0.1	1.1 ± 0.1
3i		1.1 ± 0.2	1.1 ± 0.1
3j		1.1 ± 0.1	1.2 ± 0.2

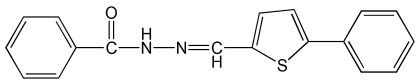
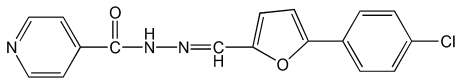
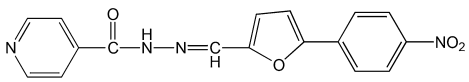
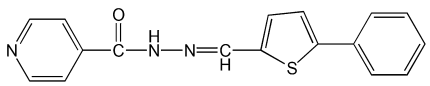
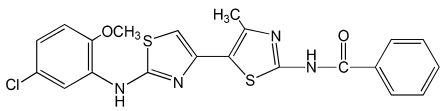
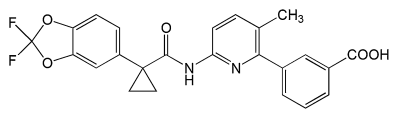
(*Continued*)

Table 1. (*Continued*)

Compound	Formula	Activity (FQR with respect to DMSO)	
		20 μM	2 μM
3k		0.9 ± 0.2	1.1 ± 0.1
3l		1.4 ± 0.1	1.2 ± 0.1
4a		1.1 ± 0.1	1.0 ± 0.1
4b		1.1 ± 0.2	1.0 ± 0.2
5a		1.5 ± 0.1	1.1 ± 0.1
5b		0.9 ± 0.1	0.9 ± 0.2
5c		1.1 ± 0.1	1.1 ± 0.1
5d		0.6 ± 0.1	1.5 ± 0.1
5e		0.7 ± 0.2	1.1 ± 0.1
6a		0.5 ± 0.1	0.9 ± 0.2
6b		0.9 ± 0.2	1.0 ± 0.1

(*Continued*)

Table 1. (***Continued***)

Compound	Formula	Activity (FQR with respect to DMSO)	
		20 μM	2 μM
6c		1.1 ± 0.1	1.0 ± 0.1
7a		0.8 ± 0.1	1.0 ± 0.1
7b		0.9 ± 0.1	1.0 ± 0.1
7c		0.8 ± 0.1	0.8 ± 0.1
corr-4a (10 μM)		1.4 ± 0.1	
VX-809 (1 μM)		2.9 ± 0.1	

[a] Activity values are the mean ± SEM of five to ten experiments.
[b] RDR-1 was synthesized by the same general procedure described in the Experimental section "General procedure for the synthesis of the phenylhydrazones **3**, **4**, **5**, **6**, and **7**" (mp 192–194°C).

The two derivatives **3b** and **5b** bearing B = CH_3 are inactive; therefore, the presence of NH seems to be essential for the searched correction activity.

Ten derivatives (**3d**, **3e**, **4**, **6**, and **7**) do not match the formula of Fig. 1. All the six compounds coming from isoniazid (**7a–c**) and benzhydrazide (**6a–c**) are toxic or ineffective, the two chloro derivatives **3d** and **3e** are inactive and the two hydrazinopyridine derivatives **4a** and **4b** are endowed with a very low activity at 20 μM: it is clear that the insertion of a carbonyl group between the aromatic ring and the NH does not attain good results.

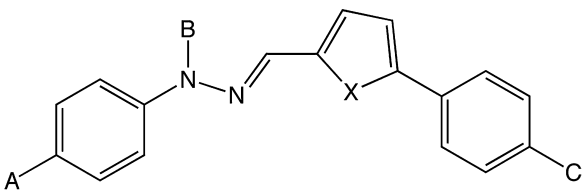


Figure 1. General structure of phenyhydrazones **3a–c, 3f–l**, and **5a–e**.

Wanting to suggest some remarks from the data here presented and referring to Fig. 1, we can deduce that a good corrector coming from these RDR-1-like derivatives should possess the methoxy or bromo substituent in the A position, the hydrogen in the B position, and the nitro group in the C position. We do not have sufficient data to establish the nature of the heteroatom X since we could take in consideration only a small number of thiophene derivatives, although the presence of sulfur seems to provide a slightly better activity together with a major toxicity in respect to furan derivatives.

Table 2. Evaluation of compounds 3c and 5d in association with VX-809 on CFBE41o- F508del-CFTR cells.[a)]

Compound	Activity (FQR with respect to DMSO)
VX-809 (1 μM) + **3c** (20 μM)	3.6 ± 0.1
VX-809 (1 μM) + **3c** (2 μM)	3.3 ± 0.1
VX-809 (1 μM) + **5d** (20 μM)	3.0 ± 0.1
VX-809 (1 μM) + **5d** (2 μM)	4.1 ± 0.1

[a)] Activity values are the mean ± SEM of four experiments.

Two compounds in our series emerge as potential F508del-CFTR correctors: **3c** with an interesting activity at 20 μM and **5d**, with a good activity at 2 μM. Therefore, we tested **3c** and **5d** together with VX-809 to search any positive effect, which should be derived by such association. The results are shown in Table 2, from which it is possible to see an additivity of action between our compounds and VX-809.

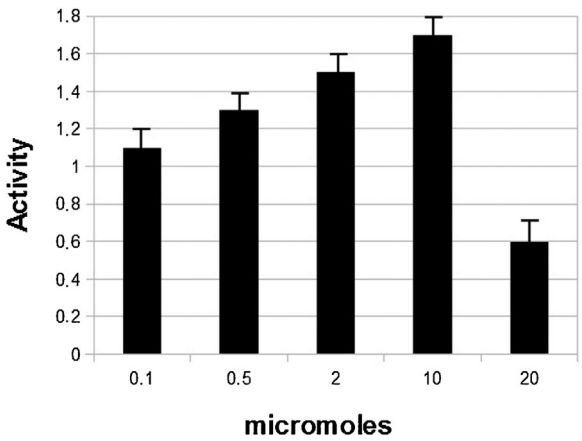


Figure 2. Dose–response graph of **5d**.

As **5d** seems to be interesting at low concentration, we tested its behavior at 0.1, 0.5, and 10.0 μM obtaining activity values (as defined in Tables 1 and 2) of 1.1, 1.3, and 1.7, respectively. Therefore, also taking in account the values from Table 1, for **5d** it was possible to draw the dose–response graph of Fig. 2. The drop of activity at 20 μM may be ascribed to sign of toxicity revealed in CFBE41o- cells.

The results described above allow us to enlarge a study done in a previous paper in which we concentrated attention to a structural design present in some F508del-CFTR correctors [21]. Briefly, in that study we compared six correctors, each of them being the lead of a greater family: besides the already cited RDR-1 and corr-4a (see Table 1), we took in account a locked bithiazole [26], a pyrazine derivative patented by Novartis [27], a pyrazolothiazole [28], and a thiazole derivative (EM-2) [29] (see Fig. 3).

Overlapping the six molecules by Chimera and MOE software [30], we could highlight a shared structural design, from which we drew the general formula depicted in Fig. 4. In this figure, great prominence was given to the presence of N (nitrogen) and X (sulfur or oxygen) whereas the other parts of the structure (the presence of one or two cycles, the presence or the absence of Y, the size of Ar and R) may be highly variable.

In our point of view, N and X in this spatial geometry may represent a signature for this type of F508del-CFTR correction.

On these grounds, in the present paper we try to validate our previous indications searching new substituents able to improve the activity of one among the above correctors (namely, RDR-1) introducing some modifications in various parts of the parent compound and also a more significant change at the level of the heteroatom X (see Fig. 4). This first synthetic approach was performed using commercial reagents: consequently, the set of the synthesized compounds is quite limited in the series of thiophene derivatives (X = S) with respect to the series of furan derivatives (X = O). Thus, the thiophene derivatives do not present any substitution on

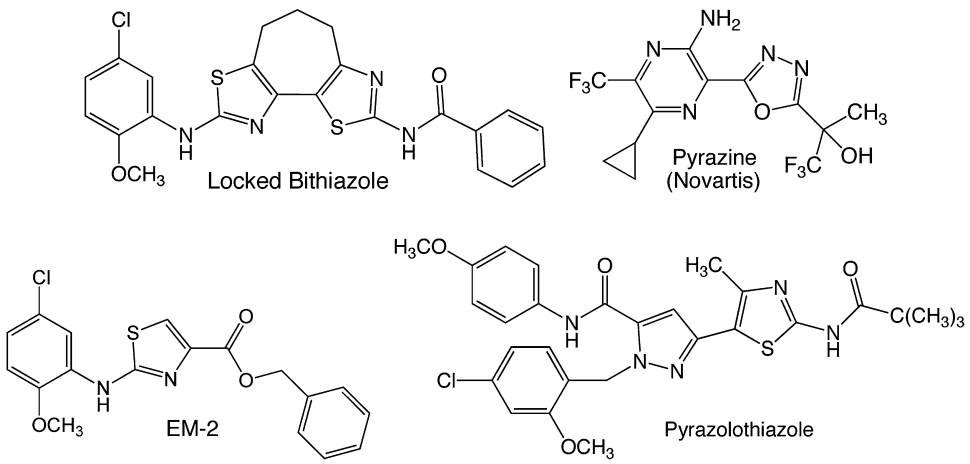


Figure 3. Structures of some F508del-CFTR correctors.

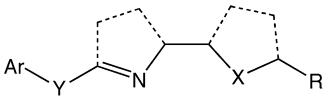


Figure 4. General formula of RDR-1-like correctors.

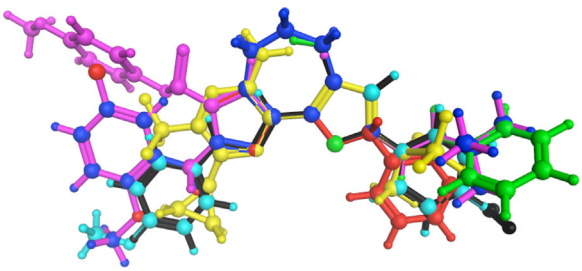


Figure 5. Superimposition of seven correctors: RDR-1 (black), corr-4a (green), locked bithiazole (blue), pyrazolothiazole (magenta), EM-2 (red), pyrazine derivative (yellow), and phenylhydrazone **5d** (light blue).

the phenyl inserted in the position 5 of the heterocycle (see Fig. 4, X = S, R = C_6H_5).

Considering the difference between **5d** and RDR-1 at the level of the heteroatom (S instead of O) in the pentacyclic ring, we were induced to amplify the figure depicted in our previous paper [21]: in Fig. 5, the superimposition of seven correctors can be seen.

Taking into account the similarity, in the central part, of the correctors displayed in Fig. 5, we can draw some observations. Firstly, it is important to stress that VX-809 does not match the correctors of Fig. 5 and it is likely, for this very active CF investigational drug, to think a binding site in the F508del-CFTR different from those of the correctors depicted in Fig. 5. Secondly, the existence of the molecular structure outlined in Fig. 4 cannot be regarded as a necessary/sufficient condition to have an F508del-CFTR correction, but may only represent an interesting support in the search of new correctors. Thirdly, the presence of furan or thiophene may drive the molecules to different targets in the mutant CFTR. Fourthly, **5d** shows at 20 μM an evident sign of toxicity, at least in CFBE41o- cells (see Fig. 2): this behavior delineates a correspondence between **5d** and corr-4a [22].

Eventually, our working assumption is, for **5d**, a mode of action similar to corr-4a, whereas the furan derivatives (for instance, **3c**) could follow the same pathway of RDR-1. This conclusion is in agreement with already published results in which it was proposed for RDR-1, corr-4a, and VX-809 different targets in F508del-CFTR [31].

Regardless of the action mechanism, we judged interesting to test the behavior of our best phenylhydrazones (**3c** and **5d**) when administered together with VX-809: this fact may be very interesting for CF patients who must take the drugs for their entire life. Indeed, the long-term cure of a CF patient will need the availability of several drugs with different chemical structures and different targets, possibly given in association with minimizing toxicity and tolerance: in this regard, the results presented in Table 2 show, at low concentration, a satisfactory additivity of action (especially for **5d**), which could be of value in succeeding association therapy.

Moreover, it must be stressed that all compounds displayed here start from cheap raw materials, rely on a very simple synthetic method, and have excellent yield. The possibility to obtain correctors in a cheap and efficient way is very interesting for this research and for clinical application, too.

We think that future research in CF drugs will be directed to the finding and assessment of clusters of F508del-CFTR modulators (correctors plus potentiators). Although phenylhydrazones present a low activity as correctors with respect to VX-809, we think that this class of compounds deserves further study since by modifying and refining the terminal endings of the basic chemical scaffold, more active compounds could be found. Eventually, the results attained in the present paper confirm that the molecular design outlined in Fig. 4 may be useful in the search of new F508del-CFTR correctors.

Experimental

Chemistry

All reagents and solvents were purchased from Sigma–Aldrich (St. Louis, MO, USA). Melting points were determined using an electrothermal apparatus and are uncorrected. Microanalyses were carried out on a Carlo Erba 1106 elemental analyzer. ^{1}H NMR and ^{13}C NMR spectra were performed on a Varian Gemini 200 (200 MHz) spectrometer using TMS as internal standard ($\delta = 0$). IR spectra were recorded on a Perkin–Elmer 398 spectrophotometer.

Compounds used in biological testing possess purity of no less than 98% as determined by elemental analysis (accuracy ±0.4%) and mass spectrometry.

*General procedure for the synthesis of the phenylhydrazones **3**, **4**, **5**, **6**, and **7***

In a 100-mL flask, to a solution of 2.3 mmoles of aldheyde A in 10 mL of ethanol, a solution of 2.3 mmoles of the suitable phenylhydrazine (or hydrazide) B in ethanol was added. The resulting mixture was refluxed for 1 h. After cooling, a red/orange precipitate separated out. Then, the solid was filtered off and crystallized from a suitable solvent. The following compounds were obtained (see also the Supporting Information providing the InChI codes).

*1-Phenyl-2-[(5-phenylfuran-2-yl)methylene]hydrazine (**3a**)*

A: 5-Phenylfurfural (0.40 g). B: Phenylhydrazine (0.25 g). Yellow crystals from toluene; yield: 48%; mp 144–146°C. ^{1}H NMR ($CDCl_3$): $\delta = 6.70$–6.80 (m, 3H, H-2,4,6 phenylhydrazine), 6.84 (d, 1H, $J = 3.6$, H-3 furan), 6.89–7.01 (m, 2H, H-3,5 phenylhydrazine), 7.12–7.60 (m, 4H, H-4 furan, H-3,4,5 phenylfuran), 7.64 (s, 1H, =CH–), 7.76 (d, 2H, $J = 8.6$, H-2,6 phenylfuran). IR (KBr): 1266,

1510, 1604, 3317 cm^{-1}. Anal. calcd. for $C_{17}H_{14}N_2O$: C, 77.84; H, 5.38; N, 10.68, found: C, 79.17; H, 5.73; N, 10.52.

*1-Methyl-2-{[5-(4-nitrophenyl)furan-2-yl]methylene}-1-phenylhydrazine (**3b**)*
A: 5-(4-Nitrophenyl)furfural (0.49 g). B: 1-Methyl-1-phenylhydrazine (0.28 g). Red purple crystals from ethyl acetate/ethanol; yield: 80%; mp 158–160°C. ^{1}H NMR (DMSO-d_6): $\delta = 3.45$ (s, 3H, N–CH_3), 6.88 (d, 1H, $J = 3.6$, H-3 furan), 6.95 (t, 1H, $J = 7.2$, H-4 phenylhydrazine), 7.30–7.47 (m, 5H, H-4 furan, H-2,3,5,6 phenylhydrazine), 7.67 (s, 1H, =CH), 7.99 (d, 2H, $J = 9.0$, H-2,6, 4-nitrophenyl), 8.31 (d, 2H, $J = 9.0$, H-3,5, 4-nitrophenyl). IR (KBr): 1331, 1497, 1598 cm^{-1}. Anal. calcd. for $C_{18}H_{15}N_3O_3$: C, 67.28; H, 4.71; N, 13.08, found: C, 67.14; H, 4.85; N, 13.11.

*1-(4-Bromophenyl)-2-{[5-(4-nitrophenyl)furan-2-yl]-methylene}hydrazine (**3c**)*
A: 5-(4-Nitrophenyl)furfural (0.50 g). B: 4-Bromophenylhydrazine (0.51 g). Red crystals from toluene; yield: 85%; mp 174–176°C. ^{1}H NMR (DMSO-d_6): $\delta = 6.93$ (d, 1H, $J = 3.6$, H-3 furan), 7.05 (d, 2H, $J = 8.8$, H-2,6 phenylhydrazine), 7.36–7.47 (m, 3H, H-4 furan, H-3,5 phenylhydrazine), 7.85 (s, 1H, =CH–), 8.01 (d, 2H, $J = 9.0$, H-2,6, 4-nitrophenyl), 8.32 (d, 2H, $J = 9.0$, H-3,5, 4-nitrophenyl), 10.76 (s, 1H, NH). ^{13}C NMR ($CDCl_3$): $\delta = 110.1$ ppm (C-3 furan), 113.2 (C-4 furan), 113.4 (C-4 phenylhydrazine), 113.6 (2C-2,6 phenylhydrazine), 122.9 (2C-3,5 nitrophenyl), 123.3 (2C-2,6 nitrophenyl), 123.5 (2C-3,5 phenylhydrazine), 123,8 (C-2 furan), 131.1 (C-1 phenylhydrazine), 131.2 (C-1 nitrophenyl), 149.3 (C-4 nitrophenyl), 150.9 (=CH–), 151.4 (C-5 furan). IR (KBr): 1329, 1510, 1586, 3320 cm^{-1}. Anal. calcd. for $C_{17}H_{12}N_3BrO_3$: C, 52.87; H, 3.13; Br, 20.69; N, 10.88, found: C, 53.16; H, 3.33; N, 10.78.

*1-(2-Chlorophenyl)-2-{[5-(4-nitrophenyl)furan-2-yl]-methylene}hydrazine (**3d**)*
A: 5-(4-Nitrophenyl)furfural (0.50 g). B: 2-Chlorophenylhydrazine (0.41 g). Red purple crystals from cyclohexane/ether; yield: 85%; mp 180–181°C. ^{1}H NMR ($CDCl_3$): $\delta = 6.80$–7.09 (m, 3H, H-3,4 furan, H-6 phenylhydrazine), 7.22–7.45 (m, 3H, H-3,4,5 phenylhydrazine), 7.67 (s, 1H, =CH), 7.94 (d, 2H, $J = 7.2$, H-2,6 4-nitrophenyl), 8.35 (d, 2H, $J = 7.2$, H-3,5 4-nitrophenyl). IR (KBr): 1330, 1510, 1580, 3317 cm^{-1}. Anal. calcd. for $C_{17}H_{12}ClN_3O_3$: C, 59.75; H, 3.54; Cl, 10.37; N, 12.30, found: C, 59.97; H, 3.,52; N.12.55.

*1-(3-Chlorophenyl)-2-{[5-(4-nitrophenyl)furan-2-yl]-methylene}hydrazine (**3e**)*
A: 5-(4-Nitrophenyl)furfural (0.50 g). B: 3-Chlorophenylhydrazine (0.41 g). Violet crystals from cyclohexane/ether; yield: 81%; mp 161–163°C. ^{1}H NMR ($CDCl_3$): $\delta = 6.77$ (d, 1H, $J = 3.6$, H-3 furan), 6.87–7.10 (m, 3H, H-4 furan, H-2,6 phenylhydrazine), 7.20–7.38 (m, 2H, H-4,5 phenylhydrazine), 7.66 (s, 1H, =CH–), 7.87 (d, 2H, $J = 8.4$, H-2,6, 4-nitrophenyl), 8.29 (d, 2H, $J = 8.4$, H-3,5, 4-nitrophenyl). IR (KBr): 1330, 1512, 1570, 3333 cm^{-1}. Anal. calcd. for $C_{17}H_{12}ClN_3O_3$: C, 59.75; H, 3.54; Cl, 10.37; N, 12.30, found: C, 59.78; H, 3.98; N, 12.58.

*1-(4-Chlorophenyl)-2-{[5-(4-nitrophenyl)furan-2-yl]-methylene}hydrazine (**3f**)*
A: 5-(4-Nitrophenyl)furfural (0.50 g). B: 4-Chlorophenylhydrazine (0.41 g). Violet crystals from cyclohexane/ether; yield: 83%; mp 172–174°C. ^{1}H NMR ($CDCl_3$): $\delta = 6.69$ (d, 1H, $J = 3.2$, H-3 furan), 6.93 (d, 1H, $J = 3.2$, H-4 furan), 7.07 (d, 2H, $J = 8.6$, H-2,6 phenylhydrazine), 7.21 (d, 2H, $J = 8.6$, H-3,5 phenylhydrazine), 7.69 (s, 1H, =CH–), 7.82 (d, 2H, $J = 8.8$, H-2,6, 4-nitrophenyl), 8.24 (d, 2H, $J = 8.8$, H-3,5, 4-nitrophenyl). IR (KBr): 1328, 1506, 1575, 3320 cm^{-1}. Anal. calcd. for $C_{17}H_{12}ClN_3O_3$: C, 59.75; H, 3.54; Cl, 10.37; N, 12,30, found: C, 60.01; H, 3.56; N, 12.32.

*4-(2-[(5-Phenylfuran-2-yl)methylene]hydrazinyl)-benzonitrile (**3g**)*
A: 5-Phenylfurfural (0.40 g). B: 4-Cyanophenylhydrazine hydrochloride (0.39 g). Yellow crystals from ethyl acetate; yield: 83%; mp 230–232°C. ^{1}H NMR (DMSO-d_6): $\delta = 6.92$ (d, 1H, $J = 3.6$, H-3 furan), 7.19–7.40 (m, 3H, H-3,5 benzonitrile, H-4 furan), 7.34 (t, 1H, $J = 7.2$, H-4 phenylfuran), 7.46 (d, 2H, $J = 7.8$, H-2,6 benzonitrile), 7.65 (dd, 2H, $J = 8.7$, $J = 7.2$, H-3,5, phenylfuran), 7.80 (d, 2H, $J = 8.7$, H-2,6 phenylfuran), 7.90 (s, 1H, =CH–). IR (KBr): 1309, 1539, 1603, 2209, 3289 cm^{-1}. Anal. calcd. for $C_{18}H_{13}N_3O$: C, 75.25; H, 4.56; N, 14.63, found: C, 75.54; H, 4.71; N, 14.73.

*4-(2-[(5-(4-Chlorophenyl)furan-2-yl)methylene]-hydrazinyl)benzonitrile (**3h**)*
A: 5-(4-Chlorophenyl)furfural (0.48 g). B: 4-Cyanophenylhydrazine hydrochloride (0.39 g). Pale yellow crystals from ethyl acetate; yield: 83%; mp 226–228°C. ^{1}H NMR (DMSO-d_6): $\delta = 6.93$ (d, 1H, $J = 3.6$, H-3 furan), 7.09–7.21 (m, 3H, H-3,5 benzonitrile, H-4 furan), 7.54 (d, 2H, $J = 8.6$, H-2,6 benzonitrile), 7.65 (d, 2H, $J = 8.7$, H-3,5 4-chlorophenyl), 7.81 (d, 2H, $J = 8.7$, H-2,6, 4-chlorophenyl), 7.90 (s, 1H, =CH–), 11.04 (s, 1H, NH). IR (KBr): 1318, 1520, 1606, 2207, 3281 cm^{-1}. Anal. calcd. for $C_{18}H_{12}ClN_3O$: C, 67.19; H, 3.76; Cl, 11.02; N, 13.06, found: C, 67.23; H, 4.01; N, 12.92.

*4-{2-[(5-(4-Nitrophenyl)furan-2-yl)methylene]hydrazinyl}-benzonitrile (**3i**)*
A: 5-(4-Nitrophenyl)furfural (0.50 g). B: 4-Cyanophenylhydrazine hydrochloride (0.39 g). Red crystals from toluene; yield: 73%; mp 246–248°C. ^{1}H NMR (DMSO-d_6): $\delta = 7.02$ (d, 1H, $J = 3.6$, H-3 furan), 7.18 (d, 2H, $J = 8.4$, H-3,5 benzonitrile), 7.46 (d, 1H, $J = 3.6$, H-4 furan), 7.67 (d, 2H, $J = 8.4$, H-2,6 benzonitrile), 7.93 (s, 1H, =CH–), 8.02 (d, 2H, $J = 8.7$, H-2,6, 4-nitrophenyl), 8.32 (d, 2H, $J = 8.7$, H-3,5, 4-nitrophenyl), 11.17 (s, 1H, NH). IR (KBr): 1336, 1512, 1608, 2209, 3272 cm^{-1}. Anal. calcd. for $C_{18}H_{12}N_4O_3$: C, 65.06; H, 3.64; N, 16.86, found: C, 65.02; H, 3.90; N, 16.73.

*1-{[5-(4-Chlorophenyl)furan-2-yl]methylene}-2-[4-(trifluoromethyl)phenyl]hydrazine (**3j**)*
A: 5-(4-Chlorophenyl)furfural (0.48 g). B: 4-(Trifluoromethyl)phenylhydrazine (0.41 g). Yellow brownish crystals

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from cyclohexane; yield: 71%; mp 122–124°C. ^{1}H NMR ($CDCl_3$): $\delta = 6.68$–7.35 (m, 4H, H-3,4 furan, H 2,6 phenylhydrazine), 7.40–7.72 (m, 7H, H-2,3,5,6 4-chlorophenyl, H-3,5 phenylhydrazine, =CH). IR (KBr): 1319, 1521, 1615, 3324 cm^{-1}. Anal. calcd. for $C_{18}H_{12}ClF_3N_2O$: C, 59.27; H, 3.32; Cl, 9.72; F, 15.63; N, 7.68, found: C, 59.32; H, 2.92; N, 7.44.

*1-{[5-(4-Nitrophenyl)furan-2-yl]methylene}-2-[4-(trifluoromethyl)phenyl]hydrazine (**3k**)*
A: 5-(4-Nitrophenyl)furfural (0.50 g). B: 4-(Trifluoromethyl)-phenylhydrazine (0.41 g). Red purple crystals from toluene; yield: 64%; mp 188–190°C. ^{1}H NMR (DMSO-d_6): $\delta = 6.97$ (d, 1H, $J = 3.8$, H-3 furan), 7.22 (d, 2H, $J = 8.4$, H-2,6 phenylhydrazine), 7.43 (d, 1H, $J = 3.8$, H-4 furan), 7.58 (d, 2H, $J = 8.4$, H-3,5 phenylhydrazine), 7.90 (s, 1H, =CH–), 8.00 (d, 2H, $J = 9.1$, H-2,6, 4-nitrophenyl), 8.30 (d, 2H, $J = 9.1$, H-3,5, 4-nitrophenyl), 11.03 (s, 1H, NH). IR (KBr): 1327, 1505, 1597, 3307 cm^{-1}. Anal. calcd. for $C_{18}H_{12}F_3N_3O_3$: C, 57.61; H, 3.22; F, 15.19; N, 11.20, found: C, 57.70; H, 3.49; N, 10.93.

*1-(4-Methoxyphenyl)-2-{[5-(4-nitrophenyl)furan-2-yl]-methylene}hydrazine (**3l**)*
A: 5-(4-Nitrophenyl)furfural (0.50 g). B: 4-Methoxyphenylhydrazine (0.40 g). Yellow brownish crystals from cyclohexane/ether; yield: 53%; mp 168–170°C. ^{1}H NMR ($CDCl_3$): $\delta = 3.84$ (s, 3H, CH_3), 6.77 (d, 1H, $J = 3.2$, H-3 furan), 6.85–7.20 (m, 5H, H-4 furan, H-2,3,5,6 phenylhydrazine), 7.84 (d, 2H, $J = 9.0$, H-2,6, 4-nitrophenyl), 8.23–8.40 (m, 3H, =CH–, H-3,5, 4-nitrophenyl). IR (KBr): 1319, 1506, 1594, 3322 cm^{-1}. Anal. calcd. for $C_{18}H_{15}N_3O_4$: C, 64.09; H, 4.48; N, 12.46, found: C, 64.00; H, 4.70; N, 12.31.

*2-{2-[(5-(4-Chlorophenyl)furan-2-yl)methylene]-hydrazinyl}pyridine (**4a**)*
A: 5-(4-Chlorophenyl)furfural (0.48 g). B: 2-Hydrazinopyridine (0.25 g). Yellow crystals from ethanol; yield: 74%; mp 211–213°C. ^{1}H NMR (DMSO-d_6): $\delta = 7.00$–7.14 (m, 1H, H-5 pyridine), 7.20–7.39 (m, 3H, H-3,4 furan, H-3 pyridine), 7.57 (d, 2H, $J = 8.6$, H-3,5 4-chlorophenyl), 7.91 (d, 2H, $J = 8.6$, H-2,6 4-chlorophenyl), 7.99–8.10 (m, 1H, H-4 pyridine), 8.14 (d, 1H, $J = 6.4$, H-6 pyridine), 8.25 (s, 1H, =CH–), 13.08 (s, 1H, NH). IR (KBr): 1474, 1606, 1653, 3376 cm^{-1}. Anal. calcd. for $C_{16}H_{12}ClN_3O$: C, 64.54; H, 4.06; Cl, 11.91; N, 14.11, found: C, 64.35; H, 3.98; N, 14.23.

*2-{2-[(5-(4-Nitrophenyl)furan-2-yl)methylene]hydrazinyl}-pyridine (**4b**)*
A: 5-(4-Nitrophenyl)furfural (0.50 g). B: 2-Hydrazinopyridine (0.25 g). Orange crystals from ethanol; yield: 76%; mp 242–244°C. ^{1}H NMR (DMSO-d_6): $\delta = 7.08$–7.20 (m, 1H, H-3 furan, H-5 pyridine), 7.37 (d, 1H, $J = 9.0$, H-3 pyridine), 7.53 (d, 1H, $J = 3.4$, H-4 furan), 8.01–8.42 (m, 7H, H-4,6 pyridine, H-3,5, 4-nitrophenyl, =CH–), 13.60 (s, 1H, NH). IR (KBr): 1329, 1506, 1595, 1650 cm^{-1}. Anal. calcd. for $C_{16}H_{12}N_4O_3$: C, 62.33; H, 3.92; N, 18.17, found: C, 62.10; H, 4.13; N, 18.02.

*1-Phenyl-2-[(5-phenylthiophen-2-yl)methylene]-hydrazine (**5a**)*
A: 5-Phenylthiophene-2-carboxaldehyde (0.43 g). B: Phenylhydrazine (0.25 g). Yellow crystals from ethanol; yield: 79%; mp 166–168°C. ^{1}H NMR ($CDCl_3$): $\delta = 6.82$–7.23 (m, 3H, H-4 phenylhydrazine, H-3,4 thiophene), 7.26–7.50 (m, 8H, H-2,3,5,6 phenylhydrazine, =CH–, H-3,4,5 phenylthiophene), 7.67 (d, 2H, $J = 7.6$, H-2,6 phenylthiophene). IR (KBr): 1493, 1602, 3312 cm^{-1}. Anal. calcd. for $C_{17}H_{14}N_2S$: C, 73.35; H, 5.07; N, 10.06; S, 11.52; found: C, 73.06; H, 5.15; N, 9.93; S, 11.24.

*1-Methyl-1-phenyl-2-[(5-phenylthiophen-2-yl)methylene]-hydrazine (**5b**)*
A: 5-Phenylthiophene-2-carboxaldehyde (0.43 g). B: 1-Methyl-1-phenylhydrazine (0.28 g). Yellow crystals from ethanol; yield: 87%; mp 114–116°C. ^{1}H NMR ($CDCl_3$): $\delta = 3.46$ (s, 3H, N–CH_3), 7.04 (t, 1H, $J = 7.4$, H-4 phenylhydrazine), 7.15 (d, 1H, $J = 3.2$, H-3 thiophene), 7.24–7.57 (m, 8H, H-4 thiophene, H-2,3,5,6 phenylhydrazine, H-3,4,5 phenylthiophene), 7.69 (d, 2H, $J = 8.4$, H-2,6 phenylthiophene), 7.89 (s, 1H, =CH). IR (KBr): 1371, 1500, 1595 cm^{-1}. Anal. calcd. for $C_{18}H_{16}N_2S$: C, 73.94; H, 5.52; N, 9.58; S, 10.97, found: C, 73.60; H, 5.41; N, 9.69; S, 10.63.

*1-(4-Bromophenyl)-2-[(5-phenylthiophen-2-yl)methylene]-hydrazine (**5c**)*
A: 5-Phenylthiophene-2-carboxaldehyde (0.43 g). B: 4-Bromophenylhydrazine (0.51 g). Yellow brownish crystals from toluene; yield: 57%; mp 163–165°C. ^{1}H NMR ($CDCl_3$): $\delta = 6.92$–7.17 (m, 3H, H-3 thiophene and H-2,6 phenylhydrazine), 7.26 (d, 1H, $J = 3.8$, H-4 thiophene), 7.34–7.58 (m, 5H, H-3,5 phenylhydrazine, H-3,4,5 phenylthiophene), 7.67 (d, 2H, $J = 6.8$, H-2,6, phenylthiophene), 7.86 (s, 1H, =CH–). ^{13}C NMR ($CDCl_3$): $\delta = 111.0$ (C-4 phenylhydrazine), 113.3 (2C-2,6 phenylhydrazine), 122.1 (2C-2,6 phenyl), 124.6 (2C-3,5 phenylthiophene), 126.7 (C-2 thiophene), 126.8 (C-4 phenylthiophene), 127.9 (C-3 thiophene), 131.0 (2C-3,5 phenylhydrazine), 131.8 (C-1 phenylthiophene), 133.0 (C-1 thiophene), 138.2 (C-4 thiophene), 142.3 (C-1 phenylhydrazine), 143.8 (=CH–). IR (KBr): 1252, 1491, 1593, 3312 cm^{-1}. Anal. calcd. for $C_{17}H_{13}BrN_2S$: C, 57.15; H, 3.67; Br, 22.37; N, 7.84; S, 8.98, found: C, 57.30; H, 3,77; N, 7.48.

*1-(4-Methoxyphenyl)-2-[(5-phenylthiophen-2-yl)-methylene]hydrazine (**5d**)*
A: 5-Phenylthiophene-2-carboxaldehyde (0.43 g). B: 4-Methoxyphenylhydrazine (0.40 g). Orange brownish crystals from toluene; yield: 63%; mp 146–148°C. ^{1}H NMR ($CDCl_3$): $\delta = 3.80$ (s, 3H, CH_3O), 6.81–6.98 (m, 3H, H-3 thiophene and H-2,6 phenylhydrazine), 7.25 (d, 1H, $J = 3.8$, H-4 thiophene), 7.30–7.55 (m, 5H, H-3,5 phenylhydrazine, H-3,4,5 phenylthiophene), 7.60–7.73 (m, 3H, H-2,6, phenylthiophene and =CH–). IR (KBr): 1236, 1505, 1520, 3311 cm^{-1}. Anal. calcd. for $C_{18}H_{16}N_2OS$: C, 70.10; H, 5.23; N, 9.08; S,10.40, found: C, 74.22; H, 5.05; N, 8.85; S, 9.43.

*1-[(5-Phenylthiophen-2-yl)methylene]-2-(4-trifluoromethyl)phenyl)hydrazine (**5e**)*
A: 5-Phenylthiophene-2-carboxaldehyde (0.43 g). B: 4-(Trifluoromethyl)phenylhydrazine (0.41 g). Yellow crystals from ethanol; yield: 89%; mp 166–167°C. ^{1}H NMR ($CDCl_3$): $\delta = 6.95$ (d, 1H, $J = 3.8$, H-3 thiophene), 7.16 (d, 2H, $J = 7.8$, H-2,6 phenylhydrazine), 7.27 (d, 1H, $J = 3.8$, H-4 thiophene), 7.35–7.60 (m, 5H, H-3,5 phenylhydrazine, H-3,4,5 phenylthiophene), 7.68 (d, 2H, $J = 7.0$, H-2,6, phenylthiophene), 7.91 (s, 1H, =CH–). IR (KBr): 1334, 1536, 1616, 3318 cm^{-1}. Anal. calcd. for $C_{18}H_{13}F_3N_2S$: C, 62.42; H, 3.78; F, 16.46; N, 8.09; S, 9.26; found: C, 62.63; H, 3,61; N, 7.90; S, 9.03.

*N′-{[5-(4-Chlorophenyl)furan-2-yl]methylene)-benzohydrazide (**6a**)*
A: 5-(4-Chlorophenyl)furfural (0.48 g). B: Benzhydrazide (0.31 g). Pale yellow crystals from toluene; yield: 60%; mp 218–220°C. ^{1}H NMR ($CDCl_3$): $\delta = 6.69$ (d, 1H, $J = 3.4$, H-3 furan), 7.80–7.97 (m, 2H, H-4 furan, H-4 benzoyl), 7.25–7.70 (m, 6H, H-3,5 benzoyl, H-2,3,5,6 4-chlorophenyl), 7.83–8.08 (m, 3H, H-2,6, benzoyl, =CH–), 11.40 (s, 1H, NH). IR (KBr): 1281, 1564, 1652, 3022 cm^{-1}. Anal. calcd. for $C_{18}H_{13}ClN_2O_2$: C, 66.57; H, 4.03; N, 8.63, found: C, 66.21; H, 4.08; N, 8.66.

*N′-{[5-(4-Nitrophenyl)furan-2-yl]methylene}-benzohydrazide (**6b**)*
A: 5-(4-Nitrophenyl)furfural (0.50 g). B: Benzhydrazide (0.31 g). Yellow crystals from toluene; yield: 90%; mp 261–263°C. ^{1}H NMR (DMSO-d_6): $\delta = 7.18$ (d, 1H, $J = 3.8$, H-3 furan), 7.48–7.63 (m, 4H, H-4 furan, H-3,4,5 benzoyl), 7.94 (d, 2H, $J = 7.6$, H-2,6 benzoyl), 8.06 (d, 2H, $J = 8.9$, H-2,6, 4-nitrophenyl), 8.34 (d, 2H, $J = 8.9$, H-3,5 4-nitrophenyl), 8.47 (s, 1H, =CH–), 11.98 (s, 1H, NH).
^{13}C NMR (DMSO-d_6): $\delta = 112.0$ ppm (C-3 furan), 115.8 (C-4 furan), 124.1 (2C-3,5 nitrophenyl), 124.2 (2C-2,6 benzoyl), 127.2 (2C-2,6 nitrophenyl), 128.1 (C-4 benzoyl), 131.5 (C-1 benzoyl), 132.8 (C-3 benzoyl), 134.7 (C-5 benzoyl), 136.6 (C-2 furan), 145.9 (2C-1,4 nitrophenyl), 151.0 (=CH–), 152.0 (C-5 furan), 162.8 (C=O). IR (KBr): 1332, 1517, 1659, 3095 cm^{-1}. Anal. calcd. for $C_{18}H_{13}N_3O_4$: C, 64.47; H, 3.91; N, 12.53, found: C, 64.22; H, 4.23; N, 12.23.

*N′-{[5-Phenylthiophen-2-yl]methylene)benzohydrazide (**6c**)*
A: 5-Phenylthiophene-2-carboxaldehyde (0.43 g). B: Benzhydrazide (0.31 g). Pale yellow crystals from toluene; yield: 22%; mp 247–248°C. ^{1}H NMR (DMSO-d_6): $\delta = 7.29$–7.60 (m, 8H, H-3,4 thiophene, H-3,4,5 phenylthiophene, H-3,4,5 benzoyl), 7.76 (d, 2H, $J = 7.6$, H-2,6 phenylthiophene), 7.92 (d, 2H, $J = 7.8$, H-2,6 benzoyl), 8.67 (s, 1H, =CH–), 11.95 (s, 1H, NH). IR (KBr): 1288, 1565, 1638, 2995 cm^{-1}. Anal. calcd. for $C_{18}H_{14}N_2OS$: C, 70.56; H, 4.61; N, 9.14; S, 10.47, found: C, 70.24; H, 4.91; N, 8.94; S, 10.79.

*N′-{[5-(4-Chlorophenyl)furan-2-yl]methylene}pyridine-4-carbohydrazide (**7a**)*
A: 5-(4-Chlorophenyl)furfural (0.48 g). B: Isoniazid (0.32 g). Pale yellow crystals from toluene; yield: 51%; mp 248–250°C. ^{1}H NMR (DMSO-d_6): $\delta = 7.15$ (d, 1H, $J = 3.7$, H-3 furan), 7.22 (d, 1H, $J = 3.7$, H-4 furan), 7.56 (d, 2H, $J = 8.6$, H-3,5 4-chlorophenyl), 7.68–7.92 (m, 4H, H-2,6, 4-chlorophenyl and H-3,5, pyridine), 8.40 (s, 1H, =CH–), 8.81 (d, 2H, $J = 5.6$, H-2,6 pyridine), 12.09 (s, 1H, NH). IR (KBr): 1291, 1549, 1658, 3189 cm^{-1}. Anal. calcd. for $C_{17}H_{12}ClN_3O_2$: C, 62.68; H, 3.71; Cl, 10.88; N, 12.90, found: C, 63.22; H, 4.47; N, 12.73.

*N′-{[5-(4-Nitrophenyl)furan-2-yl]methylene}pyridine-4-carbohydrazide (**7b**)*
A: 5-(4-Nitrophenyl)furfural (0.50 g). B: Isoniazid (0.32 g). Yellow crystals from toluene; yield: 45%; mp 207–208°C. ^{1}H NMR (DMSO-d_6): $\delta = 7.22$ (d, 1H, $J = 3.7$, H-3 furan), 7.50 (d, 1H, $J = 3.7$, H-4 furan), 7.84 (d, 2H, $J = 4.8$, H-3,5 pyridine), 8.05 (d, 2H, $J = 8.7$, H-2,6, 4-nitrophenyl), 8.33 (d, 2H, $J = 8.7$, H-3,5, 4-nitrophenyl), 8.43 (s, 1H, =CH–), 8.81 (d, 2H, $J = 4.8$, H-2,6 pyridine), 12.17 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): $\delta = 111.7$ (C-3 furan), 112.0 (2C-3,5 pyridine), 116.4 (C-4 furan), 121.0 (C-3 nitrophenyl), 124.0 (C-5 nitrophenyl), 124.2 (C-2 nitrophenyl), 125.4 (C-6 nitrophenyl), 134.6 (C-1 nitrophenyl), 137.6 (C-2 furan), 139.8 (C-4 pyridine), 145.9 (C-4 nitrophenyl), 149.9 (C-2 pyridine), 150.2 (C-6 pyridine), 152.3 (=CH–), 161.2 (C-5 furan), 178.5 (C=O). IR (KBr): 1344, 1516, 1685, 3117 cm^{-1}. Anal. calcd. for $C_{17}H_{12}N_4O_4$: C, 60.71; H, 3.60; N, 16.66, found: C, 60.96; H, 3.88; N, 16.36.

*N′-[(5-Phenylthiophen-2-yl)methylene]pyridine-4-carbohydrazide (**7c**)*
A: 5-Phenylthiophene-2-carboxaldehyde (0.43 g). B: Isoniazid (0.32 g). Pale yellow crystals from toluene; yield: 12%; mp 240–242°C. ^{1}H NMR (DMSO-d_6): $\delta = 7.31$–7.62 (m, 5H, H-3,4 furan, H-3,4,5 phenyl), 7.74–7.90 (m, 4H, H-2,6 phenyl, H-3,5, pyridine), 8.67 (s, 1H, =CH–), 8.81 (d, 2H, $J = 4.4$, H-2,6 pyridine), 12.09 (s, 1H, NH). IR (KBr): 1302, 1569, 1643, 2978 cm^{-1}. Anal. calcd. for $C_{18}H_{17}N_3OS$: C, 66.85; H, 5.30; N, 12.99; S, 9.91, found: C, 66.69; H, 5.36; N, 13.29; S, 10.29.

Biology

Cell culture
The CF bronchial epithelial cell line CFBE41o-, with stable expression of F508del-CFTR, was obtained by Dr. J. P. Clancy [32]. The cell line was subsequently transfected with the halide-sensitive yellow fluorescent protein (HS-YFP) YFP-H148Q/I152L [33]. The culture medium was minimum essential medium (MEM), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.

For fluorescence assays of CFTR activity, CFBE41o- cells were plated (50000 cells/well) on clear-bottomed 96-well black microplates (Corning Life Sciences, Acton, MA).

Fluorescence assay for CFTR activity
Measurements of CFTR activity were carried out on CFBE41o-cells expressing mutant CFTR and the HS-YFP 48 h after plating on microplates. Twenty-four hours after

plating, the cells were incubated with test compounds at 37°C for 20–24 h. At the time of assay, the cells were washed with PBS (containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$) and stimulated for 30 min with forskolin (20 μM) in the presence of genistein (50 μM). Then the cells were transferred to a microplate reader (FluoStar Galaxy; BMG Labtech GmbH, Offenburg, Germany) for CFTR activity determination. The plate reader was equipped with high quality excitation (HQ500/20X: 500 ± 10 nm) and emission (HQ535/30M: 535 ± 15 nm) filters for YFP (Chroma Technology Corp., Brattleboro, VT). Each assay consisted of a continuous 14-s fluorescence reading with 2 s before and 12 s after injection of an iodide-containing solution (PBS with Cl^- replaced by I^-; final I^- concentration in the well, 100 mM). In parallel experiments, the selective blocker $CFTR_{inh}$-172 [25] was added to the activating cocktail containing forskolin plus genistein. The data were normalized to the initial background-subtracted fluorescence. To determine fluorescence quenching rate (FQR) associated with I^- influx, the final 11 s of the data for each well were fitted with an exponential function to extrapolate initial slope (d*F*/d*t*).

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