Supporting Information

Cystic Fibrosis: a New Target for 4-Imidazo[2,1-*b*]thiazole-1,4-Dihydropyridines.

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Details For Chemistry

Aldehydes **11-15** were prepared according to the literature method.[RS1-RS5] The synthesis of imidazo[2,1-*b*]thiazoles **5-7** and aldehydes **8-10** are reported below. All the compounds prepared have a purity of at least 95% as determined by combustion analysis. The melting points are uncorrected. Analyses (C, H, N) were within (0.4% of the theoretical values.

Imidazo[2,1-*b*]thiazoles 5-7

The 2-aminothiazoles (**1** or **2**, 20 mmol) were dissolved in acetone (30 mL) and treated with the appropriate 2-bromo-1-arylethanones (**3** or **4**, 20 mmol). The reaction mixture was refluxed for 4 h and after cooling the resulting precipitate was collected, suspended in 40 mL of 2N HCl and refluxed for 1 h. The warm solution, basified with 20 % NH_4OH, after cooling at room temperature yielded the expected imidazo[2,1-*b*]thiazoles which were filtered. For **6** the basified solution was extracted with CH_2Cl_2 (3 x 20mL), the organic phase was dried (Na_2SO_4) and evaporated *in vacuo*.

Imidazo[2,1-*b*]thiazole-5-carboxaldehydes 8-10

The Vilsmeier reagent was prepared at 0-5 °C by dropping 30 mmol of $POCl_3$ into a stirred solution of DMF (33 mmol) in $CHCl_3$ (7 mL). The appropriate imidazo[2,1-*b*]thiazoles (**5-7**, 10 mmol), dissolved in $CHCl_3$ (50 mL), were added dropwise, under stirring at 0-5 °C, to the Vilsmeier reagent. After 3 h at room temperature, the reaction mixture was refluxed for 3-14 h (according to a TLC test acetone/petroleum ether 55-85 °C, 1:9 v/v, 2:8 v/v) and the solvent was evaporated under reduced pressure. The oily residue was poured into ice and the resulting precipitate was collected by filtration.

Table S1. Starting Compounds.

Comp	x-y	R	Formula	M. W.	Yield %	Mp, °C
5	HC=CH	4-(CF_3)-C_6H_4	$C_{12}H_7F_3N_2S$	268.2603	68	165-175
6	H_3CC=CCH_3	4-(CF_3)-C_6H_4	$C_{14}H_{11}F_3N_2S$	296.2705	55	185-190
7	HC=CH	4-(OCF_3)-C_6H_4	$C_{12}H_7F_3N_2OS$	296.3136	60	103-110
8	HC=CH	4-(CF_3)-C_6H_4	$C_{13}H_7F_3N_2OS$	324.3239	35	140-145
9	H_3CC=CCH_3	4-(CF_3)-C_6H_4	$C_{15}H_{11}F_3N_2OS$	284.2597	91	115-120
10	HC=CH	4-(OCF_3)-C_6H_4	$C_{13}H_7F_3N_2O_2S$	312.2699	93	101
11	HC=CH	CF_3	$C_7H_3F_3N_2OS$	219.9918	*a*	
12	H_2C-CH_2	C_6H_5	$C_{12}H_{10}N_2OS$	230.0513	*b*	
13	HC=CH	2,5-(OCH_3)-C_6H_3	$C_{14}H_{12}N_2O_3S$	288.0568	*c*	
14	H_3CC=CH	Cl	$C_7H_5ClN_2OS$	199.9811	*d*	
15	HC=CH	2,3,4-$Cl_3C_6H_2$	$C_{12}H_5Cl_3N_2OS$	329.9188	*e*	

[a] Ref.[RS1]

[b] Ref. [RS2]

[c] Ref. [RS3]

[d] Ref. [RS4]

[e] Ref. [RS5]

Table S2. IR and ^{1}H-NMR of Starting Compounds and IR for compounds **16-26**.

Comp	IR: ν_{max} cm^{-1}	^{1}H-NMR:[a] δ, ppm in DMSO-d$_6$; J, Hz
5	3140, 1614, 1189, 1103, 724	7.32 (1H, d, th, J = 4.4), 7.74 (2H, d, ar, J = 8.1), 7.98 (IH, d, th, J = 4.4), 8.05 (2H, d, ar, J = 8.1), 8.40 (1H,s, im)
6	3160, 1615, 1113, 1072, 847	2.36 (6H, s, CH_3), 7.73 (2H, d, ar, J =8.4), 8.04 (2H, d, ar, J = 8.4), 8.38 (1H, s, im)
7	3160, 1541, 1293, 1154, 722	7.29 (IH, d, th, J = 4.4), 7.38 (2H, d, ar, J = 8.4), 7.95 (3H, m, th+ar), 8.29 (1H, s, im)
8	3119, 1649, 1099, 1066, 848	7.63 (1H, d, th, J = 4.4), 7.87 (2H, d, ar, J = 8.3), 8.12 (2H, d, ar, J = 8.3), 8.42 (1H, d, th, J = 4.4), 9.93 (1H, s, CHO)
9	3400, 1666, 1111, 1014, 843	2.41 (3H, s, CH_3), 2.66 (3H, s, CH_3), 7.86 (2H, d, ar, J = 8.1), 8.03 (2H, d, ar, J = 8.1), 9.78 (1H, s, CHO)
10	3140, 1649, 1533, 1279, 1147	7.51 (2H, d, ar, J = 8.4), 7.62(1H, d, th, J = 4.3), 8.03 (2H, d, ar, J = 8.4), 8.41 (1H, d, th, J = 4.3), 9.90 (1H, s, CHO)
16	1705, 1297, 1159, 1097	
17	1705, 1658, 1211, 1114	
18	1698,1271,1125,1095	
19	1698, 1188, 1106, 854	
20	1697, 1253, 1166, 1020	
21	1743, 1265, 1152, 934	
22	1692, 1204, 1112, 718	
23	1694, 1633, 1203, 1055	
24	1696, 1670, 1202, 1020	
25	1672, 1204, 1115, 767	
26	1698, 1658, 1213, 1022	

[a] In all the dihydropyridines the NH groups give broad bands in the range 3400-3100 cm^{-1}. Abbreviations: th = thiazole, im = imidazole, ar =aromatic.

Table S3. Analytical Data.

Comp	Calcd C (found)	Calcd H (found)	Calcd N (found)
5	53.73(53.87)	2.63(2.60)	10.44(10.43)
6	56.75(56.68)	3.74(3.54)	9.45(9.54)
7	50.70(51.05)	2.48(2.60)	9.85(9.76)
8	52.70(52.24)	2.38(2.23)	9.46(9.54)
9	55.55(55.98)	3.42(3.54)	8.64(8.71)
10	50.00(50.43)	2.26(2.23)	8.97(9.02)
16	51.46(51.97)	4.55(4.42)	9.48(9.50)
17	56.21(56.04)	4.10(4.23)	8.55(8.43)
18	57.80(58.04)	4.66(4.78)	8.09(8.12)
19	60.93(61.29)	4.94(4.89)	7.35(7.43)
20	54.43(53.99)	3.97(3.87)	8.28(8.42)
21	57.95(57.87)	4.32(4.21)	7.51(7.67)
22	64.50(64.12)	5.85(5.73)	9.03(9.12)
23	62.25(62.67)	5.22(5.01)	9.90(9.98)
24	62.79(62.72)	5.46(5.32)	7.85(8.02)
25	53.83(53.65)	5.23(5.43)	9.91(10.03)
26	51.95(52.32)	4.00(3.89)	7.57(7.61)

Table S4. Cardiovascular Activity of Compounds previously described.[RS1]

	% decrease (M ± SEM)		EC_{50} of inotropic negative activity		EC_{50} of chronotropic negative activity		Vasorelaxant Activity[d]
comp	negative inotropic activity[a]	negative chronotropic activity[b]	EC_{50}[c] (μM)	95% conf lim ($x10^{-6}$)	EC_{50}[c] (μM)	95% conf lim ($x10^{-6}$)	Activity (M ± SEM)
27	90 ± 3.8[e]	54 ± 2.7[f]	0.071	0.021–0.14	0.86	0.74–1.01	34 ± 2.1[g]
28	46 ± 1.3[f]	36 ± 1.7					28 ± 1.5[h]
29	63 ± 4.7	30 ± 2.4	0.13	0.079–0.20			24 ± 1.8[h]
30	43 ± 3.3[i]	33 ± 2.6[j]					38 ± 1.5
31	77 ± 2.9	37 ± 3.5	0.18	0.13–0.23			34 ± 2.9[h]
32	63 ± 2.7[f]	22 ± 0.7	0.056	0.041–0.076			19 ± 1.3[h]
33	79 ± 3.1[f]	39 ± 1.5	0.103	0.08–0.15			47 ± 1.1[h]
34	73 ± 3.4[g]	29 ± 1.9[g]	0.081	0.056–0.11			29 ± 1.7[e]
35	54 ± 1.1[k]	95 ± 3.7[k]	1.97	1.71–2.31	4.89	3.50–6.83	28 ± 1.7
36	90 ± 3.7[k]	94 ± 2.0[g]	1.90	1.65–2.27	1.36	0.97–1.91	48 ± 1.7[e]
37	67 ± 2.0[g]	47 ± 1.4	0.075	0.050–0.093			47 ± 1.8
38	86 ± 0.7	69 ± 4.3[g]	0.83	0.60–1.04	2.41	1.85–3.15	26 ± 1.3
39	89 ± 3.3	78 ± 2.5	0.31	0.22–0.42	3.76	2.92–4.85	27 ± 1.3[h]
40	78 ± 0.9	58 ± 3.4	1.43	1.02–1.94	6.61	4.37–10.02	15 ± 0.9
41	55 ± 2.3[k]	84 ± 2.7[k]	0.90	0.56–1.44	0.98	0.73–1.34	11 ± 1.0
42	72 ± 3.6	92 ± 2.7	2.24	1.85–2.43	1.59	1.26–2.02	29 ± 2.4[h]
43	61 ± 2.7[k]	80 ± 1.9[j]	2.64	2.03–3.01	3.01	2.41–3.76	15 ± 0.9[h]
44	42 ± 0.2[f]	25 ± 1.5[g]					44 ± 3.7
45	42 ± 2.1[f]	64 ± 3.6[k]			18.52	14.19–24.16	32 ± 2.2
46	71 ± 0.9[e]	12 ± 0.9[k]	0.36	0.25–0.51			17 ± 0.9
47	91 ± 1.4	90 ± 0.8[j]	1.34	0.94–1.88	12.37	10.00–15.31	35 ± 1.7

[a] Decrease on developed tension in isolated guinea-pig left atrium at $5x10^{-5}$ M, expressed as percent changes from the control (n = 5-6). The left atria were driven at 1 Hz. [b] Decrease in atrial rate on guinea-pig spontaneously beating isolated right atrium at 10^{-5} M, expressed as percent changes from the control (n = 7-8). Pretreatement heart rate ranged from 165 to 190 beats/min. [c] Calculated from log concentration-response curves (Probit analysis by Litchfield and Wilcoxon with n = 6-7).[RS6] When the maximum effect was <50%, the EC_{50} ino., EC_{50} chrono., IC_{50} values were not calculated. [d] Percent inhibition of calcium-induced contraction on K^+-depolarized guinea-pig aortic strip at 10^{-4} M (n = 5-6). [e] At the 10^{-5} M. [f] At the 10^{-6} M. [g] At the $5x10^{-6}$ M. [h] At the $5x10^{-5}$ M. [i] At the $5x10^{-7}$ M. [j] At the $5x10^{-5}$ M. [k] At the 10^{-4} M.

Table S5. Relaxant Activity of Compounds previously described[RS1] on K^+-depolarized Guinea Pig Ileum Longitudinal Smooth Muscle.

Comp	Activity[a] (M ± SEM)	IC_{50}[b] (μM)	95% conf lim ($x10^{-6}$)
32	93 ± 0.5	2.06	1.55–2.73
36	51 ± 2.4[c]	0.083	0.066–0.103
37	81 ± 3.2[d]	1.95	1.54–2.47
44	64 ± 2.6[e]	0.55	0.43–0.69
46	86 ± 5.2[f]	11.04	8.18–14.90

[a] Percent inhibition of calcium-induced contraction on K^+-depolarized (80 mM) guinea pig longitudinal smooth muscle at 10^{-5} M. [b] Calculated from log concentration-response curves (Probit analysis by Litchfield and Wilcoxon with n = 6-7).[RS6] When the maximum effect was <50%, the IC_{50} values were not calculated. [c] At the 10^{-7} M. [d] At the $5x10^{-6}$ M. [e] At the 10^{-6} M. [f] At the 10^{-4} M.

Details for CFTR assays

1. Cell culture

Fischer rat thyroid (FRT) cells with stable experssion of F508del-CFTR and the halide-sensitive yellow fluorescent protein YFP-H148Q/I152L (12, 26) were cultured at 37 °C (5% CO_2 atmosphere) in Coon's modified Ham's F-12 medium plus 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Zeocin (0.6 mg/ml) and G418 (0.75 mg/ml) were also included in the medium to keep expression of transfected genes. For microplate reader experiments, cells were plated in clear bottom black 96-well microplates (Corning 3603) at a density of 50,000 cells per well. After 24 hours from plating, cells were incubated for further 20-24 hours at 27 °C to rescue F508del-CFTR to the cell surface.

2. Fluorescence assay for CFTR activity

Cells were washed with PBS (containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$) and stimulated for 30 min with forskolin (20 µM) in the absence or presence of genistein (50 µM). The 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 high-quality excitation (ET500/20X: 500 ± 10 nm) and emission (ET535/30M: 535 ± 15 nm) filters for YFP (Chroma Technology Corp., Brattleboro, VT). Each assay consisted of a continuous 14-s fluorescence reading with 2 s before and 12 s after injection of an iodide-containing solution (PBS with Cl^- replaced by I^-; final I^- concentration in the well: 100 mM). Data were normalized to the initial background-subtracted fluorescence. To determine fluorescence quenching rate (QR) associated with I^- influx, the final 11 s of the data for each well were fitted with an exponential function to extrapolate initial slope (dF/dt).

Details for functional assays.

1. Guinea-Pig Atrial Preparations. Guinea-pigs (300–400 g female) were sacrificed by cervical dislocation. After thoracotomy the heart was immediately removed and washed by perfusion through the aorta with oxygenated Tyrode solution of the following composition (mM): NaCl, 136.9; KCl, 5.4; $CaCl_2$, 2.5; $MgCl_2$, 1.0; $NaH_2PO_4xH_2O$, 0.4; $NaHCO_3$ 11.9; and glucose, 5.5. The physiological salt solution (PSS) was buffered at pH 7.4 by saturation with 95% O_2 – 5% CO_2 gas, and the temperature was maintained at 35 °C. The following isolated guinea-pig heart preparations were used: spontaneously beating right atria and left atria driven at 1 Hz. For each preparation, the entire left and right atria were dissected from the ventricles, cleaned of excess tissue, hung vertically in a 15 mL organ bath containing the PSS continuously bubbled with 95% O_2 – 5% CO_2 gas at 35 °C, pH 7.4. The contractile activity was recorded isometrically by means of force transducer (FT 0.3, Grass Instruments Corporation, Quincy, MA, USA) using Power Lab® software (AD-Instruments Pty Ltd, Castle Hill, Australia). The left atria were stimulated by rectangular pulses of 0.6–0.8 ms duration and about 50% threshold voltage through two platinum contact electrodes in the lower holding clamp (Grass S88 Stimulator). The right atria were in spontaneous activity. After the tissues were beating for several min, a length-tension curve was determined, and the muscle length was maintained at that which elicited 90% of maximum contractile force observed at the optimal length. A stabilization period of 45–60 min was allowed before the atria were challenged by various agents. During the equilibration period, the bathing solution was changed every 15 min and the threshold voltage was ascertained for the left atria. Atrial muscle preparations were used to examine the inotropic and chronotropic activity of the compounds (0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50 and 100 μM), first dissolved in DMSO and then diluted with PSS. According to this procedure, the concentration of DMSO in the bath solution never exceeded 0.3%, a concentration which did not produce appreciable inotropic and chronotropic effects. During the generation of cumulative concentration-response curves, the next higher concentration of the compounds was added only after the preparation reached a steady state. All data are presented as mean ± SEM. The EC_{50}, EC_{30} and IC_{50} were calculated from log concentration-response curves.[RS6]

2. Guinea-Pig Aortic Strips and Ileum Longitudinal Smooth Muscle (GPLSM).[RS1] The thoracic aorta and ileum were removed and placed in Tyrode solution of the following composition (mM): NaCl, 118; KCl, 4.75; $CaCl_2$, 2.54; $MgSO_4$, 1.20; KH_2PO_4, 1.19; $NaHCO_3$, 25; and glucose, 11; equilibrated with 95% O_2-5% CO_2 gas at pH 7.4. The vessel was cleaned of extraneous connective tissue. Two helicoidal strips (10 mm x 1 mm) were cut from each aorta beginning from the end most proximal to the heart. Vascular strips were then tied with surgical thread (6-0) and suspended in a jacketed tissue bath (15 mL) containing aerated PSS at 35 °C. Aortic strips were secured at one end to plexiglass hooks and connected via the surgical thread to a force displacement transducer (FT 0.3, Grass Instruments Corporation) for monitoring changes in isometric contraction. Aortic strips were subjected to a resting force of 1 g. The intestine was removed above the ileo-caecal junction. GPILSM segments of 2 cm length were mounted under a resting tension of 300-400 mg. Strips were secured at one end to a force displacement transducer (FT 0.3, Grass Instruments Corporation) for monitoring changes in isometric contraction and washed every 20 min with fresh PSS for 1 h. After the equilibration period, guinea-pig aortic strips were contracted by washing in PSS containing 80 mM KCl (equimolar substitution of K^+ for Na^+). When the contraction reached a plateau (about 45 min) various concentrations of the compounds (0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10 and 50 μM) were added cumulatively to the bath allowing for any relaxation to obtain an equilibrated level of force. Addition of the drug vehicle had no appreciable effect on K^+-induced contraction (DMSO for all compounds). All data are presented as mean ± S.E.M.. The IC_{50} were calculated from log concentration-response curves.[RS6]

3. Guinea-Pig Trachea.[RS7] The trachea was cut transversally between the segment of cartilage and four groups of tracheal segments, each one made up of thee rings, were tied together and mounted under a tension of 1 g at 37 °C in organ bath containing Krebs-Ringer solution of the following composition (mM): NaCl, 95; KCl, 4.7; $CaCl_2$, 2.50; $MgSO_4$, 1.0; KH_2PO_4, 1.17; $NaHCO_3$, 25; and glucose, 10.6; equilibrated with 95% O_2-5% CO_2 gas at pH 7.4. Propranolol hydrochloride (1 μM) was added in the Krebs-Ringer solution throughout the experiments outlined below to block ß-adrenoreceptors. The tissues were allowed to stabilize for 90 min. The tension was recorded isometrically. A constant level of tone was induced by the addition of carbachol

chloride (1 μM) to the organ bath. Once the sustained tension was established, the tissues were allowed to equilibrate for 30 min, a cumulative dose-response curve to the compounds were obtained.

Additional References.

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