6-Amino-2-(4-fluorophenyl)-4-methoxy-3-(4-pyridyl)-1*H*-pyrrolo[2,3-*b*]pyridine (RWJ 68354): A Potent and Selective p38 Kinase Inhibitor

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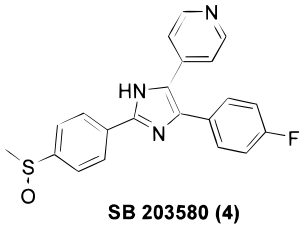
Introduction. Chronic inflammatory diseases such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD), and psoriasis affect millions of people worldwide. Current drugs treat only disease symptoms, rather than their root causes, and typically slow but do not prevent disease progression. For example, nonsteroidal antiinflammatory drugs (NSAIDs) are the most common treatment for RA patients, but these compounds do little or nothing to prevent ongoing destruction of cartilage and bone. The more powerful disease-modifying antirheumatic drugs (DMARDs), such as methotrexate and corticosteroids, are believed to slow disease progression but have more serious side effects.[1] It is now clear that inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) play important roles in these diseases.[2,3] Therapies aimed at blocking the functions of these cytokines are currently being tested in man. For example, Centocor's AVAKINE, a monoclonal antibody to TNF-α, Immunex's ENBREL, a TNF-α receptor fusion protein, and Amgen's IL-1 receptor antagonist are currently in clinical trials.

The mitogen-activated protein (MAP) kinase homologue p38 was first cloned following its identification as a kinase that was phosphorylated after stimulation of monocytes with LPS.[4] Later, a group of imidazoles binding to p38 were identified that blocked the production of TNF-α and IL-1β from LPS-stimulated monocytes.[5] Since then, it has become clear that p38 plays a key role in TNF-α and IL-1 release from monocytes as well as in the signaling cascades coupled to the TNF-α and IL-1 receptors.[6] Thus, inhibition of p38 would allow one to regulate the levels of TNF-α and IL-1, as well as prevent their actions in vivo, making it an attractive target for drug discovery. In fact, the development of p38 inhibitors is a goal of many pharmaceutical companies, creating a highly competitive and rapidly growing field. We wish to report our initial findings that RWJ 68354 (**1**) is a potent inhibitor of p38 kinase in vitro (IC_{50} 9 nM) and in vivo (ED_{50} <10 mg/kg po). This compound is also highly selective across a panel of assays and appears to be an attractive candidate for further preclinical evaluation.

Chemistry. The synthesis of **1** is quite straightforward and is outlined in Scheme 1. Condensation of siloxy ketone **2**[7] with diaminopyridine **3**[8] with H_2SO_4 as promoter gave the targeted compound **1** in 53% yield as the only detectable regioisomer.[9] A variety of analogues could be prepared using the same procedure and the appropriate diaminopyridine.

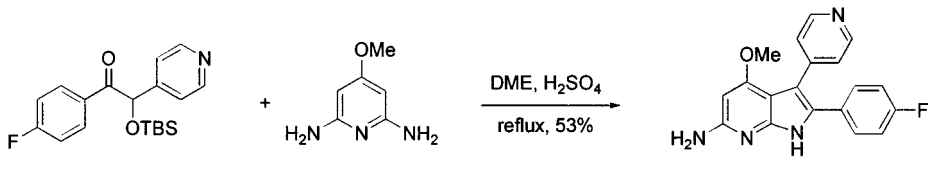
Results and Discussion. Our primary screen was an in vitro whole cell assay measuring a compound's ability to inhibit TNF-α production by human peripheral blood mononuclear cells (PBMCs). PBMCs were pretreated with various concentrations of test compound for 1 h and then stimulated with LPS. After incubating overnight, samples of cell supernatant were analyzed for TNF-α and IL-1β using commercial ELISA kits (Genzyme, Cambridge, MA). A series of compounds (Table 1) were run in this assay and compared to the literature standard SB 203580 (**4**).[10] As can be seen,



SB 203580 (4)

compound **5** (R = H) was roughly equipotent with the standard (IC_{50} 37 nM) for inhibition of TNF-α production. However, substituting an −OR group at the 4-position of the fused pyridine resulted in a significant increase in potency, with **9** having an IC_{50} of 0.91 nM. This increase in potency may be due, at least in part, to an electronic effect whereby the alkoxy group causes the electrostatic potential (ESP) at the 3-pyridyl nitrogen to become significantly more negative. The ESPs of compounds **1** and **5** were calculated at three different levels of theory (Figure 1),[11] and interestingly, the ESP at the 3-pyridyl nitrogen becomes more negative (1−4 kcal/mol) in compound **1**. Since it has been shown that the analogous pyridyl group in other p38 inhibitors participates in a critical hydrogen bond with the backbone carbonyl of Met 109,[12] and there is a direct correlation between the calculated ESP of an atom and its ability to accept a hydrogen bond,[13] increasing the ESP at the 3-pyridyl nitrogen should result in a stronger

Scheme 1



2 **3** **RWJ 68354 (1)**

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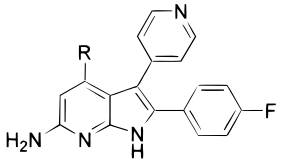
Table 1. Inhibitory Activity (IC_{50}, nM; $n \geq 3$) in Human PBMCs Stimulated with LPS or SEB



		LPS stimulus		SEB stimulus
R	compd	TNF-α release	IL-1β release	TNF-α release
	4	25 ± 11	48 ± 12	160 ± 130
H	**5**	37 ± 16	ND	450[a]
$Ph(CH_2)_3O$	**6**	9.0 ± 4.2	ND	ND
CH_3O	**1**	6.3 ± 4.4	26 ± 11	23 ± 18
$CH_3(CH_2)_3O$	**7**	4[a]	ND	ND
3-$OMePhCH_2O$	**8**	1.5 ± 0.5[b]	ND	ND
$PhCH_2O$	**9**	0.91 ± 0.12	ND	ND

[a] $n = 1$. [b] $n = 2$.

hydrogen bond and increased potency. Compound **1** was further determined to be a potent inhibitor of IL-1β release from LPS-stimulated PBMCs (IC_{50} 26 nM, Table 1), as would be expected from a p38 inhibitor.

The release of TNF-α from T-cells is an important event in immune system disorders such as rheumatoid arthritis, but p38 inhibitors are typically only tested in cellular assays stimulating monocytes or macrophages. To determine **1**'s ability to inhibit T-cell TNF-α production, human PBMCs were stimulated with staphylococcal enterotoxin B (SEB), a T-cell stimulus. Compound **1** was shown to have an IC_{50} of 23 nM in this system. In the same assay, IL-2 and IFN-γ secretion by T-cells was unchanged, indicating that **1** had no general immunosuppressive activity, as would be expected.

To confirm that the inhibition of TNF-α production in the cellular assay involved inhibition of the p38 kinase pathway, human monocytes were treated with **1** or **4** and then stimulated with LPS to activate the p38 signaling pathway. After 15 min, p38 or its in vivo substrate, MAP kinase-activated protein kinase-2 (MAPKAPK-2),[14] was immunoprecipitated from monocyte lysates and in vitro kinase assays were performed on each enzyme. Inhibition of immunoprecipitated MAPKAPK-2 activity is an indicator of the inhibition of p38 kinase within the cells and was measured by the phosphorylation of its in vivo substrate Hsp 27. Compound **1** had a mean IC_{50} of 9 nM when immunoprecipitated p38 kinase activity was measured and a mean IC_{50} of 4 nM when immunoprecipitated MAPKAPK-2 activity was measured. The corresponding values for **4** were 70 and 28 nM, respectively. These assays confirm the compounds' activity against the endogenous p38 kinase pathway. Compound **1** was further shown to directly inhibit p38 kinase in an in vitro kinase assay using p38 immunoprecipitated from LPS-treated human monocytes that were not pretreated with the compounds as the source of enzyme. In this system, **1** had a mean IC_{50} of 150 nM, while **4** had a mean IC_{50} of 800 nM. In a second system, partially activated commercial recombinant p38 was used. In this assay, the majority of p38 enzyme was in its inactive form, so accurate IC_{50} values could not be obtained since the compounds were titered out by binding to inactive enzyme. Nevertheless, compounds **1**, **5**, **7**, and **9** were confirmed to inhibit recombinant p38-mediated phosphorylation of myelin basic protein (compounds **6** and **8** were not tested). We believe it is reasonable to assume that the other compounds in this series are also potent inhibitors of p38 enzymatic activity, based on their structures and their inhibition of TNF-α release in the p38 cellular assay.

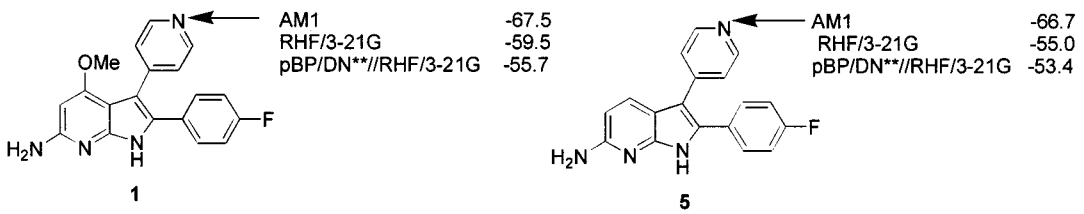
Table 2. Selectivity of p38 Inhibitors Against a Panel of Enzymes (% Inhibition at 5 μM Compound unless Otherwise Noted)

compd	$p56^{lck}$	c-src	PKA	ERK-2[a]	COX-1 IC_{50} (nM)
4	55 ± 2	54 ± 1	0	22	>5000
5	53	63	16	0	inactive
6	19	3	0	ND	ND
1	38 ± 6	43 ± 7	0	23	inactive
7	5	18	0	ND	inactive
8	56	58	0	ND	5000
9	18	19	0	ND	inactive

[a] 10 μM compound.

Compounds were then assayed for selectivity across a panel of other kinases (Table 2). Two src family tyrosine kinases, $p56^{lck}$ and c-src, were evaluated, with most compounds showing inhibition of less than 50% at 5 μM concentrations. None of the compounds significantly inhibited the unrelated serine/threonine kinase protein kinase A (PKA). Compounds **1** and **5** did not have significant activity against ERK-2, another member of the MAP kinase family, at 5 μM concentrations. Finally, all compounds were poor inhibitors of cyclooxygenase-1 (COX-1), an enzyme whose inhibition can result in serious gastrointestinal side effects, such as seen with many NSAIDs.

With potent and selective inhibitors in hand, we moved on to in vivo models. Female BALB/cJ mice were fasted and then orally dosed with a solution of the test compound or with vehicle alone. Thirty minutes after dosing, the animals were injected with LPS. After 1 h, blood serum was collected and tested by commercial ELISA (Endogen, Woburn, MA) for the presence of TNF-α. As seen in Table 3, all compounds inhibited the production of TNF-α almost completely at the 25 mg/kg dose, but large differences were seen at the 10 mg/kg dose. Compound **1** blocked TNF-α production by 85% at 10 mg/kg, while **4** inhibited by only 33%. Also, the



1 **5**

Figure 1. Calculated electrostatic potential (kcal/mol) at the 3-pyridyl nitrogens of compounds **1** and **5**.

Table 3. Percent Inhibition of TNF-α Release from Fasted BALB/cJ Mice Stimulated with 20 μg (~1 mg/kg) of LPS (n = 5/group/experiment)

compd	25 mg/kg po	no. of expts	10 mg/kg po	no. of expts
4	83 ± 11	63	33 ± 23	36
5	98 ± 0.5	2	73 ± 12	2[a]
1	99 ± 0.5	4	85 ± 6.7	7
7	71 ± 0.75	1	27 ± 0.34	1
9	86 ± 0.64	1	11 ± 2.3	1[a]

[a] n = 4.

most potent compound in the PBMC assay, **9**, inhibited by only 11% at 10 mg/kg. It appears that the larger, more lipophilic alkoxy groups are a detriment to in vivo activity, and this could be related to the bioavailability of these compounds. In two separate experiments, **1** was given orally (25 mg/kg) to male Lewis rats (n = 5/group/experiment) which were then injected with LPS. It was determined that **1** inhibited TNF-α production by 97 ± 1%. Finally, in two separate experiments, the effect of **1** was tested in the adjuvant-induced arthritis model. Male Lewis rats (n = 10/group/experiment) were dosed once daily with 50 mg/kg **1**, starting on the day of injection with adjuvant. Swelling in the rear paws was reduced by 50 ± 2% in the two experiments ($p < 0.001$ in each experiment). Similar results have been reported for **4** in the same model.[10]

Conclusion. We have shown **1** to be a potent inhibitor of cellular p38 kinase activity (9 nM), LPS-stimulated TNF-α/IL-1β production from human PBMCs (6.3 nM/26 nM), and LPS-induced TNF-α production in mice (ED_{50} <10 mg/kg) and in rats (ED_{50} <25 mg/kg). The compound was shown to directly inhibit natural activated p38 and partially activated recombinant p38 kinase. Compound **1** is more potent than the literature standard **4** in both in vitro and in vivo assays and is orally active. On the basis of these facts, **1** is a promising candidate for further preclinical evaluation as a potential phase I compound.

Supporting Information Available: Synthetic and biological procedures and characterization for all compounds (7 pages). Ordering information is given on any current masthead page.

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