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POTENT INHIBITORS OF THE MAP KINASE p38

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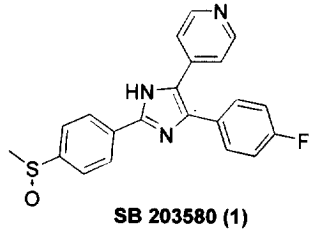
Abstract: The MAP kinase p38 plays a key role in the biosynthesis of the inflammatory cytokines TNF-α and IL-1. We have developed a novel series of potent p38 inhibitors that could lead to new methods of treatment for inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease.

It is well established that the pro-inflammatory cytokines TNF-α and IL-1β play important roles in inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, and psoriasis.[1,2] Since the mitogen-activated protein (MAP) kinase p38 was first cloned following its identification as a kinase that was phosphorylated after stimulation of monocytes with lipopolysaccharide (LPS),[3] it has become clear that p38 plays a key role in TNF-α and IL-1β release from monocytes. Because p38 is also involved in the signaling cascades coupled to the TNF-α and IL-1 receptors,[4] inhibition of p38 activity in vivo would be expected to regulate both the levels and the effects of TNF-α and IL-1β, making it an attractive target for drug discovery. The first series of compounds shown to inhibit p38, the triarylimidazoles, was reported by SmithKline Beecham, and is exemplified by SB 203580 (**1**),[5] a potent p38 inhibitor both in vitro and in vivo. We now report the development of a novel series of p38 inhibitors, the best of which are several fold more potent than SB 203580 in a p38-dependent cellular assay.



SB 203580 (1)

Since it has been established that the 4-fluorophenyl and 4-pyridyl groups on the imidazole of **1** are critical to binding, we concentrated our work on the left hand portion of the molecule. Our first compounds

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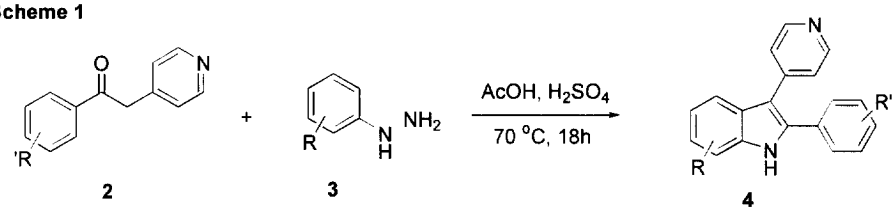
were derivatives prepared by a Fisher indole synthesis with ketone **2**[6] and hydrazine **3** (Scheme 1). The reactions were run in acetic acid as solvent with catalytic H_2SO_4 at 70 °C for 18 h to give **4** in yields ranging from 30 - 50% depending on the substituents on the hydrazine.[7,8]

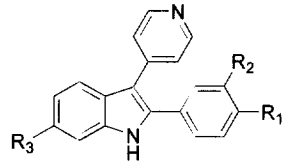
Scheme 1



2 **3** **4**

A series of these compounds were prepared and evaluated in our primary assay, where human peripheral blood mononuclear cells (PBMCs) were stimulated with LPS, and the compounds ability to inhibit TNF-α production was measured.[9] These results are summarized in Table 1. While none of these compounds were as potent as SB 203580 (**1**), several things were learned from the data. The key point is that polar groups are preferred at R_3. Compound **11** (R_3 = H) is inactive up to 10000 nM, while **9** (R_3 = MeO) is 400 nM and **7** (R_3 = NH_2) is 200 nM. It is clear from this jump in potency that R_3 is in a polar region on the enzyme (perhaps a hydrogen bond interaction). Secondly, differing halogen substituents at R_2 than R_1 affect potency. Indole **5** (R_2 = I) is 65 times more potent than **10** (R_1 = I). This difference appears to become less pronounced as the size of the halogen decreases as **8** (R_2 = F) and **9** (R_1 = F) are nearly equipotent.

Table 1. Inhibition of TNF-α production by LPS-stimulated human PBMCs.

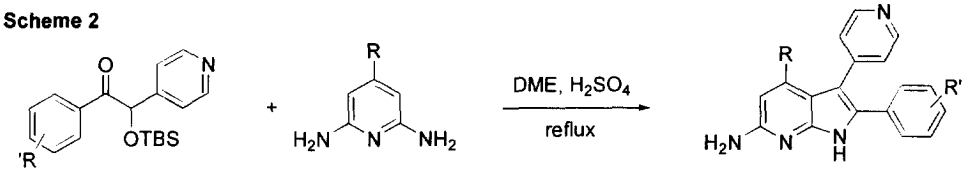


Compd	R_1	R_2	R_3	IC_{50} (nM)
1				25
5	H	I	MeO	100
6	H	Cl	MeO	125
7[a]	F	H	NH_2	200
8	H	F	MeO	300
9	F	H	MeO	400
10	I	H	MeO	6500
11	F	H	H	>10000

[a]Prepared by the method of Scheme 2 using 1,3-phenylenediamine.

We reasoned that further exploitation of the polar region of the enzyme found by R_3 could lead to more potent compounds, and so we prepared a series of pyrrolopyridines (Scheme 2).

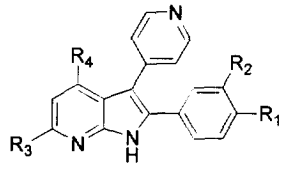
Scheme 2



12 **13** **14**

These compounds could be prepared via a modified Bischler-Möhlau indole synthesis by refluxing ketone **12**[6] with diaminopyridine **13** in DME and 5-6 equivalents of H_2SO_4 for 4 - 8 h to give the products **14** in reasonable yields.[10] The results from the PBMC assay are listed in Table 2.

Table 2. Inhibition of TNF-α production by LPS-stimulated human PBMCs.



Compd	R_1	R_2	R_3	R_4	IC_{50} (nM)
15	F	H	NH_2	$PhCH_2O$	0.91
16	F	H	NH_2	3-$OMePhCH_2O$	1.5
17	F	H	NH_2	4-$FPhCH_2O$	2.0
18	F	H	NH_2	BuO	4.0
19	F	H	NH_2	MeO	6.3
20	F	H	MeO	BuO	6.0
21	H	Cl	NH_2	H	9.0
22	F	H	NH_2	$Ph(CH_2)_3O$	9.0
23	F	H	OH	BuO	15
24	F	F	NH_2	H	30
25	F	H	NH_2	H	37
26	H	I	NH_2	H	40
27	EtO	Cl	NH_2	H	60
28	F	H	NHAc	H	100
29	F	H	MeO	H	200
30	F	H	NHBn	H	300
31[a]	F	H	H	H	400
32	F	H	NMe_2	H	400
33	F	H	OH	H	>10000

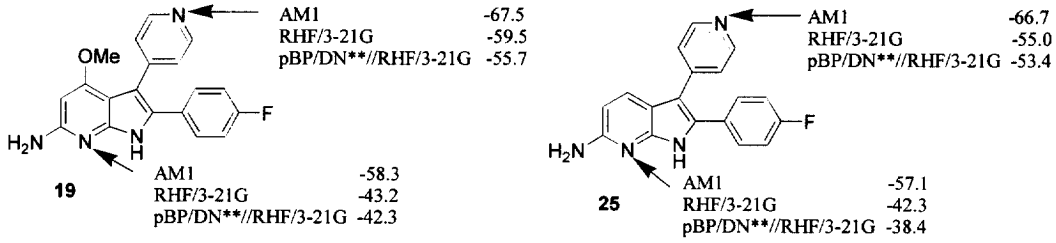
[a]Prepared by the method of Scheme 1.

Compound **25** (37 nM) was the first of the pyrrolopyridines to be prepared, resulting in a fivefold increase in potency over indole **7** (200 nM), the analogous compound from the indole series of compounds. This result further confirmed our belief that the region of the enzyme around R_3 was a hydrophilic area. Further insight was gained when we found that substituting the NH_2 of R_3 in these compounds (i.e., **28**, **30**, and **32**) resulted in a loss of potency. More striking, however, was compound **15**. By placing a benzyloxy substituent at R_4 the IC_{50} dropped to 0.91 nM. This compound is 37 times more potent than compound **25**, and 25 times more

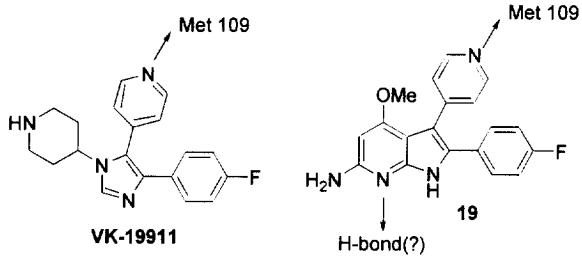
potent than the literature standard SB 203580 (compound **1**, Table 1). Other compounds with alkoxy groups at R_4 were also extremely potent, with IC_{50}s ranging from 9 nM (**22**) to 1.5 nM (**16**).[11]

We were intrigued by the increased potency of compound **15**, and set out to further analyze this result. As mentioned, we hypothesized that the R_3 region of the molecule binds in a hydrophilic pocket of the enzyme, and may be accepting a hydrogen bond. It has been shown that there is a good correlation between the calculated electrostatic potential (ESP) of a given atom in a molecule and its ability to accept a proton in a solute-solvent hydrogen bond.[12] We felt it was reasonable then, that the ESP would also correlate with an atom's ability to accept a proton in an enzyme-ligand hydrogen bond. To explore this further, the ESPs of the simple alkoxy analog **19** and the parent pyrrolopyridine **25** were calculated at three different levels of theory (Figure 1).[13] Not surprisingly, the ESP at the fused pyridine nitrogen becomes more negative (1-4 kcal/mol) by adding a methoxy group in the para position. What is surprising is that the ESP at the other pyridine nitrogen also becomes more negative by roughly the same amount.

Figure 1. Electrostatic Potential at Pyridyl Nitrogens (kcal/mol)



Workers at Vertex have reported on the crystal structure of VK-19911 bound to the p38 kinase.[14] This crystal structure shows a key hydrogen bond between the pyridyl nitrogen and Met 109. Its seems reasonable to assume that the pyridyl nitrogen in our structures hydrogen bonds to the same residue, and increasing its electrostatic potential would likewise increase its binding affinity. This electrostatic potential study also adds further credence to the idea of a hydrogen bonding interaction in the R_3 region of the molecule.



To confirm that the inhibition of TNF-α release in our assays involved p38, human monocytes were treated with **19** or **1** and then stimulated with LPS to activate the p38 signaling pathway. After 15 min, p38 or its in vivo substrate (MAPKAPK2) were immunoprecipitated and in vitro kinase assays were performed.

Decreased MAPKAPK2 activity is a direct indicator of the inhibition of p38 kinase within the cell. In this assay, **19** had a mean IC_{50} of 4 nM, while **1** had a mean IC_{50} of 28 nM. In parallel assays measuring immunoprecipitated p38 activity, **19** had a mean IC_{50} of 9 nM, while **1** had a mean IC_{50} of 70 nM. Compounds **1** and **19** were 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, **19** had a mean IC_{50} of 150 nM, while **1** had a mean IC_{50} of 800 nM. Lastly, a qualitative in vitro p38 MAP kinase assay using partially activated commercial recombinant p38 was run using some of the most active compounds. In this assay, the majority of p38 enzyme was in its inactive form, yet appeared to bind inhibitors. Thus, accurate IC_{50} values could not be obtained, since the compounds were titered out by binding to inactive enzyme. Nevertheless, all compounds tested (**5**, **6**,**15**, **18**, **19**, **20**, **21**, **23**, **25**, **26**, **28**) were confirmed to inhibit recombinant p38-mediated phosphorylation of the substrate, myelin basic protein. 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.

In summary, we have developed a series of potent p38 kinase inhibitors with excellent cellular activity. The most potent compound, **15**, has an IC_{50} of 0.91 nM for the inhibition of TNF-α release, 25 times more potent than the literature standard SB 203580 in the same assay. This marks a significant increase in activity over our earliest compounds, and is in part due to the discovery of an apparent hydrophilic binding region on the p38 enzyme which was exploited by polar groups in the area of R_3.

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7. 3-Methoxyphenylhydrazine/HCl (0.98 g, 5.6 mmol) and ketone **2** (R = 3-I)(1.8 g, 5.6 mmol) were dissolved in 20 mL of acetic acid and 5 drops H_2SO_4 and heated at 80 °C for 18 h, cooled, made basic with 15% KOH, extracted with EtOAc, dried and concentrated. Trituration of the residue with CH_2Cl_2 gave 0.58g of **5**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.58 (2H, d, J = 5.1 Hz), 7.83 (1H, s), 7.71 (1H, d, J = 9.4 Hz), 7.49 (1H, d, J = 9.4Hz), 7.39 (1H, d, J = 8.6 Hz), 7.35 (2H, d, J = 5.1 Hz), 7.09 (1H, t, J = 8.6 Hz), 6.95 (1H, s), 6.78 (1H, d, J = 8.6 Hz), 3.83 (3H, s). mp 271 - 272 °C.
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9. Peripheral blood mononuclear cells (PBMC) were harvested from human blood and suspended in low endotoxin RPMI-1640 culture medium (Sigma Chemical Co., St. Louis, MO) containing 1% fetal calf serum and 1x penicillin-streptomycin-glutamine (Life Technologies, Gaithersburg, MD) at 1.67 x 10^6 cells/ml. Cells (180 μL) were added to duplicate wells of a flat-bottom 96-well plate and allowed to settle for 1 h at 37 °C. Following cell plating, 10 μL of test compounds or vehicle (2% DMSO) were added to each well and the plate was incubated for 1 h at 37 °C. Finally, 10 μL/well of lipopolysaccharide (LPS, Sigma) (200 ng/mL) was added, for a final concentration of 10 ng/mL. Plates were incubated overnight at 37 °C, 5% CO_2. Supernatants were harvested, diluted 1/5, and assayed for TNF-α by ELISA as described by the manufacturer (Genzyme, Cambridge, MA).
10. 2,6-Diaminopyridine (0.63 g, 5.8 mmol) and ketone **12** (R = 4-F)(1.0 g, 2.9 mmol) were dissolved in 5 mL of DME and conc. H_2SO_4 (0.80 mL) was added. The mixture was then refluxed for 4 h, cooled to rt, poured into 100 mL of water and neutralized with solid K_2CO_3. The aqueous phase was extracted with ethyl acetate (3 x 50 mL). The organics were dried with sodium sulfate and concentrated. Trituration with 30 mL of ethyl acetate gave compound **25** as an off white solid (0.44 g). ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.61 (1H, s), 8.48 (2H, d, J = 7.6 Hz), 7.63 (1H, d, J = 8.6 Hz), 7.42 (2H, m), 7.23 (4H, , 6.36 (1H, d, J = 8.6 Hz), 5.87 (2H, s); mp 274 - 276 °C.
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