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3-(2-Benzyloxyphenyl)isoxazoles and Isoxazolines: Synthesis and Evaluation as CFTR Activators

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Abstract—A novel class of activators for chloride conductance in the cystic fibrosis transmembrane conductance regulator (CFTR) protein has been identified. These 3-(2-benzyloxyphenyl)isoxazoles and 3-(2-benzyloxyphenyl)isoxazolines were synthesized employing the 1,3-dipolar cycloaddition of nitrile oxides with various alkene and alkyne dipolarophiles. Utilizing a fluorescence cell-based assay of halide transport, the best compounds increased CFTR-dependent chloride transport with half-maximal stimulation at 20–50 μM.

Cystic fibrosis (CF), the most common lethal genetic disease in Caucasians, is caused by mutations in the cystic fibrosis transmembrane conductance regulator protein (CFTR).[1] CFTR is a cAMP-regulated epithelial cell membrane chloride channel that may also regulate the activities of other membrane proteins.[2] Mutations in CFTR produce chloride-impermeable epithelial cells in the airways, pancreas, and other tissues resulting in defective salt and water transport. Several chemical classes of small molecule CFTR activators have been identified, including flavones,[3] isoflavones,[4] benzimidazolones,[5] and benzoquinoliziniums[6] (Fig. 1).

Using a cell-based fluorescence screen for CFTR activators in which CFTR (wild-type or CF-causing mutants) is co-expressed in Fisher rat thyroid epithelial cells together with the halide indicator YFP-H148Q,[7] we recently identified two novel UCCF (*Univ. of Calif.-cystic fibrosis*) structural classes with CFTR activity: 7,8-benzoflavones (cf., **UCCF-029**; Fig. 2) and 1-aryl-1H-pyrazoles (cf., **UCCF-180**).[8] In addition, one isoxazole compound (**UCCF-152 = 1**) randomly screened in this same assay[8] was found to exhibit modest CFTR activity.[9] While we were apprehensive that the 'CH_2Cl' moiety of **1** might have skewed the results because this compound could potentially serve as an alkylating agent (especially so towards thiol nucleophiles), we were intrigued by this random screening result and set out to synthesize a small library of isoxazole and isoxazoline heterocyles based on **UCCF-152**. We report here that 3-(2-aryloxy-phenyl)isoxazoles and 3-(2-aryloxy-phenyl)isoxazolines do indeed represent another novel class of CFTR-activators.

Our investigation began with the preparation of isoxazoles **3** and **4** (Scheme 1). Thus, *o*-anisaldehyde was converted into oxime **2** by treatment with hydroxylamine using the bleach method for in situ nitrile oxide formation.[10] This oxime was then treated with NaOCl + propargyl chloride or propargyl alcohol in dichloromethane/water to effect the 1,3-dipolar cycloaddition.

We evaluated CFTR activation by measuring the rate of iodide influx as previously described.[8] Briefly, cells stably expressing human wildtype CFTR and the fluorescent protein YFP-H148Q were plated on 96-well microplates. After confluence, cells were washed in phosphate buffered saline, test compounds (50 μM final concentration) were added from DMSO stock solutions, and cells were subjected to a 100 mM inwardly directed iodide gradient at 37 °C. Iodide entry results in reduced YFP-H148Q fluorescence. Relative CFTR halide conductance was

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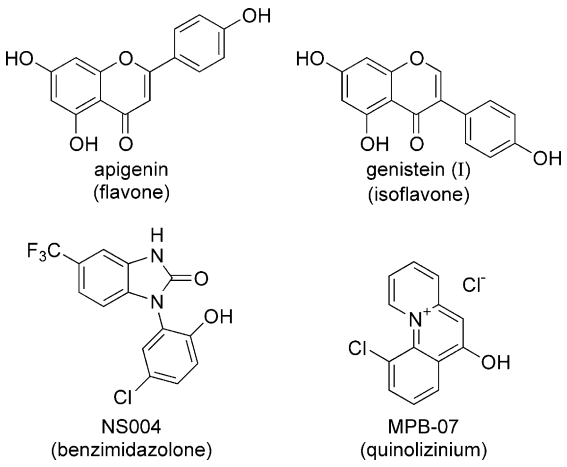


Figure 1. Activators of CFTR.

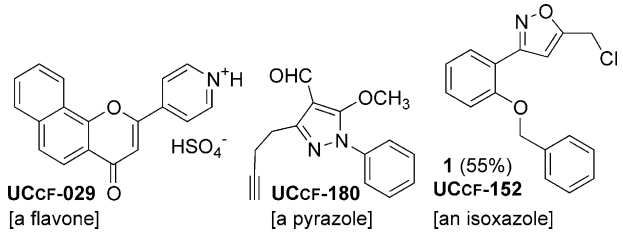
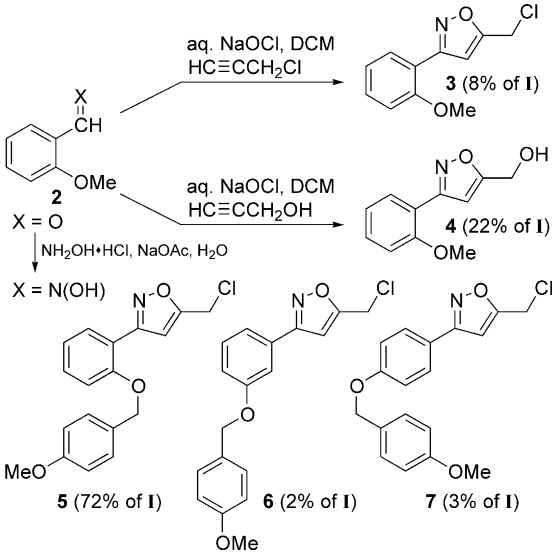


Figure 2. UCCF activators of CFTR.



Scheme 1. Pilot 3-arylisoxazole CFTR observations.

determined from the slope (fluorescence units/time) of the YFP fluorescence time course. Full dose–responses were carried out on the most active compounds.

Isoxazoles **3** and **4** gave very modest CFTR activation [8% and 22% of the activity elicited by 50 μM genistein (**I**; see Fig. 1)]. Fortunately, the next series of compounds—isoxazoles **5**–**7** gave much more encouraging activation (Scheme 1) and served to focus our further efforts. These isoxazoles were prepared from the corresponding *O*-alkyl-substituted benzaldehydes as described for the preparation of **3** and **4**.[11]

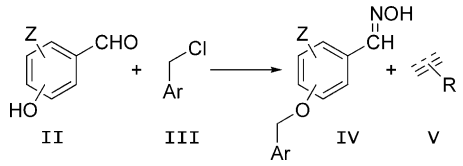


Figure 3. Components for library production.

The CFTR-activation results from isoxazoles **1** and **3**–**7** led us to conclude that: (a) a benzyloxy-type aryl substituent increases activity relative to a smaller methoxy substituent (**1** and **5** vs **3**); (b) a substituted benzyloxy increases activity relative to an unsubstituted benzyloxy (**5** vs **1**); (c) an *ortho*-substituent on the 3-phenylisoxazole increases activity relative to a *meta*- or *para*-substituent (**5** vs **6** and **7**); and (d) it appears that the isoxazole C5-substituent affects the CFTR activity (**3** vs **4**). With these initial CFTR activities in mind, we set out to use solution-phase techniques to prepare a small library of 3,5-disubstituted isoxazole derivatives to further refine these parameters.

As outlined in Figure 3, our two different hydroxy benzaldehydes (**II**) and four different benzylic alkylating agents (**III**) were incorporated to prepare the oximes (**IV**) used in 1,3-dipolar reactions with eleven different alkene/alkyne dipolarophiles (**V**). The 21 3-(2-benzyloxyphenyl)isoxazoles and 3-(2-benzyloxyphenyl)isoxazolines prepared in this way are depicted in Figure 4.[12] In nearly all cases, the reaction of **IV** with **V** (via the nitrile oxide intermediate) was highly regioselective giving only the 5-substituted isoxazoles or isoxazolines. The exception occurred with the methyl propiolate dipolarophile, which gave a 6:1 mixture of regioisomers with the major product still being 5-substituted isoxazole **13** (the 4-substituted isomer was removed by recrystallization of **13** from $CHCl_3/Et_2O$). In this specific case, electronic factors (electron withdrawing carbomethoxy substituent) begin to compete with steric factors in directing the cycloaddition regioselectivity.

Figure 4 reports the activity of the different compounds normalized to that of genistein (I; Fig. 1). One isoxazole (**10**; 106% activity relative to **I**) and two isoxazolines (**18** and **20**; 61% and 64% activity, respectively, relative to **I**) derivatives at 50 μM were found to have activity comparable to that of genistein. We determined dose-response relationships for all compounds. The K_d values obtained by fitting the results with the Hill equation are shown in (Fig. 4). On this basis, isoxazole **10** is the most potent of these compounds with a K_d of 23 μM, significantly better than that of genistein ($K_d = 40$ μM). The other compounds showed different degrees of activating potency with K_d values ranging from slightly higher than genistein to more than 300 μM.

According to functional data, we may conclude that the length and properties of the C5-substituent of the isoxazole ring are critical (compare for example compound **10** versus **8**, **9**, and **13**; the propanol is preferred over ethanol, CH_2Cl, and CO_2Me). The *meta*-bromo of compounds like **27** significantly reduce activity (compare

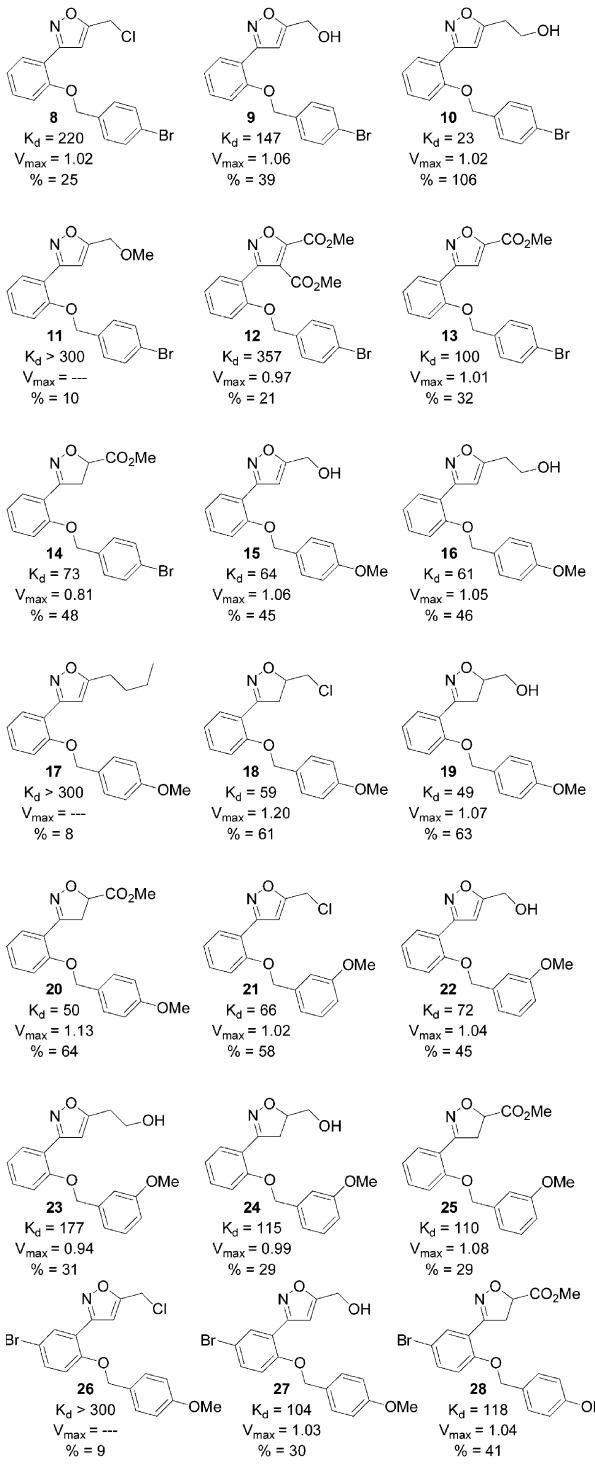


Figure 4. 3-(2-Benzyloxyphenyl)isoxazoles and 3-(2-benzyloxyphenyl)-isoxazolines; activity of compounds on CFTR is reported in terms of half effective concentration (K_d, in μM), Vmax (maximal I^- influx, in mM/s), and% of genistein activity at 50 μM. Each K_d and V_{max} value is the mean of three or more experiments and standard deviations (not shown for simplicity) were less than 10% of the corresponding mean value.

15 with **27**, and consider the very low activity of **26**). The substituents in the benzyloxy ring do not seem to consistently affect the activity (bromine seems to be better than methoxy in **10** vs **16**; however, methoxy is better than bromine in **9** vs **15**). Finally, these compounds were also tested against CFTR with the G551D mutation, but we found no activity. However, we hope that further isoxazole derivatives synthesized on the basis of this study may show improved properties against wild type as well as mutant CFTR.

In conclusion, our work has identified a new class of CFTR activators; an important result since increasing the spectrum of CFTR activators is essential in gaining a comparative perspective and in establishing possible consensus pharmacophores. This may in turn prove important in defining the mechanism of activation as well as in identifying the putative binding site.

Acknowledgements

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12. The experimental details and characterization for intermediates and final products can be provided upon request. Selected examples are included below. For **10**: IR 3279, 3083, 2951, 2878, 1602, 1468, 1245, 1014, 759 cm^{-1}; ^1H NMR δ 2.2 (br, 1H), 3.00 (t, $J=6.8$ Hz, 2H), 3.93 (t, $J=6.8$ Hz, 2H), 5.07 (s, 2H), 6.54 (s, 1H), 7.01 (m, 2H), 7.27 (d, $J=7.9$ Hz, 2H), 7.36 (td, 7.5 and 1.0 Hz, 1H), 7.49 (d, 8.3 Hz, 2H), 7.86 (dd, 7.9 and 1.8 Hz, 1H) ; ^{13}C NMR δ 30.3, 60.1, 69.7, 103.5, 112.8, 118.4, 121.4, 121.8, 128.8, 129.6, 131.0, 131.6, 135.5, 155.9, 160.0, 169.8. For **18**: IR 2998, 2953, 2836, 1598, 1513, 1450, 1241, 1031, 753 cm^{-1}; ^1H NMR (400 MHz) δ 3.36 (dd, $J=17.7$ and 6.2 Hz, 1H), 3.47 (m, 2H), 3.57 (dd, $J=11.2$ and 4.8 Hz, 1H), 3.80 (s, 3H), 4.82 (m, 1H), 5.00 (s, 2H), 6.92 (d, $J=8.6$ Hz, 2H), 6.97 (m, 2H), 7.33 (d, $J=8.6$ Hz, 2H), 7.37 (dd, $J=7.3$ and 1.8 Hz, 1H), 7.74 (dd, $J=7.6$ and 1.8 Hz, 1H) ; ^{13}C NMR (100 MHz) δ 41.0, 44.8, 55.1, 70.2, 79.6, 112.5, 113.9, 118.4, 120.9, 128.1, 129.2, 129.4, 131.4, 155.7, 156.6, 159.4. For **20**: IR 3016, 2997, 2928, 2893, 2836, 1740, 1599, 1514, 1450, 1241, 1038, 979 cm^{-1}; ^1H NMR δ 3.64 (m, 2H), 3.73 (s, 3H), 3.80 (s, 3H), 5.00 (s, 2H), 5.05 (m, 1H), 6.95 (m, 4H), 7.33 (m, 3H), 7.77 (dd, $J=7.9$ and 1.8 Hz, 1H); ^{13}C NMR δ 41.5, 52.4, 55.1, 70.2, 77.8, 112.5, 113.9, 117.9, 120.9, 128.0, 129.2, 129.6, 131.6, 155.5, 156.6, 159.4, 170.9. For **27**: IR 3417, 2947, 2835, 1612, 1514, 1450, 1243, 766, 629 cm^{-1}; ^1H NMR (400 MHz) δ 2.1 (br, 1H), 3.82 (s, 3H), 4.75 (s, 2H), 5.06 (s, 2H), 6.69 (s, 1H), 6.91 (m, 3H), 7.31 (d, $J=8.8$ Hz, 2H), 7.46 (dd, $J=9.0$ and 2.5 Hz, 1H), 8.06 (d, $J=2.5$ Hz, 1H); ^{13}C NMR (100 MHz) δ 55.3, 56.5, 70.7, 103.5, 113.4, 114.1, 114.8, 120.0, 127.9, 129.1, 132.0, 133.7, 155.4, 158.9, 159.6, 170.6.