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Asymmetric 4-Aryl-1,4-dihydropyridines Potentiate Mutant Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)

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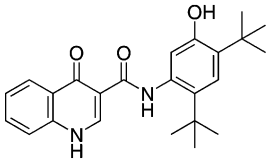
Some of the genetic mutations that cause cystic fibrosis (CF) impair the gating of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- ion channel. This defect can be corrected with pharmacological tools (potentiators) that belong to various chemical families, including the 1,4-dihydropyridines (DHPs). A small set of asymmetric 4-aryl-DHPs was synthesized, and each racemic couple was tested in a functional assay carried out on cells expressing the G1349D, ΔF508, and G551D mutants. The most active racemates were subjected to chiral separation by HPLC, and the pure enantiomers were tested to evaluate any gains in activity. Although three enantiomers demonstrated high potency (K_d values less than 0.09, 0.1, and 0.5 μM in G1349D, ΔF508, and G551D, respectively), in general, the screening of pure enantiomers did not produce a great diversity in potency values. It is probable that the degree of DHP asymmetry considered in our analysis is still insufficient with respect to that allowed in a putative DHP binding site in CFTR, so that the site could equally accommodate both enantiomers.

Introduction

Cystic fibrosis (CF) is a genetic pathology caused by mutations in the cAMP-activated cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- ion channel. Patients mainly suffer from pulmonary infection and inflammation, pancreatic insufficiency, and male sterility.[1,2] CFTR is a very complex plasma membrane protein composed of 1480 amino acids arranged in two transmembrane domains, each containing six α-helices, two nucleotide binding domains (NBD1 and NBD2), and a regulatory domain.[3] At this point, more than 1900 mutations have been discovered, with the deletion of the phenylalanine residue at position 508 (ΔF508 CFTR) being the most common and one of the most severe mutations in CF patients (http://www.genet.sickkids.on.ca/cftr/app).

The severity of disease for each CF patient depends largely on the specific set of mutations that affect the CFTR gene. Depending on the type of molecular defect, researchers have organized the CFTR mutations into five classes: lack of protein synthesis (class I), CFTR protein maturation defect (class II), channel gating defect (class III), decrease in single channel conductance (class IV), and decreased CFTR synthesis (class V).[4–6] Compounds able to repair class II CFTR mutations (among which the most relevant is ΔF508) are defined as "correctors", whereas compounds able to ameliorate the gating defect in class III mutations (which include G551D and G1349D) are defined as "potentiators".[7–10] Among these latter potential therapeutics, great interest has been placed on VX-770,[11,12] a 4-quinolone derivative very recently approved by the USFDA and marketed as Kalydeco (http://www.cff.org/treatments/Pipeline). Notably, when cells with the ΔF508 mutation are incubated at low temperature, the mutant protein may escape from degradation and reach the plasma membrane.[13] However, the "rescued ΔF508 CFTR" has decreased channel activity and low membrane stability.[14] These defects can be corrected by the same potentiators that are effective on G551D and G1349D mutations.[15]



VX-770 (Kalydeco)

The precise mechanism of action of potentiators is still unclear. Mutations of class III allow the normal trafficking of the CFTR protein to the plasma membrane, but impair channel gating by delaying channel opening and decreasing the stability of the open channel conformation.[8,9] Potentiators such as genistein,[16] benzoflavones,[17] phenylglycines,[18] and others are thought to have one or more binding sites at the NBD1/NBD2 level, the occupancy of which by the potentiator molecule leads to partial restoration of channel activity. In this regard, many theoretical studies have been performed, particularly

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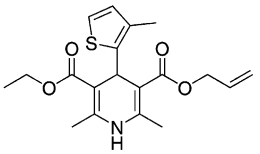
using genistein as a ligand, showing some putative binding sites on NBDs. However, to shed more conclusive light on this difficult and important aspect, additional refinements are necessary.[19–21]

The present work deals with the search for new CF potentiators. Some years ago, in order to find chemical substances useful in treating CF, the anti-hypertensive 4-phenyl-1,4-dihydropyridines (DHPs) nifedipine, felodipine, and nitrendipine were identified as compounds able to stimulate the activity of class III CFTR mutants.[22,23] Unfortunately, CFTR channel activation only becomes apparent at doses higher than those required for the anti-hypertensive activity of DHPs; such activity should be avoided in CF patients.

Recently, the challenge to obtain suitable DHPs endowed with sufficient channel potency, yet free of anti-hypertensive activity, led to the evaluation of 4-thiophenyl-1,4-dihydropyridines[24] and 4-imidazo[2,1-*b*]thiazole-1,4-dihydropyridines[25] as sound and effective potentiators. In particular, in the evaluation of 4-thiophenyl derivatives, we found that more favorable activity was present in some asymmetric DHPs bearing different substituents at the ester level (namely, at positions 3 and 5 of the 1,4-dihydropyridine ring). Among those compounds, a good activity value ($K_d = 0.3$ μM) was observed for the derivative 1,4-dihydro-2,6-dimethyl-4-(3′-methylthiophen-2′-yl)pyridine-3-carboxylic acid allyl ester, 5-carboxylic acid ethyl ester.

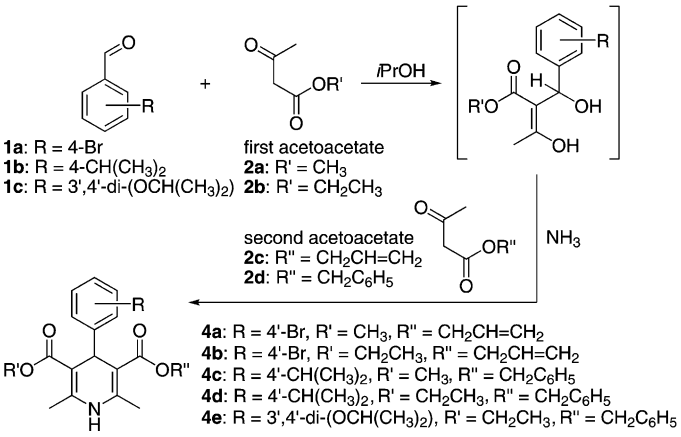


1,4-dihydro-2,6-dimethyl-4-(3'-methylthiophen-2'-yl)pyridine-3-carboxylic acid allyl ester, 5-carboxylic acid ethyl ester

In this work, we set out to evaluate the activity of 4-aryl-DHPs with dissimilar substituents at ester level toward class III mutant CFTR forms. For this purpose, we synthesized a small set of asymmetric DHPs and tested their biological activity initially as racemates; then, the more active DHPs were submitted to chiral chromatography to determine whether the activity could be assigned to a single enantiomer.

Chemistry

The synthesis of the asymmetric dihydropyridines **4a**–**l** was carried out by a modified Hantzsh method. DHPs **4a**–**e** were synthesized in three steps to minimize the formation of symmetric DHPs. Therefore, as shown in Scheme 1, the aromatic aldehydes **1a** (or **1b**, **1c**) were treated with an equimolar amount of the appropriate acetoacetate **2a** (or **2b**) in isopropanol. Upon completion of the first condensation reaction, another equimolar amount of the second acetoacetate **2c** (or **2d**) was added. After this reaction was completed, ammonia was finally added. Following the same procedure, starting from 2-naphthaldehyde **1d** and **2a** (or **2b**) as the first acetoacetate and **2c** (or **2d**, **2e**) as the second acetoacetate, DHPs **4f**–**i** were obtained (Figure 1). Finally, by using aromatic aldehydes



Scheme 1. Synthesis of asymmetric DHPs **4a**–**e** (see the Experimental Section for specific reaction conditions).

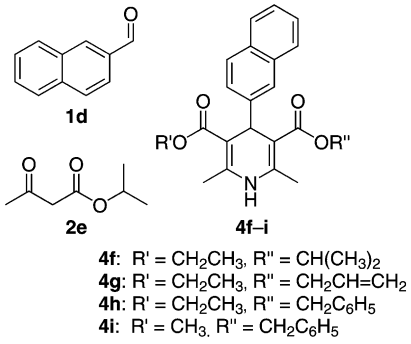
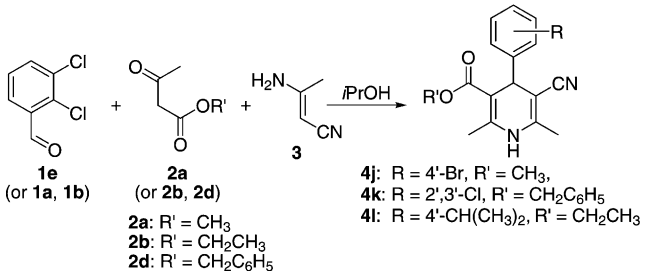


Figure 1. 4-(Naphth-2′-yl)-1,4-dihydropyridines **4f**–**i**.

1e (or **1a**, **1b**), acetoacetate **2a** (or **2b**, **2d**), and aminocrotonate **3**, DHPs **4j**–**l** were synthesized (Scheme 2).

Despite the careful addition of reagents, HPLC analyses of compounds **4a**–**l** also showed the presence of symmetric derivatives, formed as by-products through an unavoidable double reaction of the acetoacetates on a single aldehyde and transesterification reaction. Therefore, the need for several crystallization steps in the purification of asymmetric solid DHPs (and chromatographic purifications for oils) explains the low yields of final products. Compounds **4a**–**l** are white or pale-yellow crystals or pale-yellow oils. The proposed structures were confirmed by spectral data and elemental analyses.



Scheme 2. Synthesis of asymmetric DHPs **4j**–**l** (see the Experimental Section for specific reaction conditions).

Results

Biological results of racemates

Compounds **4a–l** were first tested on Fischer rat thyroid (FRT) cells expressing the G1349D mutation to evaluate, by means of the iodide influx assay, whether the synthesized DHPs could ameliorate the gating defect of mutant CFTR. As shown in Table 1, all the 4-phenyl-DHPs **4a–e** bearing two different carboxyalkyl esters elicited significant effects, whereas 4-naphthyl-DHPs **4f–i** are endowed with moderate activity. Asymmetric DHPs with a carboxyalkyl ester and a cyano group (**4j–l**) are weakly active.

Table 1. Evaluation of G1349D CFTR gating effect by compounds **4a–l** tested as racemates.[a]

Compd	K_d [μM]	E_{max} [ms^{-1}]
4a	0.29 ± 0.13	119 ± 6.3
4b	0.65 ± 0.15	115 ± 7.5
4c	0.072 ± 0.035	117 ± 6.7
4d	0.060 ± 0.017	102 ± 6.9
4e	0.087 ± 0.025	109 ± 7.7
4f	1.30 ± 0.30	112 ± 8.4
4g	0.39 ± 0.12	121 ± 9.6
4h	0.53 ± 0.15	116 ± 8.3
4i	2.94 ± 0.40	113 ± 7.8
4j	4.70 ± 0.80	115 ± 9.1
4k	10.00 ± 1.60	112 ± 7.2
4l	3.90 ± 0.60	114 ± 7.4
felodipine	0.35 ± 0.06	123 ± 8.9

[a] K_d and E_{max} values are the mean ± SEM of n = 5–10 experiments.

Five asymmetric DHPs, namely **4a**, **4c**, **4d**, **4e**, and **4g**, the K_d values of which are all < 0.4 μM (Table 1), were also tested on ΔF508 and G551D mutations (Table 2), and interesting results relative to felodipine were observed, especially in terms of potency (Figure 2A). The values of efficacy between the new DHPs and felodipine are similar. The results obtained with the YFP assay were also confirmed with short-circuit current recordings (Figure 2B). Application of asymmetric DHPs elicited dose-dependent increases in the CFTR-dependent Cl^- current.

Table 2. Evaluation of rescued ΔF508 and G551D CFTR gating effects by selected compounds tested as racemates.[a]

	Rescued ΔF508		G551D	
Compd	K_d [μM]	E_{max} [ms^{-1}]	K_d [μM]	E_{max} [ms^{-1}]
4a	0.67 ± 0.18	25 ± 1.4	1.10 ± 0.20	19 ± 1.3
4c	0.067 ± 0.025	24 ± 1.7	0.60 ± 0.19	18 ± 1.4
4d	0.17 ± 0.09	23 ± 1.5	0.62 ± 0.15	22 ± 1.9
4e	0.27 ± 0.11	22 ± 2.0	0.19 ± 0.05	16 ± 1.2
4g	0.51 ± 0.11	26 ± 2.2	1.80 ± 0.30	21 ± 2.0
felodipine	0.88 ± 0.16	29 ± 1.9	22.60 ± 2.20	21 ± 1.7

[a] K_d and E_{max} values are the mean ± SEM of n = 5–10 experiments.

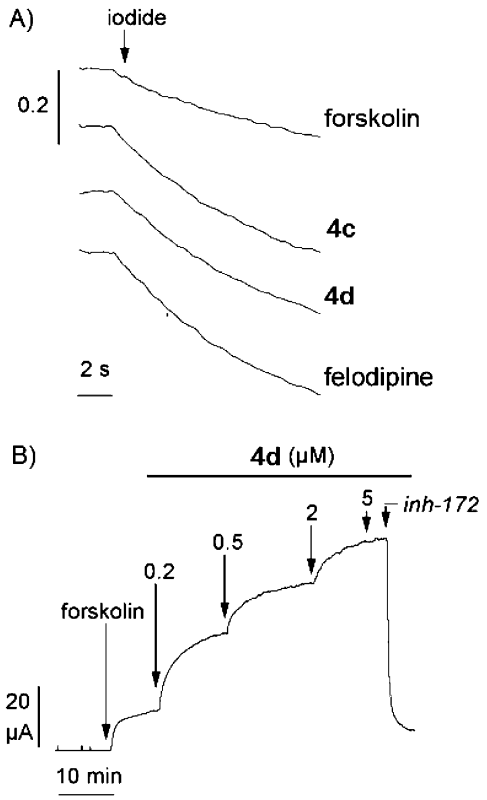


Figure 2. Functional evaluation of asymmetric DHP **4c** and **4d**. A) Representative traces from the YFP assay. FRT cells co-expressing ΔF508 CFTR and the halide-sensitive YFP were stimulated with forskolin (20 μM) alone or with indicated DHPs (2 μM). Addition of iodide-rich buffer caused YFP fluorescence quenching at a rate proportional to CFTR activity. The *y*-axis scale bar represents the fraction of normalized cell fluorescence. B) Representative short-circuit current recording from FRT cells expressing ΔF508 CFTR. Following stimulation with forskolin and with increasing concentrations of compound **4d**, total CFTR current was blocked with CFTR inhibitor-172 (*inh-172*, 10 μM).

Chiral separation of asymmetric DHPs

The above compounds **4a**, **4c**, **4d**, **4e**, and **4g** were subjected to enantiomeric separation by chiral HPLC. For each compound the stationary phase and the eluent composition were optimized to give the best results in peak separation. The stationary phases and eluents used (H = hexane, I = isopropanol) are listed in Table 3 along with retention times (t_R), retention factors (k), enantioselectivity (α), and resolution (Rs). The reported sign of the enantiomers is the sign of the optical rotation at λ 589 nm in chloroform. All experimental details are given in the Experimental Section.

Chiral separation of the above-indicated DHPs gave good results. For instance, Figure 3 shows chromatograms from the chiral separation of **4c**, together with determination of enantiomeric purity of the separated peaks obtained after semi-preparative chromatography. Enantiomeric excesses (%*ee*) were measured by integration of the UV signal at λ 254 nm and confirmed by the circular dichroism (CD) signal.

With samples (~20 mg) of the enantiomers (+)-**4a**, (−)-**4a**, (+)-**4c**, (−)-**4c**, (+)-**4d**, (−)-**4d**, (+)-**4e**, (−)-**4e**, (+)-**4g**, and (−)-**4g** available, the biological tests on FRT cells were repeated. Table 4 presents the results of these five pairs of enantio-

Table 3. Chromatographic data for selected DHPs.

Enantiomer	Column	Eluent[a]	t_{R1}/t_{R2}[b]	k_1/k_2[c]	α[d]	Rs[e]	*ee* [%]
(−)-**4a**	(*S,S*)-Whelk-O1	H/I 7:3	13.3	3.36	1.45	1.16	96
(+)-**4a**			15.0	3.90			95
(+)-**4c**	(*S,S*)-Whelk-O1	H/I 9:1	20.7	12.63	1.26	2.53	94
(−)-**4c**			25.3	15.64			95
(+)-**4d**	(*S,S*)-Whelk-O1	H/I 9:1	23.4	14.38	1.31	2.78	99
(−)-**4d**			29.6	18.47			98
(−)-**4e**	Chiralpak IC	H/I 9:1	11.2	2.60	1.15	1.21	97
(+)-**4e**			12.3	2.98			96
(−)-**4g**	(*S,S*)-Whelk-O1	H/I 7:3	7.9	4.17	1.18	1.26	99
(+)-**4g**			8.7	4.72			98

[a] H = hexane; I = *i*PrOH. [b] Retention time ratio. [c] Retention factor ratio. [d] Enantioselectivity. [e] Resolution.

Table 4. Evaluation of G1349D, rescued ΔF508, and G551D CFTR gating effects by selected DHPs tested as pure enantiomers.[a]

	G1349D		Rescued ΔF508		G551D	
Compd	K_d [μM]	E_{max} [ms^{-1}]	K_d [μM]	E_{max} [ms^{-1}]	K_d [μM]	E_{max} [ms^{-1}]
(+)-**4a**	0.362 ± 0.125	126 ± 9.8	1.0 ± 0.3	29 ± 2.2	1.8 ± 0.40	18 ± 1.6
(−)-**4a**	0.210 ± 0.085	121 ± 8.0	0.5 ± 0.2	28 ± 1.9	0.6 ± 0.20	18 ± 1.5
(+)-**4c**	0.088 ± 0.055	123 ± 8.3	0.16 ± 0.02	28 ± 2.3	1.1 ± 0.30	18 ± 1.1
(−)-**4c**	0.036 ± 0.025	120 ± 7.8	0.082 ± 0.011	27 ± 2.0	0.20 ± 0.04	18 ± 1.0
(+)-**4d**	0.046 ± 0.021	97 ± 6.7	0.061 ± 0.022	28 ± 2.4	0.46 ± 0.10	23 ± 1.9
(−)-**4d**	0.216 ± 0.031	109 ± 8.1	0.187 ± 0.025	25 ± 2.1	0.77 ± 0.12	19 ± 1.6
(+)-**4e**	0.080 ± 0.055	130 ± 10.2	0.09 ± 0.2	30 ± 2.5	0.14 ± 0.04	17 ± 1.6
(−)-**4e**	0.158 ± 0.045	125 ± 11.3	0.25 ± 0.03	29 ± 2.3	0.24 ± 0.08	16 ± 1.1
(+)-**4g**	0.559 ± 0.155	121 ± 9.4	1.4 ± 0.3	28 ± 2.0	2.3 ± 0.5	19 ± 1.2
(−)-**4g**	0.313 ± 0.075	122 ± 7.2	0.48 ± 0.17	30 ± 2.5	1.5 ± 0.4	21 ± 1.8

[a] K_d and E_{max} values are the mean ± SEM of n = 5–10 experiments.

Discussion

Previous works that describe a potential pharmacotherapy for CF stress the importance of small molecules that are able to improve the gating and/or trafficking of mutant CFTR.[26–28] For class III mutations, it was demonstrated that the 4-phenyl-DHPs act as very effective potentiators, as these compounds are able to restore the correct gating process.[22,23] Moreover, potentiators are also thought to be very important therapeutic agents for ΔF508 CFTR, because this mutant also has a gating defect in addition to impaired trafficking.[29]

To improve our knowledge of DHPs as CFTR potentiators, we synthesized a small set of asymmetric DHPs (**4a**–**l**). Such DHPs were prepared with commercial starting materials, and so they do not present great asymmetry because at the ester level we can provide only a small difference between R′ and R″ groups (Scheme 1 and Figure 1); the highest degree of asymmetry is in derivatives **4c** and **4i**, where R′ and R″ are methyl and benzyl groups, respectively. A greater degree of asymmetry would require more complex syntheses, and so may be a future development in this topic.

mers; compounds (−)-**4a**, (−)-**4c**, (+)-**4d**, (+)-**4e**, and (−)-**4g** generally show a slightly higher activity than their enantiomeric counterparts in all cell lines. In particular, the K_d ratios of the above values range from 1.7 to 4.7 for G1349D, from 2.0 to 3.1 for rescued ΔF508, and from 1.5 to 5.5 for G551D.

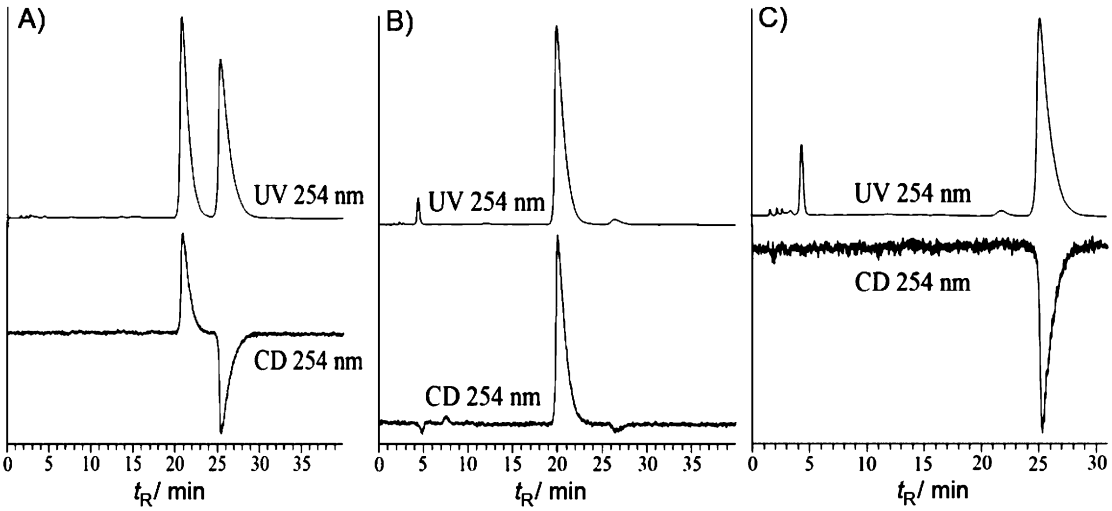


Figure 3. Enantiomeric HPLC separation of **4c** detected by UV and CD: A) analytical separation of **4c**; B) enantiomeric excess after semi-preparative separation of (+)-**4c**; C) enantiomeric excess after semi-preparative separation of (−)-**4c**.

Looking at the structure–activity relationship of the synthesized derivatives, DHPs **4a**–**e** emerged as interesting potentiators when tested as racemates, suggesting the relevance of esters at positions 3 and 5, with a phenyl group present at position 4.[22–24] In particular, compounds **4c**, **4d**, and **4e** show activity at concentrations <0.1 μM in the G1349D cell line, giving evidence, in this small series, for the importance of a branched substituent at 4′. The presence of a benzyl substituent in the ester group also seems to be valuable for this type of activity. On the other hand, 4-naphthyl derivatives **4f**–**i** showed moderate activity (only **4g** has a K_d value lower than 0.4 μM in the G1349D cell line). Cyano derivatives **4j**–**l** were weakly active (Table 1), thus confirming that both esters (at positions 3 and 5) are mandatory for high activity.

Compounds that showed the best values in the G1349D cell line ($K_d<0.4$ μM) were also tested against rescued ΔF508 and G551D mutations, demonstrating high potency (Table 2). For example, **4c**, **4d**, and **4e** have K_d values less than 0.3 and 0.7 μM in rescued ΔF508 and G551D cell lines, respectively. The above compounds show greater potency in all cell lines relative to other DHPs and the reference compound felodipine (Tables 1 and 2). Interestingly, we found that the compounds appear to be more active toward G1349D than toward the ΔF508 mutant. This is not surprising, as these two mutations affect different NBDs (NBD1 for ΔF508 and NBD2 for G1349D); therefore, they may affect the potentiator binding site(s) differently (possibly localized at the NBD1/NBD2 interface). Moreover, emphasis must be placed on the potency values of **4c**, **4d**, and **4e** with respect to felodipine in the G551D cell line: in particular, **4e** shows a gain in potency of about two orders of magnitude (Table 2).

To increase the specificity of the binding, the asymmetric DHPs **4a**, **4c**, **4d**, **4e**, and **4g** were subjected to enantiomeric separation by HPLC using chiral stationary phases. The separation of each compound was initiated by an analytical study to obtain good peak resolution; this was then followed by scaled-up separation with a semi-preparative column. The enantiomeric excess of each collected peak was evaluated by UV and CD spectroscopy. As evident in Figure 3 and in the Experimental Section, all peaks presented an enantiomeric excess between 94 and 99%.

The biological tests on FRT cells were repeated for each separated enantiomer, and the results listed in Table 4 indicate a clear, although not dramatic, increase in activity for compounds (−)-**4a**, (−)-**4c**, (+)-**4d**, (+)-**4e**, and (−)-**4g** with respect to the enantiomeric counterparts in all cell lines. In particular, compounds (−)-**4c** and (+)-**4d** showed K_d values lower than 0.050 and 0.09 μM in G1349D and rescued ΔF508, respectively, whereas compound (+)-**4e** had $K_d<0.15$ μM in G551D. On the whole, (−)-**4c**, (+)-**4d**, and (+)-**4e** can be classified among the most active derivatives of this series and also among the best potentiators identified so far.

Commercial DHPs have anti-hypertensive activity due to the blockade of Ca^{2+} channels, but such activity must be avoided in CF patients. In the present work, we did not test the activity of our DHPs on Ca^{2+} channels on the grounds that previous research with DHPs has demonstrated that the presence of bulky alkyl groups at the 4′-position of the 4-phenyl substituent is sufficient to render such derivatives inefficient as calcium blockers.[30,31] Because our most active derivatives (−)-**4c** and (+)-**4d** have an isopropyl group at 4′, and (+)-**4e** bears an isopropyloxy group at 4′, we hypothesize that these compounds possess negligible anti-hypertensive activity.

Conclusions

The synthesis and biological evaluation of some asymmetric DHPs led to racemates endowed, in some cases, with significant activity as CFTR potentiators. The more interesting racemates were subjected to enantiomeric separation: for specific pure enantiomers (−)-**4c**, (+)-**4d**, and (+)-**4e** an increase in potency was observed. Unfortunately, such increased potency for the enantiomerically pure derivatives is modest relative to the starting racemates; the maximum expected increase in activity would be twice the activity of the racemate if the activity were isolated to a single enantiomer. For this reason, the investment in terms of work and cost for the separation is very high and demanding in view of the obtained results. It is likely that a more advantageous use of enantiomers would be realized by using derivatives with a greater degree of asymmetry than that of our derivatives. In fact, the most potent DHPs, namely (−)-**4c**, (+)-**4d**, and (+)-**4e**, exhibit the greatest asymmetry in our derivatives, because at the ester level a benzyl group and a short alkyl group are present. This hypothesis is consistent with the observation that VX-770 may also be structurally considered as a 1,4-dihydropyridine with a broad asymmetry. For highly asymmetric DHPs, the synthetic procedures could be more elaborate and complex. The generation of highly asymmetric DHPs may be guided by molecular modeling provided that a suitable binding site for DHPs in mutant CFTR is identified.

Experimental Section

Chemistry

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 PerkinElmer 398 spectrophotometer. GC–MS analyses were performed on an HP 6890-5973 spectrometer; GC parameters: injector temperature 250 °C; HP5 poly(methylphenylsiloxane) column 30 m, 0.25 mm, 0.25 μm; temperature profile: from 100 to 300 °C; 10 °C min^{-1}; MS parameters mode SCAN 40–600 Da. 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 asymmetric 1,4-dihydropyridines (4a–i).

To an ice-cooled solution of an aldehyde A (5 mmol) in *i*PrOH (15 mL), the first acetoacetate B (5 mmol) was added, and the mixture was held at reflux for 3 h. The second acetoacetate C

(5 mmol) was then added, and the mixture was again held at reflux for 3 h. At the end, NH_3 (concd, 1 mL) was added, and the mixture was held at reflux for 1 h. After cooling, the solvent was evaporated under reduced pressure, and the crude reaction material obtained was crystallized from suitable solvent or purified by column chromatography on silica gel with toluene/EtOAc (9:1).

1,4-Dihydro-2,6-dimethyl-4-(4′-bromophenyl)pyridine-3-carboxylic acid allyl ester, 5-carboxylic acid methyl ester (4a): (A): 4-bromobenzaldehyde **1a**, (B): methyl acetoacetate **2a**, (C): allyl acetoacetate **2c**; pale yellow oil; yield: 39%, 0.79 g; ^{1}H NMR ($CDCl_3$): δ = 2.24 (s, 3H, CH_3-2), 2.26 (s, 3H, CH_3-6), 3.55 (s, 3H, OCH_3), 4.06 (d, J = 5.4, 2H, $CH_2CH{=}CH_2$) 4.51 (d, J = 9.6, 2H, $CH_2CH{=}CH_2$), 4.84-5.11 (m, 2H, $CH_2CH{=}CH_2$ + H-4), 5.61 (s, 1H, N*H*), 7.01–7.34 ppm (m, 4H, H-arom); ^{13}C NMR ($CDCl_3$): δ = 18.7 ppm (CH_3-2), 18.9 (CH_3-6), 32.2 (C4), 51.2 ($COOCH_3$), 70.3 ($CH_2CH{=}CH_2$), 102.4 (C3), 103.1 (C5), 116.2 (CH_2-$CH{=}CH_2$), 120.1 (C4′), 131.4 (2C-2′,6′), 131.7 (2C-3′,5′), 136.7 (C1′), 145.8 (C2), 146.5 (C6), 166.1, 166.7 ppm (COO); IR (KBr): $\tilde{\nu}$ = 3440, 2951, 2843, 1697, 1619, 1471, 1217 cm^{-1}; MS: *m/z*: 407 (100); Anal. calcd for $C_{19}H_{20}BrNO_4$: C 56.17, H 4.96, N 3.45, Br 19.67, found: C 56.40, H 4.89, N 3.48, Br 19.81.

1,4-Dihydro-2,6-dimethyl-4-(4′-bromophenyl)pyridine-3-carboxylic acid allyl ester, 5-carboxylic acid ethyl ester (4b): (A): 4-bromobenzaldehyde **1a**, (B): ethyl acetoacetate **2b**, (C): allyl acetoacetate **2c**; pale yellow solid; mp: 131–132 °C; yield: 33%, 0.69 g; crystallized from acetone/cyclohexane; ^{1}H NMR ($CDCl_3$): δ = 1.14 (t, J = 7.2, 3H, CH_2CH_3), 2.24 (s, 3H, CH_3-2), 2.25 (s, 3H, CH_3-6), 4.01 (q, J = 7.2, 2H, CH_2CH_3), 4.47 (d, J = 5.0, 2H, $CH_2CH{=}CH_2$), 4.83-5.18 (m, 3H, $CH_2CH{=}CH_2$ + H-4) 5.69-5.93 (m, 2H, $CH_2CH{=}CH_2$ + N*H*), 7.06–7.27 ppm (m, 4H, H-arom); ^{13}C NMR ($CDCl_3$): δ = 13.7 ppm (CH_3CH_2), 18.7 (CH_3-2), 18.9 (CH_3-6), 32.2 (C4), 59.5 (CH_3CH_2), 70.3 ($CH_2CH{=}CH_2$), 102.4 (C3), 103.1 (C5), 116.2 (CH_2-$CH{=}CH_2$), 120.1 (C4′), 131.4 (2C-2′,6′), 131.7 (2C-3′,5′), 136.7 (C1′), 145.8 (C2), 146.5 (C6), 166.1, 166.7 ppm (COO); IR (KBr): $\tilde{\nu}$ = 3357, 2984, 2893, 1695, 1651, 1485, 1210 cm^{-1}; MS: *m/z*: 419 (100); Anal. calcd for $C_{20}H_{22}BrNO_4$: C 57.15, H 5.28, N 3.33, Br 19.01, found: C 56.88, H 5.38, N 3.25, Br 18.84.

1,4-Dihydro-2,6-dimethyl-4-(4′-isopropylphenyl)pyridine-3-carboxylic acid benzyl ester, 5- carboxylic acid methyl ester (4c): (A): 4-isopropylbenzaldehyde **1b**, (B): methyl acetoacetate **2a**, (C): benzyl acetoacetate **2d**; pale yellow oil; yield: 31%, 0.65 g; ^{1}H NMR ($CDCl_3$): δ = 1.11-1.22 (m, 6H, $CH(CH_3)_2$), 2.30 (s, 3H, CH_3-2), 2.33 (s, 3H, CH_3-6), 2.77–2.94 (m, 1H, $CH(CH_3)_2$), 3.57 (s, 3H, OCH_3), 4.90-5.07 (m, 3H, CH_2Ph + H-4), 5.79 (s, 1H, N*H*), 6.83–7.26 ppm (m, 9H, H-arom); ^{13}C NMR ($CDCl_3$): δ = 18.8 (CH_3-2), 18.9 (CH_3-6), 24.4 (CH_3CHCH_3), 31.7 (CH_3CHCH_3), 32.1 (C4), 50.8 ($COOCH_3$), 72.6 (CH_2Ph), 102.5 (C3), 103.0 (C5), 126.2 (2C-3′,5′), 127.3 (2C-2′′,6′′), 127.8 (C4′′), 128.2 (2C-3′′,5′′), 128.9 (2C-2′,6′), 135.1 (C1′), 140.8 (C1′′), 145.7 (C4′), 145.9 (C2), 146.3 (C6), 166.2, 166.9 ppm (COO); IR (KBr): $\tilde{\nu}$ = 3440, 2961, 2871, 1692, 1617, 1470, 1227 cm^{-1}; MS: *m/z* = 419 (100); Anal. calcd for $C_{26}H_{29}NO_4$: C 74.44, H 6.97, N 3.34, found: C 74.17, H 7.06, N 3.44.

1,4-Dihydro-2,6-dimethyl-4-(4′-isopropylphenyl)pyridine-3-carboxylic acid benzyl ester, 5-carboxylic acid ethyl ester (4d): (A): 4-isopropylbenzaldehyde **1b**, (B): ethyl acetoacetate **2b**, (C): benzyl acetoacetate **2d**; pale yellow oil; yield: 42%, 0.91 g; ^{1}H NMR ($CDCl_3$): δ = 1.04–1.23 (m, 9H, $CH(CH_3)_2$ + CH_2CH_3), 2.29 (s, 3H, CH_3-2), 2.31 (s, 3H, CH_3-6), 2.78–2.94 (m, 1H, $CH(CH_3)_2$), 4.03 (q, J = 7.0, 2H, CH_2CH_3), 4.85–5.09 (m, 3H, CH_2Ph + H-4), 5.69 (s, 1H, N*H*), 6.90–7.07 ppm (m, 9H, H-arom); ^{13}C NMR ($CDCl_3$): δ = 13.7 ppm (CH_3CH_2), 18.8 (CH_3-2), 18.9 (CH_3-6), 24.4 (CH_3CHCH_3), 31.7 (CH_3CHCH_3), 32.1 (C4), 59.8 (CH_3CH_2), 72.6 (CH_2Ph), 102.5 (C3), 103.0 (C5), 126.2 (2C-3′,5′), 127.5 (2C-2′′,6′′), 127.7 (C4′′), 128.2 (2C-3′′,5′′), 128.9 (2C-2′,6′), 135.1 (C1′), 140.8 (C1′′), 145.7 (C4′), 145.9 (C2), 146.3 (C6), 166.2, 166.9 ppm (COO); IR (KBr): $\tilde{\nu}$ = 3440, 2963, 2872, 1690, 1618, 1470, 1229 ppm; MS: *m/z* = 433 (100); Anal. calcd for $C_{27}H_{31}NO_4$: C 74.80, H 7.21, N 3.23, found: C 75.03, H 7.29, N 3.16.

1,4-Dihydro-2,6-dimethyl-4-(3′,4′-diisopropoxyphenyl)pyridine-3-carboxylic acid benzyl ester, 5- carboxylic acid ethyl ester (4e): (A): 3,4-diisopropyloxybenzaldehyde **1c**, (B): ethyl acetoacetate **2b**, (C): benzyl acetoacetate **2d**; pale yellow oil; yield: 29%, 0.73 g; ^{1}H NMR ($CDCl_3$): δ = 1.04–1.35 (m, 15H, $CH(CH_3)_2$ + CH_2CH_3), 2.24 (s, 3H, CH_3-2), 2.25 (s, 3H, CH_3-6), 4.02 (q, J = 7.0, 2H, CH_2CH_3), 4.12-4.38 (m, 2H, $CH(CH_3)_2$), 4.90–5.07 (m, 3H, CH_2Ph + H-4), 5.79 (s, 1H, N*H*), 6.29–7.31 (m, 8H, H-arom); ^{13}C NMR ($CDCl_3$): δ = 13.8 ppm (CH_3CH_2), 18.8 (CH_3-2), 18.9 (CH_3-6), 22.4 (CH_3CHCH_3 in 3′), 22.6 (CH_3CHCH_3 in 4′), 32.3 (C4), 59.9 (CH_3CH_2), 70.7 (CH_3CHCH_3 in 3′), 70.8 (CH_3CHCH_3 in 4′), 72.5 (CH_2Ph), 101.7 (C3), 102.3 (C5), 114.7 (C5′), 115.5 (C2′), 121.4 (C6′), 129.9 (C1′), 127.5 (2C-2′′,6′′), 127.7 (C4′′), 128.2 (2C-3′′,5′′), 140.8 (C1′′), 141.5 (C4′), 144.4 (C3′), 145.7 (C2), 146.6 (C6), 166.5, 166.8 ppm (COO); IR (KBr): $\tilde{\nu}$ = 3440, 2980, 2874, 1690, 1618, 1500, 1227 cm^{-1}; MS: *m/z* = 507 (100); Anal. calcd for $C_{30}H_{37}NO_6$: C 70.98, H 7.35, N 2.76, found: C 71.26, H 7.30, N 2.84.

1,4-Dihydro-2,6-dimethyl-4-(naphth-2′-yl)pyridine-3-carboxylic acid ethyl ester, 5-carboxylic acid isopropyl ester (4f): (A): 2-naphthaldehyde **1d**, (B): ethyl acetoacetate **2b**, (C): isopropyl acetoacetate **2e**; white solid; mp: 154–155 °C; yield: 27%, 0.53 g; crystallized from cyclohexane; ^{1}H NMR ($CDCl_3$): δ = 0.96–1.24 (m, 9H, CH_2CH_3 + $CH(CH_3)_2$), 2.26 (s, 3H, CH_3-2), 2.28 (s, 3H, CH_3-6), 4.00 (q, J = 7.2, 2H, CH_2CH_3), 4.77–4.93 (m, 1H, $CH(CH_3)_2$), 5.08 (s, 1H, H-4), 5.70 (s, 1H, N*H*), 7.28-7.74 (m, 7H, H-arom); ^{13}C NMR ($CDCl_3$): δ = 13.5 ppm (CH_3CH_2), 18.6 (CH_3-2), 18.8 (CH_3-6), 22.0 (2C-CH_3CHCH_3), 32.2 (C4), 59.6 (CH_3CH_2), 69.0 (CH_3CHCH_3), 101.6 (C3), 101.9 (C5), 124.2 (C6′), 125.7 (C5′), 126.7 (C2′), 127.1 (C9′), 127.4 (C4′), 127.4 (C7′), 127.7 (C10′), 131.6 (C8′), 133.5 (C3′), 135.2 (C1′), 145.9 (C2), 146.7 (C6), 166.3, 166.7 (COO); IR (KBr): $\tilde{\nu}$ = 3358, 2982, 2906, 1695, 1679, 1484, 1265; MS: *m/z* = 393 (100); Anal. calcd for $C_{24}H_{27}NO_4$: C 73.26, H 6.92, N 3.56, found: C 73.02, H 7.00, N 3.48.

1,4-Dihydro-2,6-dimethyl-4-(naphth-2′-yl)pyridine-3-carboxylic acid allyl ester, 5-carboxylic acid ethyl ester (4g): (A): 2-naphthaldehyde **1d**, (B): ethyl acetoacetate **2b**, (C): allyl acetoacetate **2c**; white solid; mp: 130–131 °C; yield: 30%, 0.58 g; crystallized from acetone/cyclohexane; ^{1}H NMR ($CDCl_3$): δ = 1.15 (t, J = 7.0, 3H, CH_2CH_3), 2.30 (s, 3H, CH_3-2), 2.31 (s, 3H, CH_3-6), 3.98 (q, J = 7.0, 2H, CH_2CH_3), 4.47 (d, J = 5.4, 2H, $CH_2CH{=}CH_2$), 5.01–5.22 (m, 3H, $CH_2CH{=}CH_2$ + H-4), 5.75-5.92 (m, 2H, N*H* + $CH_2CH{=}CH_2$), 7.23–7.75 (m, 7H, H-arom); ^{13}C NMR ($CDCl_3$): δ = 13.5 ppm (CH_3CH_2), 18.6 (CH_3-2), 18.8 (CH_3-6), 32.2 (C4), 59.6 (CH_3CH_2), 70.3 ($CH_2CH{=}CH_2$), 101.8 (C3), 102.4 (C5), 115.1 ($CH_2CH{=}CH_2$), 124.2 (C6′), 125.6 (C5′), 126.7 (C2′), 127.1 (C9′), 127.4 (C4′), 127.5 (C7′), 127.7 (C10′), 131.6 (C8′), 133.5 (C3′), 135.2 (C1′), 137.5 ($CH_2CH{=}CH_2$), 145.9 (C2), 146.7 (C6), 166.3, 166.7 ppm (COO); IR (KBr): $\tilde{\nu}$ = 3340, 2968, 2849, 1701, 1652, 1485, 1210 cm^{-1}; MS: *m/z* = 391 (100); Anal. calcd for $C_{24}H_{25}NO_4$: C 73.64, H 6.44, N 3.58, found: C 73.87, H 6.41, N 3.49.

1,4-Dihydro-2,6-dimethyl-4-(naphth-2′-yl)pyridine-3-carboxylic acid benzyl ester, 5-carboxylic acid ethyl ester (4h): (A): 2-naphthaldehyde **1d**, (B): ethyl acetoacetate **2b**, (C): benzyl acetoacetate **2d**; pale yellow oil; yield: 31%, 0.68 g; ^{1}H NMR ($CDCl_3$): δ = 1.14 (t, J = 7.0, 3H, CH_2CH_3), 2.28 (s, 3H, CH_3-2), 2.30 (s, 3H, CH_3-6), 4.01 (m, 2H, CH_2CH_3), 4.96-5.17 (m, 3H, CH_2Ph + H-4), 5.66 (s, 1H, N*H*), 7.04–7.76 (m, 12H, H arom); ^{13}C NMR ($CDCl_3$): δ = 13.5 ppm (CH_3CH_2), 18.6 (CH_3-2), 18.8 (CH_3-6), 32.2 (C4), 59.6 (CH_3CH_2), 72.6 (CH_2Ph),

101.6 (C3), 101.9 (C5), 124.2 (C6′), 125.6 (C5′), 126.7 (C2′), 127.1 (C9′), 127.3 (2C-2″,6″), 127.5 (2C-4′,4″), 127.7 (C7′), 127.9 (C10′), 128.2 (2C-3″,5″), 131.6 (C8′), 133.5 (C3′), 135.2 (C1′), 140.8 (C1″), 145.9 (C2), 146.7 (C6), 166.3, 166.7 ppm (COO); IR (KBr): $\tilde{\nu}=3401$, 2931, 2891, 1692, 1619, 1471, 1227 cm^{-1}; MS: $m/z=441$ (100); Anal. calcd for $C_{28}H_{27}NO_4$: C 76.17, H 6.16, N 3.17, found: C 75.91, H 6.23, N 3.23.

1,4-Dihydro-2,6-dimethyl-4-(naphth-2′-yl)pyridine-3-carboxylic acid benzyl ester, 5-carboxylic acid methyl ester (4i): (A): 2-naphthaldehyde **1d**, (B): methyl acetoacetate **2a**, (C): benzyl acetoacetate **2d**; pale yellow oil; yield: 30%, 0.64 g; ^{1}H NMR ($CDCl_3$): $\delta=$ 2.29 (s, 3H, CH_3-2), 2.30 (s, 3H, CH_3-6), 3.61 (s, 3H, OCH_3), 4.91-5.17 (m, 3H, CH_2Ph + H-4), 5.68 (s, 1H, N*H*), 7.04–7.78 ppm (m, 12H, H-arom); ^{13}C NMR ($CDCl_3$): $\delta=18.6$ ppm (CH_3-2), 18.8 (CH_3-6), 32.2 (C4), 50.9 (COOCH_3), 72.6 (CH_2Ph), 101.6 (C3), 101.9 (C5), 124.8 (C6′), 125.7 (C5′), 126.7 (C2′), 127.1 (C9′), 127.3 (2C-2″,6″), 127.5 (2C-4′,4″), 127.7 (C7′), 127.9 (C10′), 128.2 (2C-3″,5″), 131.6 (C8′), 133.5 (C3′), 135.2 (C1′), 140.8 (C1″), 145.9 (C2), 146.7 (C6), 166.3, 166.7 ppm (COO); IR (KBr): $\tilde{\nu}=3440$, 2950, 2929, 1694, 1618, 1471, 1216 cm^{-1}; MS: $m/z=427$ (100); Anal. calcd for $C_{27}H_{25}NO_4$: C 75.86, H 5.89, N 3.28, found: C 76.09, H 6.01, N 3.21.

General procedure for the synthesis of asymmetric 1,4-dihydropyridines (4j–l).

To an ice-cooled solution of aldehyde A (5 mmol) in *i*PrOH (15 mL), acetoacetate B (5 mmol) was added, and the mixture was held at reflux for 3 h. Aminocrotonate **3** (5 mmol) was then added, and the mixture was held at reflux again for 3 h. After cooling, the solvent was evaporated under reduced pressure, and the crude reaction material obtained was crystallized from toluene.

1,4-Dihydro-2,6-dimethyl-4-(4′-bromophenyl)-5-cyanopyridine-3-carboxylic acid methyl ester (4j): (A): 4-bromobenzaldehyde **1a**, (B): methyl acetoacetate **2a**; pale yellow solid; mp: 203–204 °C; yield: 19%, 0.33 g; ^{1}H NMR ($CDCl_3$): $\delta=2.00$ (s, 3H, CH_3-2), 2.30 (s, 3H, CH_3-6), 3.85 (s, 3H, OCH_3), 4.53 (s, 1H, H-4), 5.78 (s, 1H, N*H*), 6.92–7.38 ppm (m, 4H, H-arom); ^{13}C NMR ($CDCl_3$): $\delta=18.3$ (CH_3-6), 18.7 (CH_3-2), 31.5 (C4), 51.3 (COOCH_3), 78.2 (C5), 101.9 (C3), 117.2 (CN), 120.1 (C4′), 131.4 (2C-2′,6′), 131.7 (2C-3′,5′), 136.7 (C1′), 145.8 (C2), 149.9 (C6), 166.4 ppm (COO); IR (KBr): $\tilde{\nu}=3316$, 2955, 2196, 1686, 1659, 1500, 1252 cm^{-1}; MS: $m/z=346$ (100); Anal. calcd for $C_{16}H_{15}BrN_2O_2$: C 55.35, H 4.35, N 8.07, Br 23.01, found: C 55.54, H 4.24, N 8.16, Br 22.85.

1,4-Dihydro-2,6-dimethyl-4-(2′,3′-dichlorophenyl)-5-cyanopyridine-3-carboxylic acid benzyl ester (4k): (A): 2,3-dichlorobenzaldehyde **1e**, (B): benzyl acetoacetate **2d**; white solid; mp: 169–170 °C; yield: 48%, 0.99 g; ^{1}H NMR ($CDCl_3$): $\delta=2.00$ (s, 3H, CH_3-2), 2.29 (s, 3H, CH_3-6), 4.96-5.25 (m, 3H, CH_2Ph + H-4), 5.95 (s, 1H, N*H*), 7.02–7.17 (m, 8H, H-arom); ^{13}C NMR ($CDCl_3$): $\delta=18.3$ ppm (CH_3-6), 18.7 (CH_3-2), 25.8 (C4), 72.6 (CH_2Ph), 78.2 (C5), 102.8 (C3), 117.2 (CN), 127.5 (3C-4′,2″,6″), 127.7 (C4″), 127.9 (C5′), 128.2 (3C-6′,3″,5″), 134.1 (C3′), 134.9 (C2′), 139.5 (C1′), 140.8 (C1″), 145.8 (C2), 149.9 (C6), 166.2 (COO); IR (KBr): $\tilde{\nu}=3314$, 2984, 2202, 1682, 1659, 1501, 1249 cm^{-1}; MS: $m/z=412$ (100); Anal. calcd for $C_{22}H_{18}Cl_2N_2O_2$: C 63.93, H 4.39, N 6.78, Cl 17.16, found: C 64.22, H 4.33, N 6.87.

1,4-Dihydro-2,6-dimethyl-4-(4′-isopropylphenyl)-5-cyanopyridine-3-carboxylic acid ethyl ester (4l): (A): 4-isopropylbenzaldehyde **1b**, (B): ethyl acetoacetate **2b**; white solid; mp: 82–83 °C; yield: 40%, 0.65 g; ^{1}H NMR ($CDCl_3$): $\delta=$0.93-1.28 (m, 9H, CH_2CH_3 + $CH(CH_3)_2$), 1.98 (s, 3H, CH_3-2), 2.26 (s, 3H, CH_3-6), 2.67-2.88 (m, 1H, CH(CH_3)$_2$), 3.96 (q, $J=7.0$, 2H, CH_2CH$_3$), 4.48 (s, 1H, H-4), 5.93 (s, 1H, N*H*), 6.97–7.17 ppm (m, 4H, H-arom); ^{13}C NMR ($CDCl_3$): $\delta=13.6$ (CH_3CH_2), 18.3 (CH_3-6), 18.7 (CH_3-2), 24.4 (CH_3CHCH_3), 31.5 (C4), 31.7 (CH_3CHCH_3), 59.6 (CH_3CH_2), 78.2 (C5), 101.9 (C3), 117.2 (CN), 126.2 (2C-3′,5′), 128.9 (2C-2′,6′), 134.9 (C1′), 145.7 (C4′), 145.8 (C2), 149.9 (C6), 166.4 ppm (COO); IR (KBr): $\tilde{\nu}=3022$, 2963, 2203, 1693, 1665, 1480, 1211 cm^{-1}; MS: $m/z=324$ (100); Anal. calcd for $C_{20}H_{24}N_2O_2$: C 74.04, H 7.46, N 8.64, found: C 74.29, H 7.55, N 8.52.

Chiral separation

Compounds **4a**, **4c**, **4d**, **4e**, and **4g** were subjected to chiral separation with the aim of obtaining 10–20 mg of each separated enantiomer to be tested as a potentiator for mutant CFTR. For this reason, samples were initially subjected to analytical separation and then to semi-preparative separation. In this regard, samples were injected several (~50) times until the desired amount of enantiomers was obtained. The reported sign of the enantiomers is the sign of the optical rotation at λ 589 nm in $CHCl_3$. Retention times (t_R) are given in min. The HPLC unit used was a Merck D-7000 System Manager with vacuum pump Merck-Lachrom L-7100 and oven Merck-Lachrom L-7360, UV detector Merck-Lachrom L-7400, CD detector CD-1595. Hexane and *i*PrOH (HPLC grade) were degassed and filtered (0.45 μm) before use.

Separation of 4a: Analytical: column: (*S*,*S*)-Whelk-O1 (250× 4.6 mm); mobile phase: hexane/*i*PrOH 7:3; flow: 1 mL min^{-1}; UV detector set at λ 254 nm. (−)-**4a**: $t_{R1}=13.3$, $k_1=3.36$; (+)-**4a**: $t_{R2}=$ 15.0, $k_2=3.90$, $\alpha=1.45$, $Rs=1.16$. Semi-preparative: column: (*S*,*S*)-Whelk-O1 (250×10 mm); mobile phase: hexane/*i*PrOH 7:3; flow: 5 mL min^{-1}; UV detector set at λ 254 nm. Samples of the first enantiomer were collected from 12.0 to 13.5 min. Samples of the second enantiomer were collected from 14.5 to 17.0 min. First enantiomer (−)-**4a**: 96% *ee*; $\alpha_D^{25}=-18.4$ ($CHCl_3$, $c=1.58$). Second enantiomer (+)-**4a**: 95% *ee*; $\alpha_D^{25}=+19.2$ ($CHCl_3$, $c=1.15$).

Separation of 4c: Analytical: column: (*S*,*S*)-Whelk-O1 (250× 4.6 mm); mobile phase: hexane/*i*PrOH 9:1; flow: 2 mL min^{-1}; UV detector set at λ 254 nm. (+)-**4c**: $t_{R1}=20.7$, $k_1=12.63$; (−)-**4c**: $t_{R2}=$ 25.3, $k_2=15.64$, $\alpha=1.26$, $Rs=2.53$. Semi-preparative: column: (*S*,*S*)-Whelk-O1 (250×4.6 mm); mobile phase: hexane/*i*PrOH 9:1; flow: 2 mL min^{-1}; UV detector set at λ 254 nm. Samples of the first enantiomer were collected from 19.5 to 22.0 min. Samples of the second enantiomer were collected from 25.5 to 30.0 min. First enantiomer (+)-**4c**: 94% *ee*; $\alpha_D^{25}=+12.7$ ($CHCl_3$, $c=0.70$). Second enantiomer (−)-**4c**: 95% *ee*; $\alpha_D^{25}=-12.1$ ($CHCl_3$, $c=0.73$).

Separation of 4d: Analytical: column: (*S*,*S*)-Whelk-O1 (250× 4.6 mm); mobile phase: hexane/*i*PrOH 9:1; flow: 2 mL min^{-1}; UV detector set at λ 254 nm. (+)-**4d**: $t_{R1}=23.4$, $k_1=14.38$; (−)-**4d**: $t_{R2}=$ 25.3, $k_2=18.47$, $\alpha=1.31$, $Rs=2.78$. Semi-preparative: column: (*S*,*S*)-Whelk-O1 (250×4.6 mm); mobile phase: hexane/*i*PrOH 9:1; flow: 2 mL min^{-1}; UV detector set at λ 254 nm. Samples of the first enantiomer were collected from 22.0 to 25.0 min. Samples of the second enantiomer were collected from 25.5 to 33 min. First enantiomer (+)-**4d**: 99% *ee*; $\alpha_D^{25}=+16.7$ ($CHCl_3$, $c=1.0$). Second enantiomer (−)-**4d**: 98% *ee*; $\alpha_D^{25}=-17.0$ ($CHCl_3$, $c=1.0$).

Separation of 4e: Analytical: column: Chiralpak IC (250×4.6 mm); mobile phase: hexane/*i*PrOH 9:1; flow: 1 mL min^{-1}; UV detector set at λ 254 nm. (−)-**4e**: $t_{R1}=11.2$, $k_1=2.60$; (+)-**4e**: $t_{R2}=12.3$, $k_2=$ 2.98, $\alpha=1.15$, $Rs=1.21$. Semi-preparative: column: Chiralpak IC (250×4.6 mm); mobile phase: hexane/*i*PrOH 9:1; flow: 1 mL min^{-1}; UV detector set at λ 254 nm. Samples of the first enantiomer were collected from 10.0 to 11.5 min. Samples of the second enantiomer were collected from 12.5 to 14.0 min. First enantiomer (−)-**4e**:

97% *ee*; $\alpha_D^{25} = -18.7$ ($CHCl_3$, $c = 1.0$). Second enantiomer (+)-**4e**: 96% *ee*; $\alpha_D^{25} = +14.5$ ($CHCl_3$, $c = 1.1$).

Separation of 4g: Analytical: column: (*S*,*S*)-Whelk-O1 (250× 4.6 mm); mobile phase: hexane/*i*PrOH 7:3; flow: 2 mL min^{-1}; UV detector set at λ 254 nm. (−)-**4g**: $t_{R1} = 7.9$, $k_1 = 4.17$; (+)-**4g**: $t_{R2} = 8.7$, $k_2 = 4.72$, $\alpha = 1.18$, $Rs = 1.26$. Semi-preparative: column: (*S*,*S*)-Whelk-O1 (250×10 mm); mobile phase: hexane/*i*PrOH 9:1; flow: 5 mL min^{-1}; UV detector set at λ 254 nm. Samples of the first enantiomer were collected from 7.0 to 8.5 min. Samples of the second enantiomer were collected from 9.0 to 10.5 min. First enantiomer (−)-**4g**: 99% *ee*; $\alpha_D^{25} = -24.7$ ($CHCl_3$, $c = 1.2$). Second enantiomer (+)-**4g**: 98% *ee*; $\alpha_D^{25} = +24.2$ ($CHCl_3$, $c = 1.0$).

Biology

CFTR assays

Cell culture: Fischer rat thyroid (FRT) cells, stably transfected with G1349D, G551D, or ΔF508 CFTR and the halide-sensitive yellow fluorescent protein YFP-H148Q/I152L[32] were cultured in Coon's modified Ham's F-12 medium supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin (100 U mL^{-1}), and streptomycin (100 μg mL^{-1}). For fluorescence assays of CFTR activity, cells were plated (10^5 cells per well) on clear-bottomed 96-well microplates (Corning Life Sciences, Acton, MA, USA).

Samples: Synthesized compounds **4a**–**l** were dissolved in DMSO, and for each compound a stock solution (10 mM) was prepared. Secondary plates were prepared for screening at 1 mM in DMSO using a Biomek 2000 liquid handling workstation (Beckman Coulter, Fullerton, CA, USA). All plates were stored at −70 °C.

Fluorescence assay for CFTR activity: Measurements of CFTR activity were carried out on FRT cells expressing G1349D, G551D, or ΔF508 CFTR and the halide-sensitive YFP 48 h after plating on microplates. The 96-well microplates containing FRT cells expressing ΔF508 CFTR and the halide-sensitive YFP were incubated at 27 °C for 20–24 h to allow rescue of the mutant protein to the plasma membrane. At the time of assay, cells were washed with PBS [NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (8.1 mM), KH_2PO_4 (1.5 mM), $CaCl_2$ (1 mM), and $MgCl_2$ (0.5 mM)], and stimulated for 30 min with forskolin (20 μM) in order to maximally enhance intracellular cAMP levels and allow CFTR phosphorylation, and to test compounds at the desired concentration. Cells were then transferred to a microplate reader (FluoStar Galaxy; BMG Labtech GmbH, Offenburg, Germany) for CFTR activity determination. The plate reader was equipped with excitation (HQ500/20X: 500±10 nm) and emission (HQ535/30M: 535±15 nm) filters for YFP (Chroma Technology Corp., Brattleboro, VT, USA). Each assay consisted of a continuous 14 s fluorescence reading: 2 s before and 12 s after injection of an iodide-containing solution (PBS with chloride replaced by iodide; final iodide concentration in the well: 100 mM). Data were normalized to the initial background-subtracted fluorescence. To determine the rate of iodide influx, the final 10 s of the data for each well were fitted with an exponential function to extrapolate the initial slope (dF/dt).

Short-circuit current recordings: To confirm the results obtained with the YFP assay, the most potent asymmetric DHPs were tested in short-circuit current recordings on FRT cells expressing ΔF508 CFTR as previously described.[22] Briefly, cells were plated at high density on porous membranes (Snapwell, Corning). After 4–5 days, the cells were incubated for 20–24 h at 27 °C to rescue ΔF508 CFTR. To measure CFTR activity, Snapwell supports were mounted in Ussing-chamber-like systems in the presence of a Cl^- gradient (65 mM apical/130 mM basolateral). The trans-epithelial electrical potential difference was clamped at zero with a DVC-1000 voltage-clamp amplifier (World Precision Instruments) via Ag/AgCl electrodes. The resulting short-circuit current was digitized with an analog/digital converter (PowerLab 4/25, ADInstruments).

Data analysis: Data for all experiments are presented as representative traces or as mean values ± SEM. Statistical analyses were performed with Student's *t* test for unpaired data. Values for E_{max} and K_d were obtained by fitting dose–response data with the Hill equation. The activity elicited by forskolin (20 μM) alone was subtracted from the E_{max} value. Felodipine properties are reported for comparison.

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Keywords: asymmetric 1,4-dihydropyridines · chiral separation · cystic fibrosis transmembrane conductance regulator (CFTR) · potentiators

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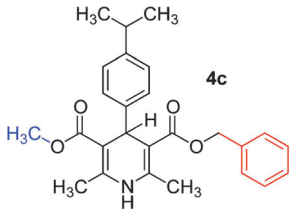
Asymmetric 4-Aryl-1,4-dihydropyridines Potentiate Mutant Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)



The racemate for a cure: 1,4-Dihydropyridines (DHPs) are already known as potential drugs for cystic fibrosis caused by class III mutations. Asymmetric DHPs were synthesized and tested as racemates on Fischer rat thyroid cells expressing three mutations. Some DHPs are effective potentiators at the nanomolar level. The most active DHPs (e.g., **4c**) were subjected to chiral separation and retested, but the resulting difference in activity between enantiomers was modest.

