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Synthesis of 4-thiophen-2′-yl-1,4-dihydropyridines as potentiators of the CFTR chloride channel

Francesca Cateni [a], Marina Zacchigna [a], Nicoletta Pedemonte [b], Luis J. V. Galietta [b], Marco T. Mazzei [c], Paola Fossa [d], Michele Giampieri [d], Mauro Mazzei [d,*]

[a] Department of Pharmaceutical Sciences, Piazzale Europa 1, 34127 Trieste, Italy
[b] Molecular Genetics Laboratory, Giannina Gaslini Institute, Largo Gerolamo Gaslini 5, 16147 Genova, Italy
[c] Department of Experimental Medicine, Viale Benedetto XV 1, 16132 Genova, Italy
[d] Department of Pharmaceutical Sciences, Viale Benedetto XV 3, 16132 Genova, Italy

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ABSTRACT

The gating of the CFTR chloride channel is altered by a group of mutations that cause cystic fibrosis. This gating defect may be corrected by small molecules called potentiators. Some 1,4-dihydropyridine (DHP) derivatives, bearing a thiophen-2-yl and a furanyl ring at the 4-position of the nucleus, were prepared and tested as CFTR potentiators. In particular, we evaluated the ability of novel DHPs to enhance the activity of the rescued ΔF508-CFTR as measured with a functional assay based on the halide-sensitive yellow fluorescent protein. Most DHPs showed an effect comparable to or better than that of the reference compound genistein. The potency was instead significantly improved, with some compounds, such as **3g, 3h, 3n**, **4a**, **4b**, and **4d**, having a half effective concentration in the submicromolar range. CoMFA analysis gave helpful suggestions to improve the activity of DHPs.

1. Introduction

About 55,000 people in Europe and the US are affected by cystic fibrosis (CF), an autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene.[1,2] CF is characterized by abnormalities of chloride transport in many organs (pancreas, lungs, intestine, etc.). In particular, the defective chloride transport in the lungs causes airway surface dehydration with the production of an abnormally thick, sticky mucus that helps to initiate a cycle of lung inflammation and infection. This fact ultimately leads to respiratory failure in most patients.

The increasing knowledge regarding CFTR function and structure is helping to discover small molecules able to correct the CF basic defect, that is, the defective chloride transport. These molecules may be the basis for a specific pharmacotherapy of CF.[3,4]

In this regard, the search for novel drugs to treat CF has to take into account the large number of mutations affecting the gene and the variety of molecular mechanisms through which these mutations cause the functional defect. For instance, deletion of phenylalanine at position 508 (ΔF508), the most frequent mutation (occurring in more than 50–70% of patients),[5] causes both a severe CFTR processing defect (the protein being trapped and degraded at the endoplasmic [ER] level) and a decrease of its channel activity.[6–8] Other mutations, like G551D and G1349D, produce only a gating defect. Compounds able to deliver the ΔF508-CFTR from the ER to plasma membrane are called 'correctors' whereas compound able to increase the channel activity are called 'potentiators'. Generally, correctors and potentiators have been identified by high-throughput screening (HTS) of large compound libraries.[9–12]

CF patients homozygote for ΔF508 mutation do not have the ΔF508-CFTR in the plasma membrane but, in vitro, the mutant protein may be helped to escape the endoplasmic reticulum and reach the plasma membrane by incubating the cells at low temperature (27 °C).[13] Under this condition, the 'rescued' ΔF508-CFTR protein respond to potentiators with a significant increase in activity and, therefore, in anion transport.

The treatment of CF patients carrying the ΔF508 mutation probably requires two drugs: a corrector to transport the CFTR from the ER to the plasma membrane, and a potentiator to improve the gating activity.

In this regard, we have found that the 1,4-dihydropyridines (DHPs) used to treat hypertension are acting as potentiators and so are able to stimulate the activity of some CFTR mutants (in particular, G551D, G1349D and rescued ΔF508-CFTR).[14,15] Antihypertensive DHPs act by blocking L-type voltage-dependent Ca^{2+} channels (L-VDCC) and therefore cause the relaxation of arterial

* Corresponding author. Tel.: +39 010 3538371; fax: +39 010 3538358.
E-mail address: mauro.mazzei@unige.it (M. Mazzei).

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smooth muscle cells.[16] We have obtained evidence that mutant CFTR is activated by DHPs through a mechanism not involving the modulation of Ca^{2+} channels, but by a probable direct interaction with the CFTR protein itself.[14]

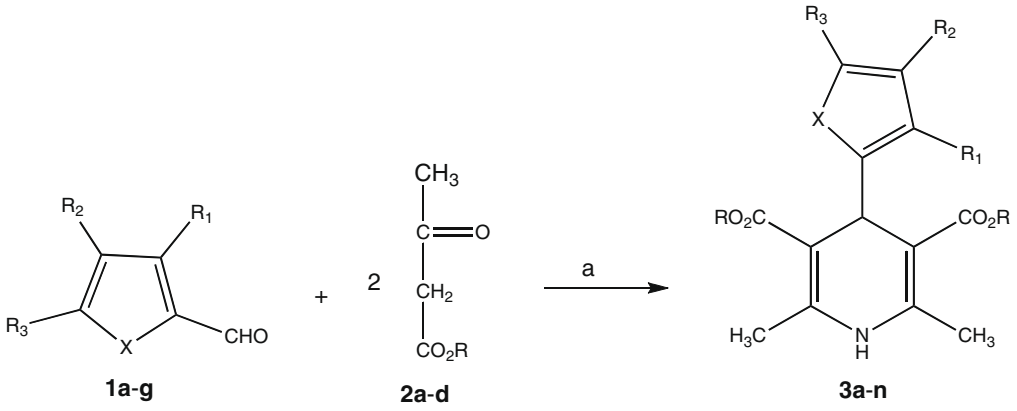
A more recent study, has shown the possibility to generate DHPs with enhanced selectivity towards CFTR relative to calcium channels.[15] To further elucidate the structure–activity relationships of DHPs as CFTR potentiators, we have now analyzed novel DHP derivatives in which the phenyl substituent in position 4 is replaced by heterocycles like thiophene and furan. The new DHP derivatives have shown a significant efficacy, comparable to the reference compound genistein, and improved potency. The activity on Ca^{2+} channels was in general similar to that one of felodipine, with some important differences.

Finally, the CoMFA investigation on the synthesized compounds gives some helpful suggestions to ameliorate the design of new potentially active DHPs for a possible CF therapy.

2. Chemistry

The synthesis of the symmetrical dihydropyridines **3a–n** was accomplished by the Hantzsh method[17] as shown in Scheme 1, starting from the corresponding aldehydes **1a–g** and the appropriate acetoacetate **2a–d** in the presence of ammonia.

For the synthesis of asymmetrical DHPs we slightly changed the above method to avoid the formation of symmetrical DHPs. In this regard, the synthesis begins by reacting the aldehyde with the first acetoacetate. When the condensation reaction is over, the second



	X	R_1	R_2	R_3
1a	S	H	H	NO_2
1b	S	H	H	Me
1c	S	Me	H	H
1d	S	H	H	H
1e	O	H	H	Me
1f	S	H	Ph	H
1g	S	SCH_2CO_2Me	H	H

	R	X	R_1	R_2	R_3
3a	Me	S	H	H	NO_2
3b	Me	S	H	H	Me
3c	Me	S	Me	H	H
3d	Me	S	H	H	H
3e	Et	S	Me	H	H
3f	Me	O	H	H	Me
3g	CH_2-CH=CH_2	S	Me	H	H
3h	CH_2Ph	S	Me	H	H
3i	CH_2-CH=CH_2	S	H	H	NO_2
3j	CH_2Ph	S	H	H	NO_2
3k	CH_2-CH=CH_2	S	H	Ph	H
3l	CH_2Ph	S	H	Ph	H
3m	CH_2-CH=CH_2	S	SCH_2CO_2Me	H	H
3n	CH_2Ph	S	SCH_2CO_2Me	H	H

	R
2a	Me
2b	Et
2c	CH_2-CH=CH_2
2d	CH_2Ph

Scheme 1. Reagents and conditions: (a) NH_3, MeOH, reflux, 6 h.

acetoacetate is added and, finally, ammonia is added, as shown in Scheme 2.

Compounds **3a**–**l** and **4a**,**b** are white or pale yellow crystals. Compounds **3m**,**n** and **4c**,**d** are oils. All structures were confirmed by spectral data and elemental analyses.

3. Experimental protocols

Nuclear magnetic resonance spectra were recorded with a Varian Gemini 200 MHz spectrometer. ^{13}C NMR: 90.5 MHz, Gemini 200 spectrometer. NMR spectra were obtained by using $CDCl_3$ as solvent; chemical shifts are expressed as δ units (ppm) relative to tetramethylsilane (TMS) as internal standard. The abbreviations s, d, t, m and sb refer to singlet, doublet, triplet, multiplet and singlet broad signal, respectively. The EI-MS spectra were measured with a VG-ZAB 2F spectrometer. The ionizing energy was 70 eV in all cases and compounds were introduced by direct insertion. Elemental analyses were carried out on a Carlo Erba model 1016 analyzer. The column chromatography was performed by using silica gel (Kieselgel 60, 230–400 Mesh, 60 Å Merck). TLC: Kieselgel 60 F_{254} (20 × 20 cm; 0.2 mm, Merck). Melting points were determined on a Büchi 510 micromelting point apparatus and are uncorrected. Reagents used were AR grade and all solvents for synthesis, extraction and column chromatography were distilled and dried before use.

3.1. General procedure for the synthesis of symmetrical 1,4-dihydropyridines (3a–n)

To an ice-cooled solution of 5 mmol of aldehyde **1a**–**g** and 12 mmol of acetoacetate **2a**–**d** in 15 ml of dry methanol were added dropwise 0.7 ml of concd ammonia. The ice bath was removed, the mixture was allowed to stir at room temperature for 15 min and then refluxed for 6 h. After cooling, the solvent was evaporated and the crude reaction material obtained was purified by chromatography on silica gel with petrol ether/AcOEt (7:3).

3.1.1. 1,4-Dihydro-2,6-dimethyl-4-(5′-nitro-thiophen-2′-yl)-pyridine-3,5-dicarboxylic acid dimethyl ester (3a)

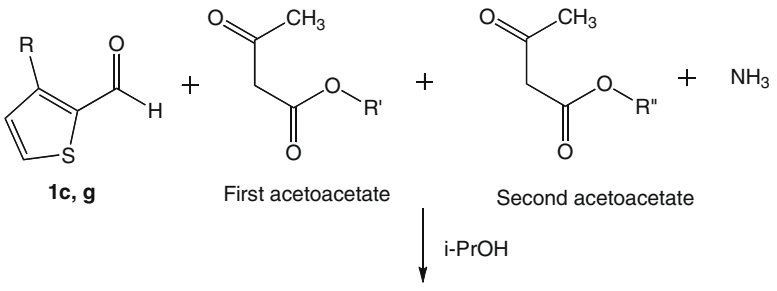
Prepared from 5 mmol of **1a** and 12 mmol of methyl acetoacetate **2a**. The crude reaction material obtained was purified by chromatography on silica gel with petrol ether/AcOEt (7:3) to give the compound **3a** as pale yellow microcrystalline precipitate: mp 155–156 °C; yield 70%.

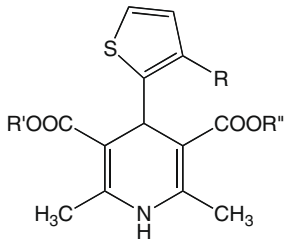
^1H NMR ($CDCl_3$): δ = 2.40 ppm (s br; 6H, 2CH_3-2,6), 3.78 (s br; 6H, 2COOCH_3), 5.35 (s; 1H, H-4), 5.90 (s br; 1H, N*H*), 6.80 (d; 1H, *J* = 4.15 Hz, H-3′), 7.77 (d; 1H, *J* = 4.17 Hz, H-4′). ^{13}C NMR ($CDCl_3$): δ = 19.6 ppm (2CH_3-2,6), 35.4 (C-4), 51.0 (COOCH_3), 102.3 (C-3, C-5), 123.0 (C-3′), 128.9 (C-4′), 145.8 (C-2, C-6), 148.5 (C-2′), 150.0 (C-5′), 165.9 (2*C*OO). EI-MS (70 eV): *m*/*z* = 352 (M, 20%)$^+$, 337 (M−15, 18%)$^+$, 321 (M−31, 22%)$^+$, 293 (M−59, 100%)$^+$. Anal. Calcd for $C_{15}H_{16}O_6N_2S$: C, 50.91; H, 4.54; N, 7.95; S, 9.09. Found: C, 50.91; H, 4.39; N, 8.02; S, 9.15.

3.1.2. 1,4-Dihydro-2,6-dimethyl-4-(5′-methyl-thiophen-2′-yl)-pyridine-3,5-dicarboxylic acid dimethyl ester (3b)

Prepared from 5 mmol of **1b** and 12 mmol of methyl acetoacetate **2a**. The crude reaction material obtained was purified by chromatography on silica gel with petrol ether/AcOEt (7:3) to give the compound **3b** as pale yellow microcrystalline precipitate: mp 179–180 °C; yield 75%.

^1H NMR ($CDCl_3$): δ = 2.30 ppm (s; 3H, CH_3-5′), 2.40 (s br; 6H, 2CH_3-2,6), 3.79 (s br; 6H, 2COOCH_3), 5.34 (s; 1H, H-4), 5.89 (s br; 1H, N*H*), 6.62 (d; 1H, *J* = 4.5 Hz, H-3′), 6.63 (d; 1H, *J* = 4.51 Hz, H-





4a-d

4a: R= CH_3, R'= allyl, R"= ethyl

4b: R = CH_3, R'= benzyl, R"= ethyl

4c: R = SCH_2COOCH_3, R' = allyl, R" = ethyl

4d: R = SCH_2COOCH_3, R' = benzyl, R" = ethyl

Scheme 2.

4′). ^{13}C NMR ($CDCl_3$): δ = 14.2 ppm (*C*H_3-5′), 19.4 (2*C*H_3-2,6), 33.4 (C-4), 51.2 (COO*C*H_3), 104.0 (C-3, C-5), 127.3 (C-3′, C-4′), 137.0 (C-5′), 137.5 (C-2′), 146.1 (C-2, C-6), 166.2 (2*C*OO). EI-MS (70 eV): *m/z* = 321 (M, 28%)$^+$, 306 (M−15, 21%)$^+$, 290 (M−31, 14%)$^+$, 262 (M−59, 93%)$^+$, 224 (M−C_5H_5S, 100%)$^+$, 165 (M−C_5H_5S−59, 9%)$^+$. Anal. Calcd for $C_{16}H_{19}O_4NS$: C, 59.81; H, 5.92; N, 4.36; S, 9.97. Found: C, 59.73; H, 5.90; N, 4.29; S, 9.81.

3.1.3. 1,4-Dihydro-2,6-dimethyl-4-(3′-methyl-thiophen-2′-yl)-pyridine-3,5-dicarboxylic acid dimethyl ester (3c)

Prepared from 5 mmol of **1c** and 12 mmol of methyl acetoacetate **2a**. The crude reaction material obtained was purified by chromatography on silica gel with petrol ether/AcOEt (7:3) to give the compound **3c** as pale yellow microcrystalline precipitate: mp 216–217 °C; yield 75%.

^{1}H NMR ($CDCl_3$): δ = 2.26 ppm (s; 3H, C*H*$_3$-3′), 2.35 (s br; 6H, 2C*H*$_3$-2,6), 3.35 (s br; 6H, 2COOC*H*$_3$), 5.33 (s; 1H, H-4), 5.80 (s br; 1H, N*H*), 6.61 (d; 1H, *J* = 5.04 Hz, H-4′), 6.91 (d; 1H, *J* = 5.05 Hz, H-5′). ^{13}C NMR ($CDCl_3$): δ = 13.6 ppm (*C*H_3-3′), 19.4 (2*C*H_3-2,6), 32.8 (C-4), 51.0 (COO*C*H_3), 104.1 (C-3, C-5), 124.3 (C-5′), 124.8 (C-4′), 132.4 (C-3′), 143.8 (C-2′), 146.1 (C-2, C-6), 167.6 (2*C*OO). EI-MS (70 eV): *m/z* = 321 (M, 28%)$^+$, 306 (M−15, 21%)$^+$, 290 (M−31, 14%)$^+$, 262 (M−59, 93%)$^+$, 224 (M−C_5H_5S, 100%)$^+$, 165 (M−C_5H_5S−59, 9%)$^+$. Anal. Calcd for $C_{16}H_{19}O_4NS$: C, 59.81; H, 5.92; N, 4.36; S, 9.97. Found: C, 59.69; H, 6.01; N, 4.47; S, 10.02.

3.1.4. 1,4-Dihydro-2,6-dimethyl-4-(thiophen-2′-yl)-pyridine-3,5-dicarboxylic acid dimethyl ester (3d)

Prepared from 5 mmol of **1d** and 12 mmol of methyl acetoacetate **2a**. The crude reaction material obtained was purified by chromatography on silica gel with petrol ether/AcOEt (7:3) to give the compound **3d** as pale yellow microcrystalline precipitate: mp 189–190 °C; yield 77%.

^{1}H NMR ($CDCl_3$): δ = 2.40 ppm (s br; 6H, 2C*H*$_3$-2,6), 3.60 (s br; 6H, 2COOC*H*$_3$), 5.34 (s; 1H, H-4), 5.80 (s br; 1H, N*H*), 6.60 (d; 1H, *J* = 5.0 Hz, H-3′), 6.76 (m; 1H, H-4′), 7.01 (d; 1H, *J* = 5.2 Hz, H-5′). ^{13}C NMR ($CDCl_3$): δ = 19.3 ppm (2*C*H_3-2,6), 32.8 (C-4), 51.3 (COO*C*H_3), 104.2 (C-3, C-5), 124.2 (C-5′), 126.8 (C-4′), 126.4 (C-3′), 140.1 (C-2′), 146.4 (C-2, C-6), 167.3 (2*C*OO). EI-MS (70 eV): *m/z* = 307 (M, 32%)$^+$, 292 (M−15, 23%)$^+$, 276 (M−31, 17%)$^+$, 248 (M−59, 100%)$^+$. Anal. Calcd for $C_{15}H_{17}O_4NS$: C, 58.63; H, 18.06; N, 4.56; S, 10.42. Found: C, 58.49; H, 17.97; N, 4.61; S, 10.53.

3.1.5. 1,4-Dihydro-2,6-dimethyl-4-(3′-methyl-thiophen-2′-yl)-pyridine-3,5-dicarboxylic acid diethyl ester (3e)

Prepared from 5 mmol of **1c** and 12 mmol of ethyl acetoacetate **2b**. The crude reaction material obtained was purified by chromatography on silica gel with petrol ether/AcOEt (7:3) to give the compound **3e** as pale yellow microcrystalline precipitate: mp 135–137 °C; yield 74%.

^{1}H NMR ($CDCl_3$): δ = 1.25 ppm (t; 6H, *J* = 7.5 Hz, 2C*H*$_3$CH$_2$), 2.20 (s; 3H, C*H*$_3$-3′), 2.38 (s br; 6H, 2*CH*$_3$-2,6), 4.15 (m; 4H, CH$_3$*CH*$_2$-3,5), 5.40 (s; 1H, H-4), 6.15 (s br; 1H, N*H*), 6.63 (d; 1H, *J* = 5.04 Hz, H-4′), 6.95 (d; 1H, *J* = 5.05 Hz, H-5′). ^{13}C NMR ($CDCl_3$): δ = 13.9 ppm (*C*H_3-3′), 14.0 (2*C*H_3CH$_2$–), 19.3 (2*C*H_3-2,6), 32.7 (C-4), 64.5 (2*C*H_2CH$_3$), 103.8 (C-3, C-5), 124.4 (C-5′), 124.9 (C-4′), 132.6 (C-3′), 135.4 (C-2′), 146.0 (C-2, C-6), 168.9 (2*C*OO). EI-MS (70 eV): *m/z* = 349 (M, 31%)$^+$, 334 (M−15, 14%)$^+$, 304 (M−C_2H_5O, 100%)$^+$. Anal. Calcd for $C_{18}H_{23}O_4NS$: C, 61.89; H, 6.59; N, 4.01; S, 9.17. Found: C, 61.77; H, 6.48; N, 4.11; S, 9.28.

3.1.6. 1,4-Dihydro-2,6-dimethyl-4-(5′-methyl-furan-2′-yl)-pyridine-3,5-dicarboxylic acid dimethyl ester (3f)

Prepared from 5 mmol of **1e** and 12 mmol of methyl acetoacetate **2a**. The crude reaction material obtained was purified by chromatography on silica gel with petrol ether/AcOEt (7:3) to give the compound **3f** as pale yellow microcrystalline precipitate: mp 180–181 °C. yield 70%.

^{1}H NMR ($CDCl_3$): δ = 2.29 ppm (s; 3H, C*H*$_3$-5′), 2.41 (s br; 6H, 2*CH*$_3$-2,6), 3.79 (s br; 6H, 2COOC*H*$_3$), 5.33 (s; 1H, H-4), 5.88 (s br; 1H, N*H*), 5.93 (d; 1H, *J* = 4.9 Hz, H-3′), 6.00 (d; 1H, *J* = 4.92 Hz, H-4′). ^{13}C NMR ($CDCl_3$): δ = 14.3 ppm (*C*H_3-5′), 19.4 (2*C*H_3-2,6), 33.4 (C-4), 51.4 (COO*C*H_3), 104.2 (C-3, C-5), 109.4 (C-3′, C-4′), 149.8 (C-5′), 150.0 (C-2′), 146.3 (C-2, C-6), 166.3 (2*C*OO). EI-MS (70 eV): *m/z* = 305 (M, 40%)$^+$, 290 (M−15, 30%)$^+$, 274 (M−31, 100%)$^+$. Anal. Calcd for $C_{16}H_{19}O_5N$: C, 62.95; H, 6.23; N, 4.59. Found: C, 62.78; H, 6.11; N, 4.66.

3.1.7. 1,4-Dihydro-2,6-dimethyl-4-(3′-methyl-thiophen-2′-yl)-pyridine-3,5-dicarboxylic acid diallyl ester (3g)

Prepared from 5 mmol of **1c** and 12 mmol of allyl acetoacetate **2c**. The crude reaction material obtained was purified by chromatography on silica gel with petrol ether/AcOEt (7:3) to give the compound **3g** as pale yellow microcrystalline precipitate: mp 82–83 °C; yield 75%.

^{1}H NMR ($CDCl_3$): δ = 2.22 ppm (s; 3H, C*H*$_3$-3′), 2.38 (s br; 6H, 2*CH*$_3$-2,6), 4.60 (d; 4H, 2*CH*$_2$CH=CH$_2$), 5.21 (s; 2H, 2CH$_2$CH=C*H*$_2$), 5.22 (s; 2H, 2CH$_2$CH=C*H*$_2$), 5.40 (s; 1H, H-4), 5.89 (m; 2H, 2CH$_2$*CH*=CH$_2$), 6.40 (s br; 1H, N*H*), 6.60 (d; 1H, *J* = 5.04 Hz, H-4′), 6.95 (d; 1H, *J* = 5.05 Hz, H-5′). ^{13}C NMR ($CDCl_3$): δ = 14.1 ppm (*C*H_3-3′), 19.5 (2*C*H_3-2,6), 35.5 (C-4), 64.9 (*C*H_2CH=CH$_2$), 101.8 (C-3, C-5), 117.8 (CH$_2$CH=*C*H$_2$), 124.1 (C-5′), 124.8 (C-4′), 132.1 (CH$_2$*C*H=CH$_2$), 133.1 (C-3′), 136.2 (C-2′), 145.8 (C-2, C-6), 165.9 (*C*OO). EI-MS (70 eV): *m/z* = 373 (M, 10%)$^+$, 358 (M−15, 9%)$^+$, 331 (M−C_3H_6, 50%)$^+$, 288 (M−$C_4H_6O_2$, 53%)$^+$, 203 (M−2$C_4H_6O_2$, 100%)$^+$. Anal. Calcd for $C_{20}H_{23}O_4NS$: C, 64.34; H, 6.16; N, 3.75; S, 8.58. Found: C, 64.21; H, 6.09; N, 3.83; S, 8.67.

3.1.8. 1,4-Dihydro-2,6-dimethyl-4-(3′-methyl-thiophen-2′-yl)-pyridine-3,5-dicarboxylic acid dibenzyl ester (3h)

Prepared from 5 mmol of **1c** and 12 mmol of benzyl acetoacetate **2d**. The crude reaction material obtained was purified by chromatography on silica gel with hexane/AcOEt (7:3) to give the compound **3h** as pale yellow microcrystalline precipitate: mp 109–110 °C; yield 77%.

^{1}H NMR ($CDCl_3$): δ = 2.00 ppm (s; 3H, C*H*$_3$-3′), 2.38 (s br; 6H, 2*CH*$_3$-2,6), 5.18 (s; 4H, C*H*$_2$Ph), 5.41 (s; 1H, H-4), 6.17 (s br; 1H, N*H*), 6.60 (d; 1H, *J* = 5.04 Hz, H-4′), 6.97 (d; 1H, *J* = 5.05 Hz, H-5′), 7.20–7.34 (m; 10H, arom.H). ^{13}C NMR ($CDCl_3$): δ = 13.2 ppm (*C*H_3-3′), 18.9 (2*C*H_3-2,6), 32.2 (C-4), 65.0 (*C*H_2Ph), 103.4 (C-3, C-5), 121.2 (C-5′), 122.3 (C-4′), 127.0–128.5 (C-arom.), 131.9 (C-3′), 135.8 (C-2′), 143.6 (C-1"), 145.4 (C-2, C-6), 166.4 (*C*OO). EI-MS (70 eV): *m/z* = 473 (M, 10%)$^+$, 382 (M−C_7H_7, 18%)$^+$, 338 (M−$C_8H_7O_2$, 43%)$^+$, 232 (M−$C_8H_7O_2$−C_7H_7−CH_3, 72%)$^+$, 91 (C_7H_7, 100%)$^+$. Anal. Calcd for $C_{28}H_{27}O_4NS$: C, 71.03; H, 5.71; N, 2.96; S, 6.76. Found: C, 70.91; H, 5.60; N, 3.03; S, 6.84.

3.1.9. 1,4-Dihydro-2,6-dimethyl-4-(5′-nitro-thiophen-2′-yl)-pyridine-3,5-dicarboxylic acid diallyl ester (3i)

Prepared from 5 mmol of **1a** and 12 mmol of allyl acetoacetate **2c**. The crude reaction material obtained was purified by chromatography on silica gel with petrol ether/AcOEt (7:3) to give the compound **3i** as red microcrystalline precipitate: mp 124–125 °C; yield 72%.

^{1}H NMR ($CDCl_3$): δ = 2.40 ppm (s br; 6H, 2C*H*$_3$-2,6), 4.62 (d; 4H, 2*CH*$_2$CH=CH$_2$), 5.23 (s; 2H, 2CH$_2$CH=C*H*$_2$), 5.24 (s; 2H, 2CH$_2$CH=C*H*$_2$), 5.40 (s; 1H, H-4), 5.90 (m; 2H, 2CH$_2$*CH*=CH$_2$), 6.20 (s br; 1H, N*H*), 6.83 (d; 1H, *J* = 4.15 Hz, H-3′), 7.78 (d; 1H, *J* = 4.17 Hz, H-4′). ^{13}C NMR ($CDCl_3$): δ = 19.5 ppm (2*C*H_3-2,6), 35.5 (C-4), 64.9 (2CH$_2$CH=CH$_2$), 101.8 (C-3, C-5), 117.8 (2CH$_2$CH=*C*H$_2$), 123.0 (C-3′), 128.9 (C-4′), 132.1 (2CH$_2$*C*H=CH$_2$), 145.8 (C-2′), 146.4 (C-2, C-6), 149.6 (C-5′), 165.9 (*C*OO). EI-MS (70 eV): *m/z* = 404 (M,

37%)$^+$, 363 (M−C_3H_5, 76%)$^+$, 347 (M−C_3H_5O, 18%)$^+$, 319 (M−$C_4H_5O_2$, 92%)$^+$, 276 (M−$C_4H_2NO_2S$, 100%)$^+$. Anal. Calcd for $C_{19}H_{20}O_6N_2S$: C, 56.43; H, 4.95; N, 6.93; S, 7.92. Found: C, 56.29; H, 4.78; N, 6.99; S, 8.01.

3.1.10. 1,4-Dihydro-2,6-dimethyl-4-(5′-nitro-thiophen-2′-yl)-pyridine-3,5-dicarboxylic acid dibenzyl ester (3j)

Prepared from 5 mmol of **1a** and 12 mmol of benzyl acetoacetate **2d**. The crude reaction material obtained was purified by chromatography on silica gel with hexane/AcOEt (7:3) to give the compound **3j** as pale yellow microcrystalline precipitate: mp 117–118 °C; yield 70%.

^{1}H NMR ($CDCl_3$): δ = 2.35 ppm (s br; 6H, 2CH_3-2,6), 5.20 (s; 4H, CH_2Ph), 5.40 (s; 1H, H-4), 6.15 (s br; 1H, N*H*), 6.82 (d; 1H, *J* = 4.14 Hz, H-3′), 7.20–7.34 (m; 10H, H-arom.), 7.77 (d; 1H, *J* = 4.16 Hz, H-4′). ^{13}C NMR ($CDCl_3$): δ = 18.7 ppm (2CH_3-2,6), 32.4 (C-4), 65.2 (CH_2Ph), 103.2 (C-3, C-5), 123.4 (C-3′), 127.0–128.5 (C-arom.), 128.8 (C-4′), 145.7 (C-2′), 146.4 (C-2, C-6), 149.5 (C-5′),166.2 (*C*OO). EI-MS (70 eV): *m/z* = 504 (M, 23%)$^+$, 413 (M−C_7H_7, 100%)$^+$, 376 (M−$C_4H_2NO_2S$, 83%)$^+$. Anal. Calcd for $C_{27}H_{24}O_6N_2S$: C, 64.28; H, 4.76; N, 5.55; S, 6.35. Found: C, 64.07; H, 4.54; N, 5.68; S, 6.47.

3.1.11. 1,4-Dihydro-2,6-dimethyl-4-(4′-phenyl-thiophen-2′-yl)-pyridine-3,5-dicarboxylic acid diallyl ester (3k)

Prepared from 5 mmol of **1f** and 12 mmol of allyl acetoacetate **2c**. The crude reaction material obtained was purified by chromatography on silica gel with petrol ether/AcOEt (7:3) to give the compound **3k** as white microcrystalline precipitate: mp 117–118 °C; yield 68%.

^{1}H NMR ($CDCl_3$): δ = 2.40 ppm (s br; 6H, 2CH_3-2,6), 4.61 (d; 4H, 2CH_2CH=CH_2), 5.22 (s; 2H, 2CH_2CH=CH_2), 5.23 (s; 2H, 2CH_2CH=CH_2), 5.40 (s; 1H, H-4), 5.89 (m; 2H, 2CH_2C*H*=CH_2), 6.20 (s br; 1H, N*H*), 7.10 (m; 1H, H-3′), 7.20–7.51 (m; 5H, H-arom), 7.50 (m; 1H, H-5′). ^{13}C NMR ($CDCl_3$): δ = 19.4 ppm (2CH_3-2,6), 35.6 (C-4), 65.0 (2CH_2CH=CH_2), 102.0 (C-3, C-5), 117.6 (2CH_2CH=CH_2), 120.2 (C-5′), 123.4 (C-3′), 132.5 (2CH_2*C*H=CH_2), 127.0–129.3 (C-arom.), 136.7 (C-1″), 143.0 (C-2′), 143.2 (C-4′), 146.3 (C-2, C-6), 166.2 (*C*OO). EI-MS (70 eV): *m/z* = 435 (M, 22%)$^+$, 394 (M−C_3H_5, 90%)$^+$, 378 (M−C_3H_5O, 14%)$^+$, 350 (M−$C_4H_5O_2$, 100%)$^+$, 336 (M−$C_4H_5O_2$−CH_3, 10%)$^+$, 276 (M−$C_{10}H_7S$, 43%)$^+$. Anal. Calcd for $C_{25}H_{25}O_4NS$: C, 68.96; H, 5.75; N, 3.22; S, 7.36. Found: C, 68.71; H, 5.60; N, 3.31; S, 7.40.

3.1.12. 1,4-Dihydro-2,6-dimethyl-4-(4′-phenyl-thiophen-2′-yl)-pyridine-3,5-dicarboxylic acid dibenzyl ester (3l)

Prepared from 5 mmol of **1f** and 12 mmol of benzyl acetoacetate **2d**. The crude reaction material obtained was purified by chromatography on silica gel with hexane/AcOEt (7:3) to give the compound **3l** as white microcrystalline precipitate: mp 148–150 °C; yield 73%.

^{1}H NMR ($CDCl_3$): δ = 2.38 ppm (s br; 6H, 2CH_3-2,6), 4.92 (s; 4H, CH_2Ph), 5.20 (s; 1H, H-4), 6.00 (s br; 1H, N*H*), 6.98 (m; 1H, H-3′), 7.19–7.50 (m; 15H, H-arom., H-5′). ^{13}C NMR ($CDCl_3$): δ = 18.8 ppm (2CH_3-2,6), 32.5 (C-4), 65.5 (CH_2Ph), 103.1 (C-3, C-5), 120.1 (C-5′), 123.5 (C-3′), 127.2–129.3 (C-arom.), 137.3, 140.9, 143.0 (C-2′), 143.1 (C-4′), 146.5 (C-2, C-6), 166.2 (*C*OO). EI-MS (70 eV): *m/z* = 535 (M, 24%)$^+$, 444 (M−C_7H_7, 100%)$^+$, 400 (M−$C_8H_7O_2$, 43%)$^+$, 376 (M−$C_{10}H_7S$, 75%)$^+$. Anal. Calcd for $C_{33}H_{29}O_4NS$: C, 7.40; H, 5.42; N, 2.62; S, 5.98. Found: C, 7.32; H, 5.36; N, 2.78; S, 6.03.

3.1.13. Synthesis of Methyl 3-[2-(2-formylthiophene-3-yl)]-2-thioacetate (1g)

Pyridinium tosylate (0.4 mmol) was added to a mixture of 3-bromo-thiophene-2-carboxaldehyde (9.8 mmol), ethylene glycol (40 mmol) and dry toluene (10 ml) as described in the literature.[18] The crude product was distilled to give 1.76 g (yield 76%) of 3-bromo-2-(2-dioxolanyl) thiophene. ^{1}H NMR ($CDCl_3$): δ = 4.0–4.17 ppm (m; 4H), 6.14 (s; 1H), 6.96 (d; H-5, *J* = 5.50 Hz), 7.30 (d; 1H, *J* = 5.50 Hz). EI-MS (70 eV): *m/z* = 235 (M, 45%)$^+$.

3-Bromo-2-(2-dioxolanyl) thiophene (1.1 g; 0.005 mmol) in THF was treated with *n*-Butyllithium and sulphur at −100 °C to give 1.3 g of crude product which was used in the next step without purification. Methyl 3-[2-(2-dioxolanyl) thiophene-3-yl]-*2-thioacetate* (1.17 g, 0.005 mol) was dissolved in acetone (10 mL), and *p*-toluenesulfonic acid hydrate (0.01 g; 0.068 mmol) was added and stirred over 1 h. The usual work up gave 0.5 g of crude product which was purified by chromatography on silica gel with hexane/ether (7:3) to give 0.45 g of **1g** (yield 41%). ^{1}H NMR ($CDCl_3$): δ = 3.70 ppm (s; 1H, SCH_2), 3.77 (s; 3H, COOCH_3), 7.07 (s; 2H, C*H*=C*H*), 9.83 (s; 1H, C*H*O). EI-MS (70 eV): *m/z* = 216 (M, 37%)$^+$.

3.1.14. 1,4-Dihydro-2,6-dimethyl-4-(3′-methyl 2-thioacetate-thiophen-2′-yl)-pyridine-3,5-dicarboxylic acid diallyl ester (3m)

Prepared from 5 mmol of **1g** and 12 mmol of allyl acetoacetate **2c**. The crude reaction material obtained was purified by chromatography on silica gel with petrol ether/AcOEt (7:3) to give the compound **3m** as an oil: yield 63%.

^{1}H NMR ($CDCl_3$): δ = 2.39 ppm (s br; 6H, 2CH_3-2,6), 3.48 (s; 1H, SCH_2), 3.68 (s; 3H, COOCH_3), 4.60 (d; 4H, 2CH_2CH=CH_2), 5.22 (s; 2H, 2CH_2CH=CH_2), 5.23 (s; 2H, 2CH_2CH=CH_2), 5.40 (s; 1H, H-4), 5.89 (m; 2H, 2CH_2C*H*=CH_2), 6.20 (s br; 1H, N*H*), 6.79 (m; 2H, H-3′, H-4′). ^{13}C NMR ($CDCl_3$): δ = 19.4 ppm (2CH_3-2,6), 34.9 (C-4), 40.5 (CH_2S), 51.3 (COOCH_3), 65.1 (2CH_2CH=CH_2), 102.7 (C-3, C-5), 117.4 (2CH_2CH=CH_2), 123.7 (C-5′), 128.3 (C-4′), 131.6 (C-3′), 133.4 (C-2′), 136.4 (2CH_2*C*H=CH_2), 146.4 (C-2, C-6), 166.2, 171.3 (*C*OO). EI-MS (70 eV): *m/z* = 463 (M, 12%)$^+$, 422 (M−C_3H_5, 92%)$^+$, 378 (M−$C_4H_5O_2$, 100%)$^+$, 276 (M−$C_7H_7S_2O_2$, 67%)$^+$. Anal. Calcd for $C_{22}H_{25}O_6NS_2$: C, 57.02; H, 5.39; N, 3.02; S, 13.8. Found: C, 56.89; H, 5.26; N, 3.11; S, 14.07.

3.1.15. 1,4-Dihydro-2,6-dimethyl-4-(3′-methyl 2-thioacetate-thiophen-2′-yl)-pyridine-3,5-dicarboxylic acid dibenzyl ester (3n)

Prepared from 5 mmol of **1g** and 12 mmol of benzyl acetoacetate **2d**. The crude reaction material obtained was purified by chromatography on silica gel with petrol ether/AcOEt (7:3) to give the compound **3n** as an oil: yield 71%.

^{1}H NMR ($CDCl_3$): δ = 2.40 ppm (s br; 6H, 2CH_3-2,6), 3.49 (s; 1H, SCH_2), 3.69 (s; 3H, COOCH_3), 4.95 (s; 4H, CH_2Ph), 5.22 (s; 1H, H-4), 6.10 (s br; 1H, N*H*), 6.78 (m; 2H, H-4′, H-5′), 7.17–7.30 (m; 10H, H-arom.). ^{13}C NMR ($CDCl_3$): δ = 19.5 ppm (2CH_3-2,6), 34.5 (C-4), 40.6 (CH_2S), 51.2 (COOCH_3), 65.7 (CH_2Ph), 103.3 (C-3, C-5), 124.6 (C-5′), 128.3 (C-4′), 127.2–129.4 (C-arom.), 133.1 (C-3′), 133.6 (C-2′), 140.6 (C-1″), 146.3 (C-2, C-6), 166.2, 171.3 (*C*OO). EI-MS (70 eV): *m/z* = 563 (M, 29%)$^+$, 472 (M−C_7H_7, 72%)$^+$, 428 (M−$C_8H_7O_2$, 100%)$^+$, 376 (M−$C_7H_7S_2O_2$, 51%)$^+$. Anal. Calcd for $C_{30}H_{29}O_6NS_2$: C, 63.94; H, 5.15; N, 2.48; S, 11.37. Found: C, 63.81; H, 5.06; N, 2.54; S, 11.42.

3.2. General procedure for the synthesis of asymmetrical 1,4-dihydropyridines (4a–d)

To an ice-cooled solution of 5 mmol of an aldehyde (A) in 15 ml of isopropanol, 5 mmol of the first acetoacetate (B) were added and the mixture is refluxed for 3 h. Then 3 mmol of the second acetoacetate (C) is added and the mixture is refluxed for 3 h. At the end, 1 ml of concd ammonia is added and the mixture is refluxed for 1 h. After cooling, the solvent is evaporated under reduced pressure and the crude reaction material obtained is purified by column chromatography on silica gel with toluene/AcOEt (9:1).

3.2.1. 1,4-Dihydro-2,6-dimethyl-4-(3′-methyl-thiophen-2′-yl)-pyridine-3-carboxylic acid allyl ester, 5- carboxylic acid ethyl ester (4a)

Prepared from **1c** (A), ethyl acetoacetate (B) and allyl acetoacetate (C). Crystallized from cyclohexane; mp: 157–158 °C; yield: 32%.

IR (KBr) ν (cm^{-1}): 3313, 2983, 2927, 1697, 1645, 1487, 1206.

^{1}H NMR (δ, $CDCl_3$): 1.25 ppm (t, 3H, CH_2CH_3), 2.21–2.39 (m, 9H, CH_3-3′ + CH_3-2 + CH_3-6), 4.10 (q, 2H, CH_2CH_3), 4.57 (d, 2H, $CH_2CH{=}CH_2$), 5.04–5.14 (m, 2H, $CH_2CH{=}CH_2$), 5.35 (s, 1H, H-4), 5.79–5.93 (m, 2H, NH + $CH_2CH{=}CH_2$), 6.60 (d, 1H, H-4′), 6.91 (d, 1H, H-5′).

^{13}C NMR ($CDCl_3$): δ = 13.4 ppm (CH_3-3′), 13.7 (CH_3CH_2), 18.8 (CH_3-2), 18.9 (CH_3-6), 32.5 (C-4), 59.5 (CH_2CH_3), 63.9 ($CH_2CH{=}CH_2$), 103.3 (C-3), 103.7 (C-5), 116.7 ($CH_2CH{=}CH_2$), 120.8 (C-5′), 128.3 (C-4′), 131.5 ($CH_2CH{=}CH_2$), 131.5 (C-3′), 132.1 (C-2′), 143.5 (C-2), 145.5 (C-6), 166.2, 166.6 (COO). Anal. Calcd for $C_{19}H_{23}O_4NS$: C, 63.14; H, 6.41; N, 3.88; S, 8.87. Found: C, 63.38; H, 6.35; N, 3.96; S, 8.76.

3.2.2. 1,4-Dihydro-2,6-dimethyl-4-(3′-methyl-thiophen-2′-yl)-pyridine-3-carboxylic acid benzyl ester, 5- carboxylic acid ethyl ester(4b)

Prepared from **1c** (A), ethyl acetoacetate (B) and benzyl acetoacetate (C). Crystallized from cyclohexane; mp: 186–187 °C; yield: 35%.

IR (KBr) ν (cm^{-1}): 3018, 2953, 2930, 1691, 1617, 1470, 1221.

^{1}H NMR ($CDCl_3$): δ = 1.23 (t, 3H, CH_2CH_3), 2.21–2.38 (m, 9H, CH_3-3′ + CH_3-2 + CH_3-6), 4.11 (q, 2H, CH_2CH_3), 5.05–5.19 (m, 3H, CH_2Ph + H-4), 5.87 (s, 1H, NH), 6.57 (d, 1H, H-4′), 6.91 (d, 1H, H-5′), 7.13–7.26 (m, 5H, H-arom).

^{1}H NMR ($CDCl_3$): δ = 2.00 ppm (s; 3H, CH_3-3′), 2.38 (s br; 6H, 2CH_3-2,6), 5.18 (s; 4H, CH_2Ph), 5.41 (s; 1H, H-4), 6.17 (s br; 1H, NH), 6.60 (d; 1H, J = 5.04 Hz, H-4′), 6.97 (d; 1H, J = 5.05 Hz, H-5′), 7.20–7.34 (m; 10H, arom.H). ^{13}C NMR ($CDCl_3$): δ = 13.2 ppm (CH_3-3′), 13.6 (CH_3CH_2–), 18.7 (CH_3-2), 18.9 (CH_3-6), 32.1 (C-4), 59.5. Anal. Calcd for $C_{23}H_{25}O_4NS$: C, 67.13; H, 6.12; N, 3.40; S, 7.79. Found: C, 66.90; H, 6.14; N, 3.46; S, 7.70.

3.2.3. 1,4-Dihydro-2,6-dimethyl-4-(3′-methyl 2-thioacetate-thiophen-2′-yl)-pyridine-3-carboxylic acid allyl ester, 5-carboxylic acid ethyl ester (4c)

Prepared from **1g** (A), ethyl acetoacetate (B) and allyl acetoacetate (C). Pale yellow oil; yield: 33%.

IR (KBr) ν (cm^{-1}): 3020, 2955, 2934, 17634, 1690, 1471, 1225.

^{1}H NMR ($CDCl_3$): δ = 1.26 ppm (t, 3H, CH_2CH_3), 1.97–2.08 (m, 6H, CH_3-2 + CH_3-6), 3.33 (s, 1H, SCH_2), 3.67 (s, 3H, $COOCH_3$), 4.18 (q, 2H, CH_2CH_3), 4.61 (d, 2H, $CH_2CH{=}CH_2$), 5.08–5.25 (m, 2H, $CH_2CH{=}CH_2$), 5.38 (s, 1H, H-4), 5.79–6.05 (m, 2H, NH + $CH_2CH{=}CH_2$), 6.87 (d, 1H, H-5′), 7.12–7.26 (m, 5H, H-arom).

^{13}C NMR ($CDCl_3$): δ = 13.6 ppm (CH_3CH_2–), 18.7 (CH_3-2), 18.9 (CH_3-6), 33.4 (C-4), 40.3 (CH_2S), 51.3 ($COOCH_3$), 59.1 (CH_2CH_3), 64.8 ($CH_2CH{=}CH_2$), 102.1 (C-3), 102.4 (C-5), 117.7 ($CH_2CH{=}CH_2$), 125.6 (C-5′), 126.3 (C-4′), 133.5 (C-3′), 135.7 (C-2′), 136.5 ($CH_2CH{=}CH_2$), 146.3 (C-2), 146.9 (C-6), 166.2, 166.7, 171.4 (COO). Anal. Calcd for $C_{21}H_{25}O_6NS_2$: C, 55.86; H, 5.58; N, 3.10; S, 14.20. Found: C, 55.97; H, 5.68; N, 3.03; S, 14.32.

3.2.4. 1,4-Dihydro-2,6-dimethyl-4-(3′-methyl 2-thioacetate-thiophen-2′-yl)-pyridine-3-carboxylic acid benzyl ester, 5-carboxylic acid ethyl ester (4d)

Prepared from **1g** (A), ethyl acetoacetate (B) and benzyl acetoacetate (C). Pale yellow oil; yield: 36%.

IR (KBr) ν (cm^{-1}): 3019, 2955, 2929, 1735, 1693, 1470, 1225.

^{1}H NMR ($CDCl_3$): δ = 1.23 (t, 3H, CH_2CH_3), 2.24–2.38 (m, 6H, CH_3-2 + CH_3-6), 3.47 (s, 1H, SCH_2), 3.69 (s, 3H, $COOCH_3$), 4.15 (q, 2H, CH_2CH_3), 5.04–5.23 (m, 3H, CH_2Ph + H-4), 5.95 (s, 1H, NH), 6.87 (s, 1H, H-5′), 7.16–7.38 (m, 10H, H-arom).

^{13}C NMR ($CDCl_3$): δ = 13.6 ppm (CH_3CH_2–), 18.8 (CH_3-2), 18.9 (CH_3-6), 33.6 (C-4), 40.5 (CH_2S), 51.2 ($COOCH_3$), 59.5 (CH_2CH_3), 64.9 (CH_2Ph), 102.3 (C-3), 102.4 (C-5), 125.6 (C-5′), 126.8 (C-4′), 127.0–129.4 (C-arom.), 133.2 (C-3′), 135.9 (C-2′), 141.2 (C-1″), 146.5 (C-2), 146.9 (C-6), 166.3, 166.5, 171.5 (COO). Anal. Calcd for $C_{25}H_{27}O_6NS_2$: C, 59.86; H, 5.43; N, 2.79; S, 12.78. Found: C, 60.08, H, 5.38, N, 2.85; S, 12.86.

4. Methods

4.1. CFTR assays

4.1.1. Cell culture

Fischer rat thyroid (FRT) cells, stable transfected with ΔF508-CFTR and the halide-sensitive yellow fluorescent protein YFP-H148Q/I152L[19] were cultured in Coon′s modified Ham′s F-12 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin. For fluorescence assays of CFTR activity, cells were plated (100,000 cells/well) on clear-bottomed 96-well microplates (Corning Life Sciences, Acton, MA).

H9C2 cells, a cell line derived from rat heart tissue with endogenous expression of L-type voltage-dependent Ca^{2+} channels (L-VDCCs),[20] were grown in Dulbecco′s modified Eagle′s medium/Ham′s F-12 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin. For fluorescence assays of L-VDCC activity, cells were plated (100,000 cells/well) on clear-bottomed 96-well microplates. After 24 h, cells received fresh medium containing all-*trans*-retinol (10 nM) to increase L-VDCC expression.[21] The functional assay was performed after additional 24 h.

4.1.2. Samples

The synthesized compounds **3a–n** and **4a–d** were dissolved in dimethyl sulfoxide and for each compound 10 mM stock solutions were prepared.

Secondary plates were prepared for screening at 1 mM concentration in dimethyl sulfoxide using a Biomek 2000 liquid handling workstation (Beckman Coulter, Fullerton, CA). All plates were stored at −70 °C.

4.1.3. Fluorescence assay for CFTR activity

Measurements of CFTR activity were carried out on FRT cells expressing ΔF508-CFTR and the halide-sensitive YFP 48 h after plating on microplates. Before the assay, ΔF508-CFTR-expressing cells were incubated at 27 °C for 20–24 h to allow targeting of the mutant protein to the plasma membrane.

At the time of assay, 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 20 μM forskolin (to maximally enhance intracellular cAMP levels and allow CFTR phosphorylation) and test compounds at the desired concentration. Then, cells were 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). 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 iodide influx rate, the final 10 s of the data for each well were fitted with an exponential function to extrapolate the initial slope (dF/dt).

4.1.4. Fluorescence assay for VDCC activity

Cells were incubated with a loading solution containing the calcium-sensitive fluorescent probe Fluo-4 AM (4 μM), glucose (10 mM) and probenecid (2 mM). After 1 h, cells were washed with phosphate-buffered saline and then incubated for 20 min in the presence of test compounds or known VDCC modulators (probenecid and glucose were maintained in the solution). Then, cells were transferred to the microplate reader (485 nm excitation; 520 nm emission). Each assay consisted of a continuous 8-s fluorescence reading, after which a solution containing high-potassium (PBS with Na^+ replaced by K^+) was injected into the well to depolarize the cell membrane and activate VDCCs. The fluorescence was continuously monitored for additional 27 s. The activity of VDCCs was determined as the increase in Fluo-4 fluorescence upon injection, normalized to the initial background-subtracted fluorescence.

4.2. 3D-QSAR analysis

4.2.1. CoMFA procedure

Comparative Molecular Field Analysis (CoMFA) method is a widely used 3D-QSAR technique to relate the biological activity of a series of molecules with their steric and electrostatic fields which are calculated placing the aligned molecules, one by one, in a 3D cubic lattice with 2 Å grid spacing. The column-filtering threshold value in this study was set to 2.0 kcal/mol, to improve the signal-noise ratio. A methyl probe with a +1 charge is used to calculate steric and electrostatic fields, represented by van der Waals potential and coulombic term, respectively. A 30 kcal/mol energy cut-off was applied to avoid infinity of energy values inside the molecule. Regression analyses were performed applying the partial least squares (PLS) algorithm in SYBYL; the combination of the steric and electrostatic fields were used as structural descriptors to evaluate their correlation with the biological activity (expressed as pK_d) data. PLS with cross-validation, performed using five cancellation groups was used to identify the optimal number of components to be used in the subsequent analyses. The final CoMFA model was generated using non-cross-validated PLS analysis. To further assess the statistical confidence and robustness of derived model, a 100-cycle bootstrap analysis was performed.

The alignment procedure was carried out using the Molecular Operating Environment (MOE; version 2006.08) suite. CoMFA was performed using SYBYL.[22] All calculations were carried out using a PC, with operative system windows XP and an SGI O2 Silicon Graphics.

5. Results

FRT cells expressing ΔF508-CFTR were incubated at 27 °C to rescue the mutant protein from the ER, thus improving its targeting to the plasma membrane. Subsequent stimulation of cells with forskolin plus genistein evoked a threefold increase in halide transport relative to cells treated with forskolin alone. Most DHPs elicited a similar increase in ΔF508-CFTR activity. We determined dose–response relationships for symmetrical (**3a–n**) and asymmetrical (**4a–d**) DHPs and derived apparent K_d values and maximal effect by fitting the data with a Hill equation (Table 1).

The affinity calculated for our DHPs was high for compounds **3g, 3h, 3n**, **4a**, **4b**, and **4d**, with K_d values in the 0.3–1.0 μM range. The other compounds showed different degrees of affinity. Compounds **3j** and **3l** were the least potent, the corresponding K_d values being close to that of genistein (~16 μM).

From the data reported in Figure 1 it is possible to see that the efficacy of symmetrical DHPs **3** is, in general, better than the reference compound genistein, whereas the asymmetrical DHPs **4** present a CFTR activity less than genistein.

Table 1
K_d values for DHPs **3** and **4**

Compound	K_d (μM)
3a	8.3 ± 2.1
3b	6.0 ± 0.8
3c	3.1 ± 0.3
3d	9.5 ± 1.2
3e	5.7 ± 1.2
3f	6.0 ± 1.8
3g	1.0 ± 0.3
3h	0.7 ± 0.1
3i	3.8 ± 0.6
3j	13.1 ± 0.7
3k	1.5 ± 0.4
3l	16.5 ± 2.2
3m	1.3 ± 0.3
3n	0.3 ± 0.1
4a	0.3 ± 0.1
4b	0.5 ± 0.2
4c	1.9 ± 0.5
4d	0.3 ± 0.1
Genistein	16.7 ± 0.1

About the substituents on thiophene ring of DHPs, the presence of substituents at the 3′ position seems to favour the activity on CFTR. Thus, the 3′-methyl 2-thioacetate derivatives **3m** and **3n** show an outstanding efficacy and also good results are obtained by the 3′-methyl derivatives **3g** and **3h** (the only exception to this trend is **3c**). The phenyl derivatives at the position 4′ (**3k** and **3l**) show a good activity on CFTR. On the other hand, compounds with substituents at the position 5′ (**3a** and **3b**) are less effective. Also the furanyl isoster **3f** presents a modest degree of efficacy. Nevertheless, this compound may deserve a further development (see later).

In general, not only the kind of substituents of the thiophen-2-yl ring is important for potency on CFTR, but also the esterification of the carboxyl groups with allyl and benzyl, instead of methyl and ethyl, shows improved properties with the exception of the compounds **3i**, **3j** and **3l**.

The synthesized DHPs **3** and **4** were also tested in the calcium channel assay and compared to felodipine (see Fig. 2). The symmetrical DHPs **3a–n** had different levels of activity on Ca^{2+} channels. In particular, **3f** bearing a furanyl substituent at the C-4 position of the dihydropyridine moiety, was the weakest Ca^{2+}

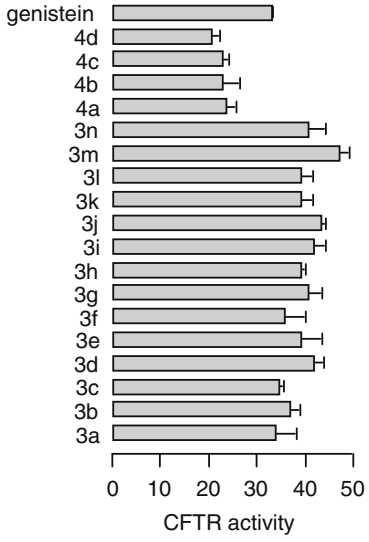


Figure 1. Compound efficacy on ΔF508-CFTR. The efficacy is obtained from dose–response relationships of YFP fluorescence experiments (mean ± SD, $n = 3$).

blocker. Also the compounds **3b** and **3d** had reduced activity on calcium channels. On the contrary, the behaviour of asymmetrical DHPs **4a–d** on Ca^{2+} channel activity is quite similar to that of the reference compound felodipine.

6. Discussion

6.1. Activity of 1,4-dihydropyridines on CFTR

Identification of selective potentiators of the CFTR Cl^- channel is important to develop effective drugs for the treatment of the basic defect in cystic fibrosis patients. In particular, potentiators may be useful to enhance the activity of the ΔF508 mutant, once the trafficking defect is corrected, at least partially, by suitable pharmacological chaperones.[23,24] In 2005, we showed that DHPs possess a notable activity in potentiating the conductance in cells carrying CFTR gating mutants as G551D or when the cells with ΔF508-CFTR were rescued by incubation at low temperature.[11] In this way more than 300 DHPs with a phenyl group in position 4 were evaluated in order to investigate the structure–activity relationship.

4-Thiophen-2-yl-1,4-dihydropyridines, the object of the present work, represent an extension of our knowledge on this topic. In general, the behaviour of the present DHPs are similar to the previous ones, supporting the idea that the activity of such compounds is unbound from the quality of the substituent present in position 4 (also the isosteric furanyl derivative **3f** has a certain degree of activity). Regarding the affinity of our DHPs (see Table 1) we can say that both compounds **3** and **4** present K_d values better than the reference compound genistein, but activity is distributed in a large array. In this regard, the asymmetrical derivatives **4a–d**, tested as racemates, showed better affinity with K_d values ranging from 0.3 to 1.9 μM and for this reason constitute a sub-class deserving a future wider study. Symmetrical DHPs **3a–n** present K_d values spreading in a broad range in comparison to compounds **4**. In particular, the derivative **3n** possesses the lowest K_d value of this group (0.3 μM), and such value is identical to that of **4a** and **4d**. Moreover, it must be noted that DHPs **3n** and **4d** have similar substituents, the only difference being the presence at position 3 of a carboxybenzyl ester in **3n** and the presence at position 3 of a carboxyethyl ester in **4d**. Regarding the efficacy of our DHPs (see Fig. 1), the asymmetrical DHPs **4a–d** are less active than the reference compound genistein, whereas the symmetrical DHPs **3a–n** present an activity comparable or better than genistein. As the efficacy of DHPs **3** is confined in a narrow range, it causes difficulties to give an order of importance to the substituents of the DHPs present in this work, however we can affirm that, regarding the substituents at positions 3 and 5 of these DHPs, carboxybenzyl and carboxyallyl esters are more active than carboxymethyl and carboxyethyl esters. Regarding the substituents on the thiophene ring, remarkable results are attained when the 3′-methyl 2-thioacetate group is present: indeed, compounds **3m** and **3n** (Table 1 and Fig. 1) have both a good affinity and a notable efficacy.

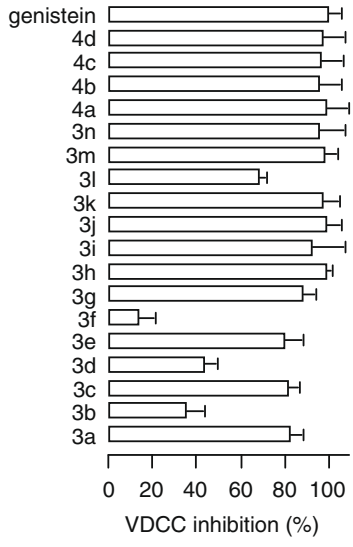


Figure 2. Compound activity on VDCC. Activity on VDCC is reported as the percentage of block obtained at a single concentration (10 μM) on H9C2 cells (mean ± SD, $n = 3$).

6.2. Activity of 1,4-dihydropyridines on Ca^{2+} channels

The lack of activity on Ca^{2+} channels is a very important characteristic in order to avoid negative consequences on the cardiovascular system in CF patients. As already observed in previous work,[15] the DHPs potentially useful for CF are often endowed of a VDCC block. Now, the only derivatives that may represent an exception to this propensity are **3d**, **3f** and **3l**. These three compounds each presents at position 3 and 5 a carboxymethyl group, but this fact does not represent a selectiveness because other Ca^{2+} channel blocking compounds (e.g., **3a**, **3c**) also possess this group. As the substitutions on esters by allyl and benzyl groups give a great VDCC inhibition (the only partial exception being **3l**), we can conclude that a carboxymethyl group could be useful in lowering the Ca^{2+} blocking activity. Also the substitutions on the thiophene ring do not seem to have a great significance in modifying the activity of DHPs on Ca^{2+} channels. Interestingly, the only compound without activity on Ca^{2+} channels is the isosteric furanyl derivative **3f**, having a relatively good activity on CFTR. This fact deserves further investigation: indeed, it will be very interesting to examine the behaviour of other furan derivatives possessing more interesting groups at the ester level (as benzyl or allyl) or on the furan ring as the 3′-methyl-2-thioacetate group, to test if it will be possible maximize the activity on CFTR maintaining a low VDCC inhibition.

6.3. CoMFA analysis

In order to give further support to the biological results obtained and to better address the future synthesis, a preliminary quantitative evaluation of the structure–activity relationship study inside this series of CFTR chloride channel potentiators has been performed. This in silico investigation is important because the true binding site of potentiators in CFTR is unclear. Some studies indicate that genistein binds CFTR at nucleotide binding domains (NBDs) level and inside NBDs three or four binding sites, at different degree of affinity, are proposed.[25,26] Compounds **3a–n** and **4a–d** were manually divided into a training set (**3b**–**h**, **3j**–**m**, **4a**–**d**) for model generation and into a test set (**3a, 3i, 3n**) for model validation. The analysis was developed using CoMFA steric and electrostatic fields as independent variables and activity (expressed as pK_d) as the dependent one.

CoMFA steric and electrostatic fields effect on the target property can be viewed as 3D coefficient contour plots, thus they could be helpful to identify important regions where any change in these fields may affect the biological activity. The final CoMFA model (Tables 2–4) was generated using non-cross-validated PLS analysis with the optimum number of components (4) to give an r^2_{ncv} 0.93, Standard Error of Estimate, SEE = 0.165, steric contribution = 0.486 and electrostatic contribution = 0.514. The results of the CoMFA approach qualitatively points out that the activity of DHPs **3a–n, 4a–d** is almost equally affected by the steric and electronic properties of the ligands. More in detail, the steric contour map shows

Table 2
Summary of CoMFA results

No. of compounds	18
Opt. No. components	4
Cross-validated r^2	0.716
Std. error of estimate	0.165
Non cross-validated r^2_{ncv}	0.930
F values	136.565
Steric contribution	0.486
Electrostatic contribution	0.514
Bootstrap r^2	0.861
Std. Error of estimate$_{(bootstrap\ r2)}$	0.212
r^2_{pred} [a]	0.973

[a] Correlation coefficient for the test set.

favourable interaction polyhedra (green region) in the area around the 1,4-dihydropyridine ring and around one of the two substituents at positions 3 and 5, thus suggesting the possibility of a non-symmetric substitution. In addition, a large yellow polyhedron (unfavourable interaction) underlines the importance of a small substituent on positions 2 and 6 in order to avoid negative interactions with a bulky moiety at position 3 and 5. Moreover, a second yellow area is detected around position 4 of the dihydropyridine ring (Fig. 3).

As concern the electrostatic maps, all the compounds display a red region, decoding for a favourable electrostatic interaction, behind the substituent on position 4 of the dihydropyrine ring, while two blue regions (unfavourable interactions) suggest the importance to limit the polar portion of substituents on position 3 and 5 to the carbonyl group directly linked to the scaffold (Fig. 4).

7. Conclusions

4-Thiophen-2′-yl-1,4-dihydropyridines **3** and **4** were found to act as valuable potentiators of rescued ΔF508-CFTR. In this regard, the activity of DHPs **3** and **4** is consistent with the activity of the 4-phenyl-1,4-dihydropyridines already tested.[14,15] About the activity on Ca^{2+} channels, our data show that it is difficult to separate a positive action on CFTR from a block of Ca^{2+} channels; that said, there is the chance that a single DHP may not cause a significant Ca^{2+} channel block, while maintainig a notable degree on CFTR activation. The CoMFA analysis may help in improving the activity of these potentiators as, even if the number of the tested compounds is small, it gives some interesting suggestions for further research. In particular, it must be stressed that the CoMFA indications about the asymmetry of the molecule and the changes at the position 4 of the DHP ring join the structure of our set of DHPs to the compound Vx-770, a potential drug now in pharmacological trial (phase III) as potentiator for CF.[27] Vx-770 is a quinolin-4-

Table 3
Experimental and predicted binding affinities of the training set molecules

Compound	pK_d exp	pK_d pred
3b	5.22	5.23
3c	5.51	5.35
3d	5.02	5.10
3e	5.24	5.54
3f	5.22	5.19
3g	6.00	6.21
3h	6.15	6.34
3j	4.88	4.95
3k	5.82	6.00
3l	4.80	4.89
3m	5.89	5.94
4a	6.52	6.50
4b	6.30	6.69
4c	5.72	5.66
4d	6.52	6.61

Table 4
Experimental and predicted binding affinities of the test set molecules

Compound	pK_d exp	pK_d pred
3a	5.08	5.92
3i	5.42	5.42
3n	6.52	6.39

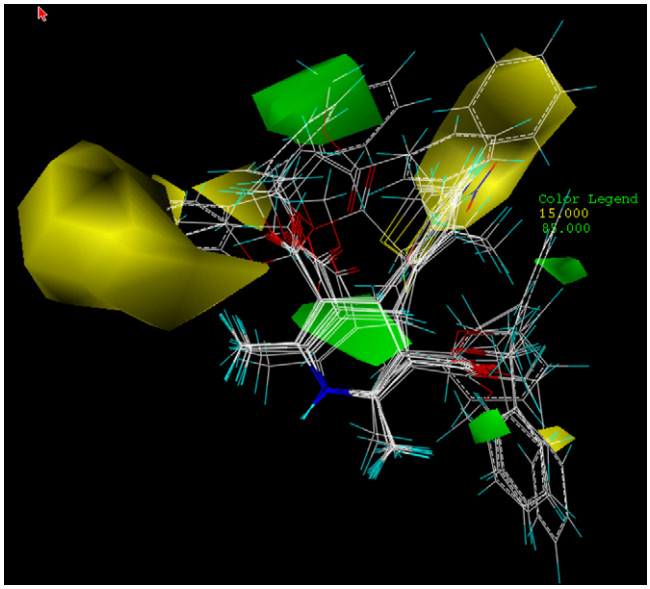


Figure 3. Contour maps of CoMFA steric regions (green, favoured; yellow, disfavoured) are shown around the studied compounds.

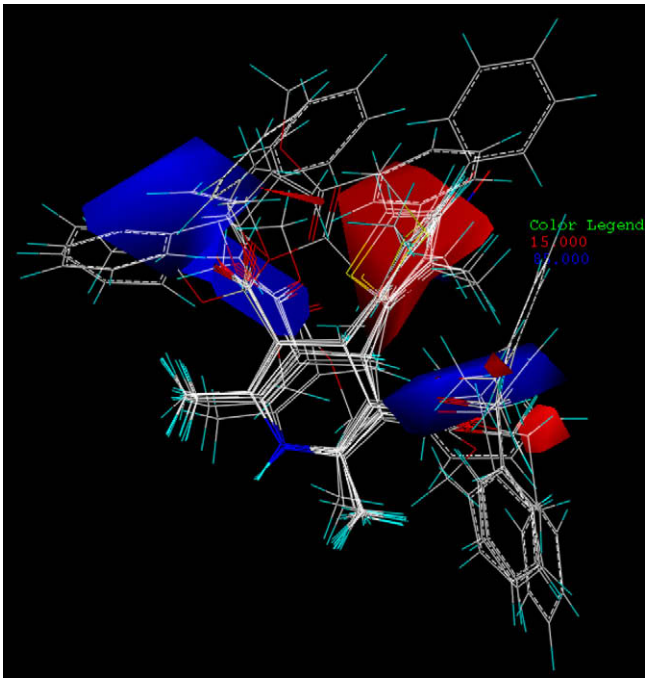


Figure 4. Contour maps of CoMFA electrostatic regions are shown around the studied compounds. Blue regions are favourable for more positively charged groups; red regions are favourable for less positively charged groups.

one derivative substituted at position 3 with a carboxyanilide;[28] therefore, Vx-770 has tight structural relation with our asymmetric DHPs. We believe these observations deserve further development.

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