

Isoxazolopyrimidines as Novel Δ F508-CFTR Correctors

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Abstract: Using a cell-based high-throughput screen, we identified isoxazolo[5,4-*d*]pyrimidines as novel small-molecule correctors of the cystic fibrosis mutant protein Δ F508-CFTR. 22 Isoxazolo[5,4-*d*]pyrimidine analogues were synthesized and tested. Synthesis of the key intermediate, 5-amino-3-arylisoazole-4-carboxamide, was accomplished by nitrile oxide cycloaddition to (2-amino-1-cyano-2-oxoethyl)sodium. Formation of 3-arylisoazolo-[5,4-*d*]pyrimidin-4(5*H*)-one and chlorination gave 4-chloro-3-arylisoazolo[5,4-*d*]pyrimidine. Finally, functionalization at C-4 of the pyrimidine ring by nucleophilic substitution gave the targeted isoxazolo[5,4-*d*]pyrimidines. Six of the reported analogues had low micromolar potency for increasing halide transport in Δ F508-CFTR cells.

Key words: cystic fibrosis, Δ F508-CFTR, corrector, isoxazolopyrimidine

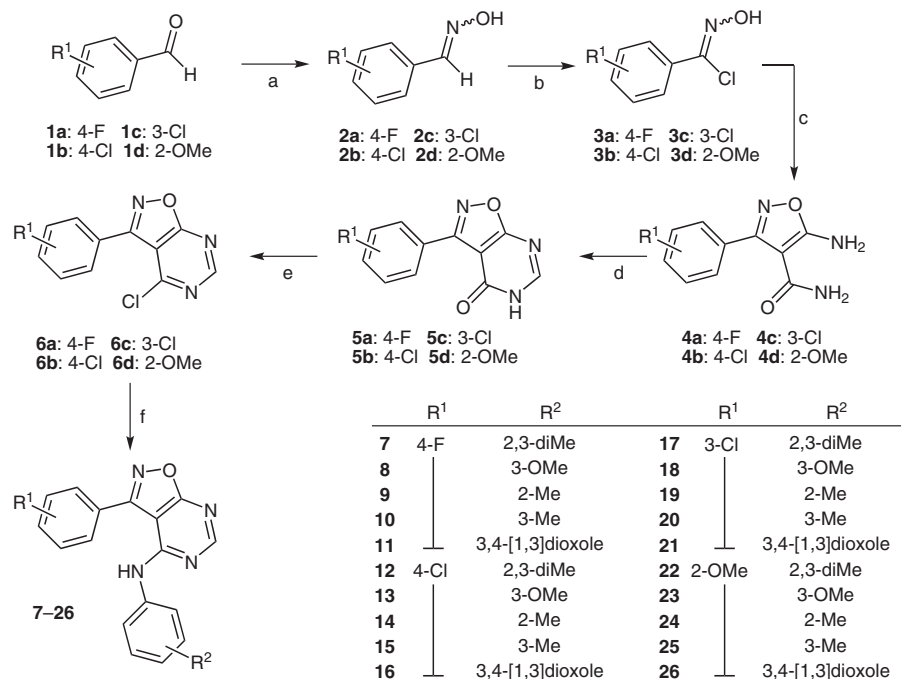
Cystic fibrosis (CF) is a relatively common inherited disease caused by mutations in the CF transmembrane conductance regulator protein (CFTR). The most common CFTR mutation, deletion of phenylalanine at residue 508, Δ F508, results in a defective CFTR protein that fails to traffic to the plasma membrane and fails to activate as a chloride channel.^{1–3} As a consequence a viscous mucus accumulates in the lung, which promotes bacterial growth and progressive deterioration of lung function.^{4,5} Although CF patient care has improved considerably, there is no cure for CF. While available CF-relevant drugs target the various symptoms of CF, many current studies are focused on the discovery of compounds that restore normal Δ F508-CFTR processing, which are called correctors.^{6–14}

Using screening methods we developed to discover bithiazoles as the first Δ F508-CFTR correctors,¹⁴ we screened a new collection of 50,000 diverse, drug-like small molecules (from Chemdiv Inc., San Diego CA). The goal was to identify new corrector scaffolds as potential drug candidates. Screening and secondary verification studies indicated isoxazolopyrimidines as Δ F508-CFTR correctors. Fused pyrimidines have broad-ranging biological activities, including antibacterial,¹⁵ CRF (corticotropin-releasing factor) antagonist,¹⁶ antimalarial,¹⁷ and antitumor activities.¹⁸ While isoxazolopyrimidines are not well studied, pyrimidine-based synthesis and application studies have been the focus of numerous research groups, includ-

ing Laitonjam et al.,¹⁹ Badiger et al.,²⁰ Wentrup et al.,²¹ Peinador et al.,²² and Sankyo Co., Ltd., Japan.²³ Herein, we report the synthesis and Δ F508-CFTR corrector activity of twenty-two novel isoxazolopyrimidines. To the best of our knowledge, this is the first report of these heterocycles as Δ F508-CFTR correctors.

Our synthetic route, illustrated in Scheme 1, began with hydroximoyl chloride formation from aryl aldehyde **1** in two steps in quantitative yield.²⁴ Hydroximoyl chlorides are well-established nitrile oxide precursors (via dehydrochlorination) that readily participate in 1,3-dipolar cycloadditions. Adapting a protocol reported by Rajagopalan and Talaty,²⁵ hydroximoyl chloride **3** was dissolved in ethanol and added dropwise to an ethanol suspension of (2-amino-1-cyano-2-oxoethyl)sodium to produce 5-amino-3-arylisoazole-4-carboxamide **4** (35–89% yield). Next, carboxamide **4** and triethyl orthoformate were dissolved in acetic anhydride, and the solution was refluxed for 2.5 hours to give 3-isoxazolo[5,4-*d*]pyrimidin-4(5*H*)-one **5** in 48–75% yield. Pyrimidinone **5** was treated with phosphorous oxychloride (0.83 equiv) in dry acetonitrile (alternatively, toluene can be used as solvent) at reflux in the presence of *N,N*-dimethylaniline (2 equiv) for 3 hours in a sealed tube under a N₂ atmosphere to afford 4-chloropyrimidine **6**.²⁶ The chlorination of **5a** to **6a** was also attempted with excess phosphorous oxychloride as described by Rajagopalan and Talaty.²⁵ However, after washing with water, only starting material **5a** was recovered suggesting that, under acidic conditions, water displaces the chloride to reverse the transformation (e.g., **6a** → **5a**). We also found that treating chloropyrimidine **6a** with 2,3-dimethylaniline under reflux in methanol for 24 hours in the presence of K₂CO₃ failed to deliver isoxazolopyrimidine **7** (4-chloro- → 4-hydroxypyrimidine occurred instead). Fortunately, target molecules **7–26** were obtained by refluxing an isopropanol solution of **6** with the appropriate aniline (2 equiv) in the presence of a catalytic amount of HCl gas.²⁷

Twenty isoxazolo[5,4-*d*]pyrimidine analogues (**7–26**) were synthesized using the method outlined in Scheme 1 by varying R¹ (four inputs) and R² (five inputs). Δ F508-CFTR corrector data for six active compounds are summarized in Table 1. Compounds were tested using a cell-based fluorimetry assay of iodide influx as described previously.³⁰ Corrector activity of each compound was verified by the lack of compound effect on FRT-null cells (not expressing Δ F508-CFTR) and by inhibition of the in-



Scheme 1 Reagents: (a) $\text{NH}_2\text{OH}\cdot\text{HCl}$, H_2O – EtOH – THF , NaOAc , 66–100%; (b) NCS , DMF – MeCl , Et_3N , pyridine (cat.), 60–95%; (c) i) 2-cyanoacetamide, NaOEt , EtOH , ii) **3**, EtOH , 35–89%; (d) triethyl orthoformate, Ac_2O , reflux, 2.5 h, 48–75%; (e) POCl_3 (0.83 equiv), N,N -dimethylaniline (2 equiv), $\text{Et}_3\text{N}\cdot\text{HCl}$ (2 equiv), MeCN , reflux under N_2 , sealed tube, 2.5 h, 83–92%; (f) 2,3-dimethylaniline, i - PrOH , HCl (gas), 30–76%.

creased iodide influx by the thiazolidinone CFTR inhibitor CFTR_{inh}-172. Compound **7** exhibited a greater V_{max} value than *s-cis* locked bithiazole **27** reported in our earlier studies.¹⁴ Compounds **10**–**14** also had comparable corrector activities (Table 1). An SAR trend is observed when the location of substituent *R* on the phenyl ring of the isoxazole portion of the molecule is varied. For instance, when $\text{R}^1 = 3\text{-Cl}$ or 2-OMe , corrector activity is diminished. Of the isoxazolopyrimidines tested, only those with a *para* substituent on the isoxazole phenyl moiety (4-F and 4-Cl) were active. This dramatic change in corrector activity in the 4-F or 4-Cl versus the 3-Cl or 2-OMe suggests that the aldehyde reagent employed in Scheme 1 is best fixed as 4-chloro- or 4-fluorobenzaldehyde. When R^2 is fixed as 2,3-dimethyl (e.g., employing 3,4-dimethylaniline in Scheme 1), isoxazolopyrimidines **7** and **12** had comparable IC_{50} values, although the V_{max} of **7** is much larger. The best IC_{50} value was obtained when 3-methoxyaniline (**13**) was employed.

The hydrogen bond donor/acceptor profile of these isoxazolopyrimidines was then considered. For example, we investigated changing the NH of isoxazolopyrimidine **7** to an NMe (Figure 1; **28** was prepared by N-methylating **7** with iodomethane in methanol + NaH). We also modified **7** (starting from **6a**) by replacing the 2,3-dimethylaniline moiety with a 3-methylpyridin-2-amine moiety to give analogue **29**. When comparing the LogP values of compound **7**, **28**, and **29** (Figure 1), there is no significant difference between **7** and **28**, while **29** has a lower LogP value (**7**: LogP 5.4813; **28**: LogP 5.7171; **29**: LogP 4.3731; data generated with ChemPropPro 8.03). Unfor-

Table 1 IC_{50} and V_{max} Data for Active Isoxazolopyrimidines^a

| Compound | R ¹ | R ² | IC ₅₀ μM | V _{max} μM/s |
|-----------|----------------|------------------|---------------------|-----------------------|
| 7 | 4-F | 2,3-diMe | 3.9 | 161 |
| 10 | 4-F | 3-Me | 5.8 | 113 |
| 11 | 4-F | 3,4-[1,3]dioxole | 5.7 | 90 |
| 12 | 4-Cl | 2,3-diMe | 3.9 | 109 |
| 13 | 4-Cl | 3-OMe | 3.2 | 100 |
| 14 | 4-Cl | 2-Me | 6.9 | 103 |

^a For comparison, IC_{50} and V_{max} of reference compound corr-4a¹¹ were 0.92 μM and 152 μM/s.

tunately, isoxazolopyrimidines **28** and **29** showed little corrector activity compared to **7**.

In summary, a collection of 4-aniline substituted derivatives of isoxazole[5,4-*d*]pyrimidines was synthesized. The synthetic protocol outlined in Scheme 1 is suitable to high-volume combinatorial analogue generation. The six isoxazolopyrimidine-fused heterocycles listed in Table 1 demonstrate corrector activity with low micromolar potency. Isoxazolopyrimidines might thus serve as novel

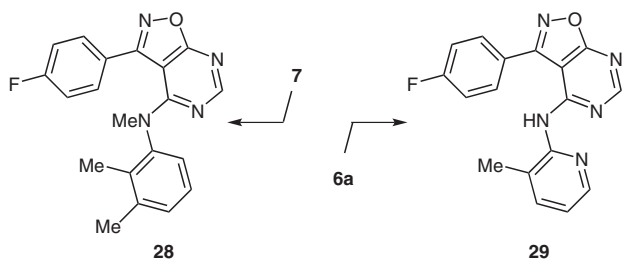


Figure 1 *N*-Methyl and pyridyl analogues of **7**

correctors to probe defective $\Delta F508$ -CFTR cellular processing and for further preclinical development.

4-Fluorobenzaldehyde Oxime (**2a**)

To a stirred solution of hydroxylamine-HCl (1.85 g, 26.6 mmol) in THF-EtOH-H₂O (30 mL:75 mL:15 mL) was added 4-fluorobenzaldehyde (**1a**, 3.0 g, 24.2 mmol), and the mixture was stirred at r.t. for 25 min at which time EtOH and THF were removed in vacuo. The residue was extracted with Et₂O (3 × 30), washed with brine, dried over anhyd Na₂SO₄, and filtered. Evaporation of the solvent afforded **2a** (3.36 g, 100%), which was used in the next step without further purification. ¹H NMR matches the literature data;²⁸ ESI-MS: *m/z* = 139.99 [M + H]⁺. Oximes **2b–d** were prepared following analogous procedures with or without NaOAc.

4-Fluoro-*N*-hydroxybenzimidoyl Chloride (**3a**)

To a stirred solution of NCS (3.55 g, 26.6 mmol) in DMF-CH₂Cl₂ (20 mL:120 mL) was added dropwise a CH₂Cl₂ (120 mL) solution of pyridine (200 μ L, 2.42 mmol), Et₃N (3.37 mL, 24.2 mmol), and 4-fluorobenzaldehyde oxime (**2a**; 3.36 g, 24.2 mmol). The solution was stirred at r.t. for 12 h at which time it was washed with H₂O (5 × 100 mL) and concentrated to afford **3a** (4.2 g, 100%). ¹H NMR matches the literature data.²⁸ Hydroxymoyl chloride **3b–d** were prepared following analogous procedures.

5-Amino-3-(4-fluorophenyl)isoxazole-4-carboxamide (**4a**)

A freshly prepared NaOEt in EtOH solution, made at r.t. from Na metal (977 mg, 42.5 mmol) in abs. EtOH (150 mL), was added to a stirred solution of 2-cyanoacetamide (3.57 g, 42.5 mmol) in abs. EtOH (50 mL) at 50 °C. To the resulting clear solution cooled to 0 °C was added dropwise a solution of hydroxymoyl chloride **3a** (7.38 g, 42.5 mmol) in abs. EtOH (100 mL). The resulting suspension was stirred at r.t. for 30 min and then refluxed overnight. EtOH was removed in vacuo, and the resulting residue was washed with H₂O and recrystallized from MeOH to yield **4a** as light yellow crystals (2.91 g, 31%).^{29a} Carboxamides **4b–d** were prepared following analogous procedures.

3-(4-Fluorophenyl)isoxazolo[5,4-*d*]pyrimidin-4(5*H*)-one (**5a**)

A mixture of **4a** (2.88 g, 13 mmol), triethyl orthoformate (2.16 mL, 13 mmol), and Ac₂O (15 mL) was refluxed overnight. The solution was cooled on ice, and the resulting precipitate was collected by filtration, washed with H₂O, and air dried to yield **5a** as an off white powder (2.05 g, 68%).^{29b} Pyrimidinones **5b–d** were prepared following analogous procedures refluxing for from 2.5–12 h.

4-Chloro-3-(4-fluorophenyl)-4,5-dihydroisoxazolo[5,4-*d*]pyrimidine (**6a**)

POCl₃ (167 μ L, 1.79 mmol) was added to a solution of **5a** (0.54 g, 2.16 mmol), *N,N*-dimethylaniline (411 μ L, 3.24 mmol), and Et₃N-HCl (0.6 g, 4.33 mmol) in dry MeCN (1.7 mL) under N₂ in a sealable tube. The mixture was refluxed for 3 h, cooled, and the

MeCN was removed in vacuo. The resulting residue was subjected to flash column chromatography purification (hexane-EtOAc = 9:1) to provide **6a** as off-white crystals (480 mg, 92%).^{29c} Pyrimidines **6b–d** were prepared following analogous procedures.

N-(2,3-Dimethylphenyl)-3-(4-fluorophenyl)-4,5-dihydroisoxazolo[5,4-*d*]pyrimidin-4-amine (**7**)

A mixture of **6a** (111 mg, 0.443 mmol), 2,3-dimethylbenzenamine (108 μ L, 0.885 mmol), and catalytic HCl (g) in 2-PrOH (2 mL) was sealed in a tube and refluxed overnight. Cooling on ice produced white crystals which were collected by filtration and washed with cold 2-PrOH to afford **7** (112 mg, 76%).^{29d} Isoxazolopyrimidines **8–26** and **29** were prepared following analogous procedures.

N-(2,3-dimethylphenyl)-3-(4-fluorophenyl)-4,5-dihydroisoxazolo[5,4-*d*]pyrimidin-4-amine (**28**)

To a solution of **7** (95 mg, 0.284 mmol) in DMF (3 mL) was added 60% NaH (26 mg, 0.643 mmol) in mineral oil. The suspension was stirred at r.t. for 30 min; MeI (40 μ L, 0.643 mmol) was added and the mixture stirred at r.t. overnight. The resulting mixture was washed with H₂O (3 × 10 mL), extracted with EtOAc (10 mL), washed with brine, dried over anhyd Na₂SO₄, and filtered. Removal of EtOAc afforded crude **28** which was subjected to flash chromatography (hexanes-EtOAc = 9:1 → 4:1) to deliver pure **28** (58 mg, 61%).^{29e}

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- (29) **Representative Spectral Data** (a) Compound **4a**: ^1H NMR (600 MHz, DMSO): δ = 7.65 (br s, 2 H), 7.63–7.58 (m, 2 H), 7.38–7.32 (m, 2 H). ^{13}C NMR (15 MHz, DMSO): δ = 171.70, 164.11, 163.85, 162.22, 159.78, 131.09, 131.04, 125.46, 125.44, 115.93, 115.79, 86.79. ESI-MS: m/z = 222.08 $[\text{M} + \text{H}]^+$. (b) Compound **5a**: ^1H NMR (300 MHz, DMSO): δ = 13.17 (br s, 1 H), 8.46 (s, 1 H), 8.38 (m, 2 H), 7.42 (m, 2 H). ESI-MS: m/z = 232.06 $[\text{M} + \text{H}]^+$. (c) Compound **6a**: ^1H NMR (600 MHz, CDCl_3): δ = 9.02 (s, 1 H), 7.92–7.77 (m, 2 H), 7.28 (m, 2 H). ^{13}C NMR (150 MHz, CDCl_3): δ = 175.19, 165.60, 164.12, 158.07, 157.03, 156.72, 132.17, 132.11, 122.57, 116.39, 116.24, 110.62, 77.37, 77.16, 76.95. (d) Compound **7**: ^1H NMR (600 MHz, CDCl_3): δ = 8.59 (s, 1 H), 7.85–7.69 (m, 2 H), 7.45 (d, J = 7.9 Hz, 1 H), 7.38–7.29 (m, 2 H), 7.16 (t, J = 7.7 Hz, 1 H), 7.10 (d, J = 7.5 Hz, 1 H), 6.81 (br s, 1 H), 2.31 (s, 3 H), 2.03 (s, 3 H). ^{13}C NMR (150 MHz, CDCl_3): δ = 176.12, 165.31, 163.63, 159.45, 157.18, 156.18, 138.28, 134.79, 131.06, 130.70, 130.64, 128.85, 126.29, 124.84, 123.31, 117.45, 117.30, 95.73, 20.71, 14.21. ESI-MS: m/z = 335.10 $[\text{M} + \text{H}]^+$. (e) Compound **28**: ^1H NMR (600 MHz, CDCl_3): δ = 8.67 (s, 1 H), 6.79 (m, 7 H), 6.56 (br s, 1 H), 3.35 (s, 3 H), 2.00 (s, 2 H), 1.96 (s, 3 H). ^{13}C NMR (150 MHz, CDCl_3): δ = 176.07, 163.97, 162.25, 160.25, 157.52, 144.82, 139.36, 133.03, 130.43, 129.88, 127.08, 123.56, 114.55, 96.77, 40.97, 20.28, 15.68. ESI-MS: m/z = 349.12 $[\text{M} + \text{H}]^+$.
- (30) **General Procedure for Bioassays – $\Delta 508$ -CFTR Corrector Activity Assay**
Assays were performed by utilizing FRT epithelial cells stably coexpressing human ΔF508 -CFTR and the high-sensitivity halide-sensing fluorescent protein YFP-H148Q/I152L used as described previously.¹¹ Cells were grown at 37 °C (95% air/5% CO_2) for 24 h and then incubated for 16–20 h with 50 μL of medium containing the test compound. At the time of the assay, cells were washed with PBS and then incubated with PBS containing forskolin (20 μM) and genistein (50 μM) for 20 min. Measurements were carried out using FLUOstar fluorescence plate readers (Optima; BMG LABTECH GmbH), each equipped with 500 ± 10 nm excitation and 535 ± 15 nm emission filters (Chroma Technology Corp.). Each well was assayed individually for I^- influx by recording fluorescence continuously (200 ms per point) for 2 s (baseline) and then for 12 s after rapid (<1 s) addition of 165 μL PBS in which 137 mM Cl^- was replaced by I^- . I^- influx was computed by fitting the final 11.5 s of the data to an exponential for extrapolation of initial slope. All experiments contained negative control (DMSO vehicle) and positive control corr-4a¹¹ ($\{N$ -[2-(5-chloro-2-methoxyphenylamino)-4'-methyl-4,5'-bithiazol-2'-yl]benzamide}). Background I^- influx (from DMSO control) was subtracted to report the increase in I^- influx in Table 1.

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