Identification of Novel Inhibitors of the Transforming Growth Factor β1 (TGF-β1) Type 1 Receptor (ALK5)

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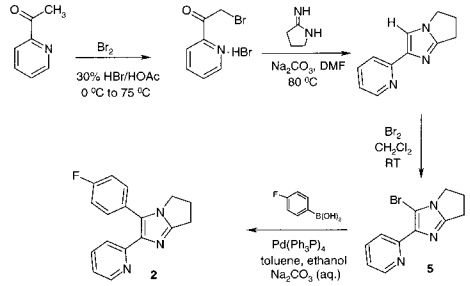
Abstract: Screening of our internal compound collection for inhibitors of the transforming growth factor β1 (TGF-β1) type I receptor (ALK5) identified several hits. Optimization of the dihydropyrroloimidazole hit **2** by introduction of a 2-pyridine and 3,4-methylenedioxyphenyl group gave **7**, a selective ALK5 inhibitor. With this information, optimization of the triarylimidazole hit **8** gave the selective inhibitor **14**, which inhibits TGF-β1-induced fibronectin mRNA formation while displaying no measurable cytotoxicity in the 48 h XTT assay.

Progressive fibrosis in the kidney, liver, heart, lung, bone marrow, and skin is a major cause of morbidity and mortality. A central player in this progressive fibrosis is transforming growth factor β1 (TGF-β1), which enhances extracellular matrix production by both increasing the transcription of matrix proteins, e.g., fibronectin and collagen, and inhibiting enzymes responsible for matrix degradation.[1] TGF-β1 signals through two highly conserved single transmembrane receptors with intracellular serine/threonine kinase domains.[2] Upon TGF-β1 binding, the type II receptor phosphorylates threonine residues in the GS domain of the ligand-occupied type I receptor or activin-like kinase (ALK5), which results in the activation of the type I receptors. The TGF-β type I receptor in turn phosphorylates Smad2 and Smad3 proteins, which translocate to the nucleus and mediate intracellular signaling. We propose that inhibition of ALK5 phosphorylation of Smad3 will reduce TGF-β1 induced extracellular matrix production.

Scheme 1. Synthesis of Dihydropyrroloimidazole Template



To identify inhibitors of the ALK5 kinase, a flashplate-based assay was developed with GST-tagged ALK5 as the kinase and GST-tagged full-length Smad3 as the immobilized substrate.[3] Screening of our internal compound collection for inhibitors of ALK5 resulted in the identification of several substituted imidazole inhibitors that were originally developed as inhibitors of p38 kinase.[4] Although these hits are good inhibitors of ALK5, they are in general much better inhibitors of p38. The substituted imidazole hits contain a pyridine ring that includes a 4′-nitrogen that is involved in a required hydrogen bond to the ATP site of p38.[5,6] An inhibitor, SKF-104365 (**1**), that contained a 2-pyridyl substituent was also identified in the screen. SKF-104365 (**1**) is a modest, ATP-competitive[7] inhibitor of ALK5 that does not inhibit p38. Although the corresponding carbon analogue **2** is also a selective inhibitor of ALK5, analogues containing either a 3- or 4-pyridyl substitution, e.g., **3** and **4**, lack ALK5 inhibitory activity. The lack of a 4′-nitrogen in **1** and **2**, which makes an essential hydrogen bonding interaction in p38 as well as other related kinases,[5,6] suggests that there may be an alternative binding site available to ALK5 inhibitors involving the 2′-pyridine that is not accessible in p38. In an attempt to increase the potency of these initial hits and to explore this novel pharmacophore, analogues that varied in the 2-phenyl substituent of **2** were synthesized utilizing the Suzuki coupling[8] of aryl boronic acids to the 2-bromoimidazole **5** (Scheme 1). Although the 4′-methoxyphenyl analogue **6** retains ALK5 activity, the 3,4-methylenedioxyphenyl analogue **7** displays significantly improved ALK5 inhibition (Table 1). This ALK5 inhibition translates into significant cellular activity. The inhibitor **7** inhibits TGF-β1-induced fibronectin (FN) mRNA (IC_{50} = 0.50 uM) in A498 cells.[9]

Taking these results into consideration, we initiated the exploration of the related triarylimidazole template. The screening hit, SB-202620 (**8**), that contains a 4-pyridyl substituent and is an essentially equipotent inhibitor of both ALK5 and p38 (Table 2) was the starting point for this lead optimization effort. A key feature of **8** that contributes to ALK5 inhibitory activity is the 4′-carboxyphenyl substituent because the corresponding sulfoxide-containing analogue, SB-203580[10] (**9**), displays both significantly reduced ALK5 activity and improved p38 inhibition. Replacement of the 4-pyridyl with the 2-pyridyl substituent derived from the dihydropyrroloimidazole series above gave analogue **10**

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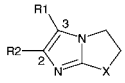
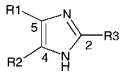
Table 1. Inhibitory Activity of Dihydropyrroloimidazole Analogues



Compound	R_1	R_2	X	ALK5 Inhibition IC_{50} (uM)	p38 Inhibition IC_{50} (uM)
1			S	1.6	> 50[b]
2			C	5.8	NA[b,c]
3			C	>50	ND[d]
4			S	>50	10[a]
6			C	5.1	NA[b]
7			C	0.46	NA[b]

[a] Inhibition of binding to p38. [b] Inhibition of p38 kinase activity. [c] NA = no significant inhibition at 16.7 μM. [d] ND = not determined.

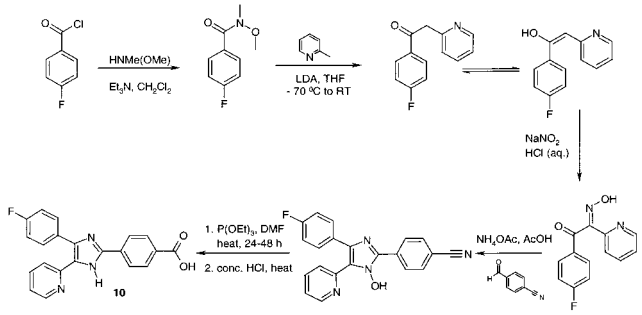
Table 2. Inhibitory Activity of Triarylimidazole Analogues



Compound	R_1	R_2	R_3	ALK5 Inhibition IC_{50} (uM)	p38 Inhibition IC_{50} (uM)
8				0.40	0.49[a]
9				6	0.042[a] 0.048[b]
10				1.5	NA[b,c]
11				0.17	NA[b]
12				2.95	15
13				0.26	NA[b]
14				0.094	NA[b]

[a] Inhibition of binding to p38. [b] Inhibition of p38 kinase activity. [c] NA = no significant inhibition at 16.7 μM.

Scheme 2. Synthesis of Triarylimidazole Template



(Scheme 2). Although **10** has reduced ALK5 inhibitory activity, it does not inhibit p38. As in the dihydropyrroloimidazole series, introduction of a 3,4-methylenedioxyphenyl ring, analogue **11**, significantly increased ALK5 activity without affecting selectivity vs p38. Although **11** is a better inhibitor than **7** in the ALK5 kinase assay (Table 2), **11** only exhibited modest activity in the cellular assay ($IC_{50} \approx 4$ uM, TGF-β1-induced FN mRNA).[9] This lower than expected cellular activity is most likely due to the presence of the carboxylic acid, a functional group that is known to limit cellular permeability. A series of related analogues were subsequently investigated that replaced the ionizable carboxyl group with various substituents capable of H-bond donor or acceptor interactions with the kinase. Analogue **12**, which lacks an H-bond-donating carboxylic acid, displays reduced ALK-5 inhibitory activity, while analogues **13** and **14** with carboxylic acid replacements that allow for H-bond donation retain the ALK-5 inhibition exhibited by **11**. The carboxamide-containing analogue, **14**, which is the most potent inhibitor of the series,[7] exhibits good cellular activity, inhibiting TGF-β1-induced (FN) mRNA formation in A498 cells with $IC_{50} = 0.05$ uM.[9] To further evaluate the cellular activity of **14**, TGF-β1-induced nuclear localization of Smad3 was examined. TGF-β1 causes the translocation of Smad proteins from the cytoplasm to the nucleus.[11] The Smad proteins were visualized in A498 cells by immunofluorescent antibodies raised against Smad3. Inhibitor **14** significantly reduced the TGF-β-induced nuclear accumulation of Smad proteins with an IC_{50} value of 0.04 μM. Of equal importance, **14** exhibits no measurable cytotoxicity in the 48 h XTT assay (LD_{50} > 30 uM).[12]

In conclusion, novel inhibitors of ALK5 have been identified that exhibit no measurable inhibition for p38 kinase, allowing for differentiation of the respective activation pathways. This class of inhibitors lacks the 4-pyridyl characteristic of related p38 inhibitors, suggesting the identification of a novel binding mode for these ALK5 inhibitors. In addition, **14** has been shown to inhibit TGF-β1-stimulated matrix protein mRNA without measurable cytotoxicity. These inhibitors are currently being used as pharmacological tools to examine various aspects of the TGF-β1 signaling pathway.

Supporting Information Available: Representative experimental procedures and spectral data for the preparation and characterization of the dihydropyrroloimidazole and triarylimidazole inhibitors are presented. This material is available free of charge via the Internet at http://pubs.acs.org.

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(3) A brief description of the flashplate assay for ALK5 follows. The kinase domain of TGFβRI, amino acid 200 to the C-terminus, and the full-length Smad3 protein were expressed as N-terminal glutathion S-transferase (GST) fusion proteins in baculovirus expression system. Proteins were purified with glutathion Sepharose beads 4B (Pharmacia Biotech, Sweden). Basic FlashPlates (NEN Life Sciences, Boston, MA) were coated with 0.1 M sterile filtered sodium bicarbonate, pH 7.6, containing 700 ng of GST-Smad3 per 100 μL. Assay buffer contained 50 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM DTT, 100 mM GTP, 3 μM ATP plus 0.5 μCi/well g33P-ATP, and 85 ng of

GST-ALK5 with or without compounds. Plates were incubated for 3 h at 30 °C. The assay buffer was removed by aspiration, and the plate was counted on a Packard TopCount 96-well scintillation plate reader.

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(12) A498 cells were seeded at 5000−10000 cells/well in 96-well plates. The cells were serum-deprived for 24 h and then treated with compounds for 48 h to assess the cellular toxicity. Cell viability is determined by incubating cells for 4 h with XTT labeling and electron coupling reagent according to the manufacturer's directions (Boehringer Mannheim). Live cells with active mitochondria produce an orange product, formazan, which is detected using a plate reader at 450−500 nm absorbance with a reference wavelength greater than 600 nm. The absorbance values correlates with the number of viable cells.

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