Letters

Development of Orally Bioavailable Bicyclic Pyrazolones as Inhibitors of Tumor Necrosis Factor-α Production

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Abstract: 2-Aryl-3-pyrimidinyl based tumor necrosis factor-α (TNF-α) inhibitors, which contain a novel bicyclic pyrazolone core, are described. Many showed low-nanomolar activity against lipopolysaccharide-induced TNF-α production in monocytic cells. Secondary screening data are presented for the pyrimidinyl bicyclic pyrazolones. Several of these analogues showed good oral bioavailability in rat and efficacy in the rat iodoacetate in vivo model.

In our continuing efforts toward the development of disease-modifying treatments for osteoarthritis, we are targeting the development of novel mitogen-activated protein (MAP) kinase inhibitors. MAP kinases constitute a part of the signal transduction pathway responsible for the relay of information from the cell surface to the nucleus. MAP kinases, such as the p38 and JNK (c-Jun amino terminal kinase) families, are activated in response to infection, cellular stress, and the proinflammatory cytokines TNF-α (tumor necrosis factor) and IL-1β (interleukin-1).[1] The overexpression of these cytokines has been implicated in the pathogenesis of a number of serious inflammatory disorders such as rheumatoid arthritis (RA),[2] osteoarthritis (OA),[3] and Crohn's disease. The effective therapeutic treatment of inflammatory diseases such as RA with the monoclonal anti-TNF-α antibody Remicade (infliximab), the TNF-α receptor fusion protein Enbrel (etanercept), and the soluble IL-1 receptor antagonist Anakinra (kineret) has been hampered by the high cost and inconveniences associated with these biologics. Therefore, a low molecular weight, orally bioavailable molecule that suppresses TNF-α production remains an attractive target. It has been established that agents that inhibit the enzyme p38 MAP kinase can decrease levels of these proinflammatory cytokines[1] and thereby reduce inflammation and prevent further tissue destruction.

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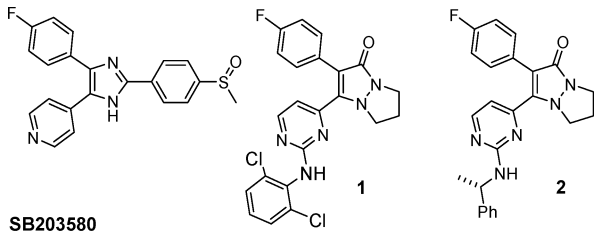

Figure 1. Vicinal aryl/pyridinyl (pyrimidinyl) TNF-α inhibitors.

The prototypical p38 inhibitor (SB-203580)[4] containing the 4-pyridinylimidazole motif was found to be competitive with ATP and to interact with the p38 ATP binding site. It is known that many other relevant kinases, including the JNK and ERK MAP kinases, that share structural and functional homology with p38 can be modulated with ATP mimetics as inhibitors.[5] Additionally, it is estimated that 518 kinases exist in the human genome.[6] Therefore, kinase specificity could prove to be a major hurdle in designing safe MAP kinase inhibitors.

Our research strategy involves designing kinase inhibitors that will result in the inhibition of TNF-α formation. Since multiple kinases exist within the signaling cascade, there exist several points at which one may effectively inhibit TNF-α formation. By optimization of the TNF-α inhibitory activity rather than utilizing a single kinase assay, the end result is establishing efficacy by shutting down the overall production of TNF-α. Therefore, our primary screening assay entails evaluating analogues for lipopolysaccharide (LPS) stimulated TNF-α inhibition in the THP-1 cellular assay.[7] Herein, we report a new structural class of 2-aryl-3-pyrimidinyl-based TNF-α inhibitors that contain a novel bicyclic pyrazolone core. Our investigations have yielded amino-substituted pyrimidine bicyclic pyrazolone analogues, such as phenylaminopyrimidine **1** and alkylaminopyrimidine **2**, as extremely potent inhibitors of TNF-α production (Figure 1).

Synthesis of bicyclic pyrazolone (**1**) was accomplished in seven steps from the known pyrazolidine **3**[8] (Scheme 1). Removal of the Boc protecting group, acylation with 4-fluorophenylacetyl chloride, and hydrogenolysis of the Cbz protecting group affords the amine **4**. Acylation of amine **4** with the pyrimidine acid chloride **5**[9] and base-induced cyclization of the resulting bisamide provide the intermediate bicyclic pyrazolone **6**. Oxidation of the sulfide **6** to the sulfone allows for facile displacement with 2,6-dichloroaniline to afford bicyclic pyrazolone **1**.

Table 1 summarizes a preliminary survey of phenylamino moieties introduced to the 2-position of the pyrimidine ring of the bicyclic pyrazolones. Examination of the results demonstrates that small electron-withdrawing substituents at the ortho position of the aniline ring tend to increase potency (**1** and **7**) relative to the

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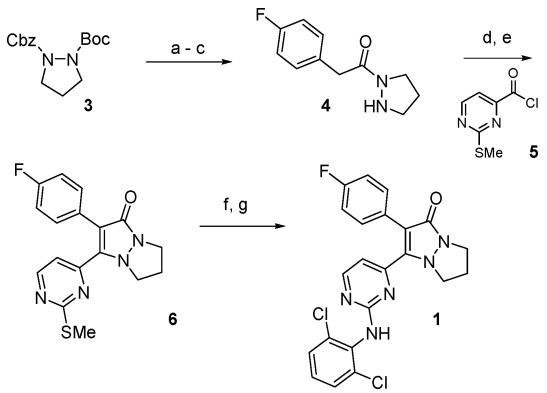
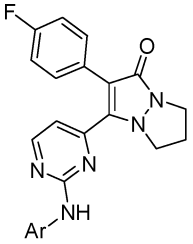
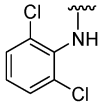
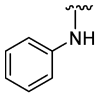
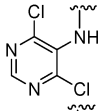
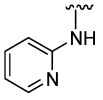
Scheme 1. Synthetic Scheme for Phenylaminopyrimidine Bicyclic Pyrazolone **1**[a]



[a] Reagents and conditions: (a) $SOCl_2$, MeOH, 90%; (b) 4-fluorophenylacetyl chloride, NaOH, H_2O/CH_2Cl_2, 96%; (c) H_2, Pd/C, methanol, 83%; (d) acid chloride **5**, NaOH, H_2O, CH_2Cl_2, 90%; (e) NaH, DMF, 0 °C, 52%; (f) oxone, THF/MeOH/H_2O; (g) NaH, 2,6-dichloroaniline, THF, 50%, two steps.

Table 1. TNF-α Inhibition Values of the Phenylaminopyrimidine Bicyclic Pyrazolone Analogues



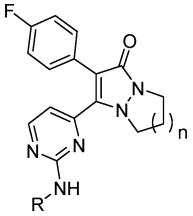
Compound	NH-Ar	TNF-α IC_{50} (nM)[a]
1		4
7		6
8		80
9		476
10		600

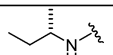
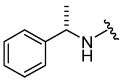
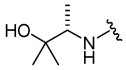
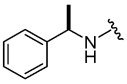
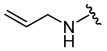
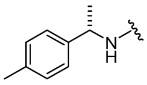
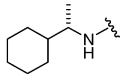
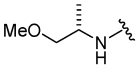
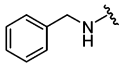
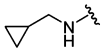
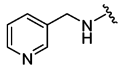
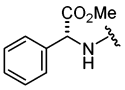
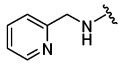
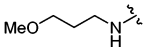
[a] IC_{50} of LPS-stimulated TNF-α production in human monocytic cells (THP-1). Standard deviation for assays is typically ±30% of the mean or less.

meta and para positions (data not shown) and to the unsubstituted aniline (**8**). Replacement of the phenyl ring with a heterocycle, such as a pyridine or pyrimidine ring, dramatically reduced the potency (**9** and **10**) relative to the aniline (**8**) derivative.

The related alkylaminopyrimidine bicyclic pyrazolone series was also synthesized from the intermediate **6**. Nucleophilic displacement of the sulfone functionality with a wide array of alkylamines was carried out at higher temperatures in toluene. Yields for the sulfone displacement ranged from 30% to 65%.

Table 2. TNF-α Inhibition Values of the Alkylaminopyrimidine Bicyclic Pyrazolone Analogues



Compound	Amine (NH-R)	n	TNF-α IC_{50} (nM)[a]
11		1	2
12		2	106
2		1	3
13		2	29
14		1	22
15		2	260
16		1	24
17		1	27
18		1	30
19		1	37
20		1	38
21		1	50
22		2	543
23		1	74
24		1	78
25		1	94
26		1	121
27		1	147
28		1	263
29		1	301

[a] IC_{50} of LPS-stimulated TNF-α production in human monocytic cells (THP-1). Standard deviation for assays is typically ±30% of the mean or less.

Table 2 summarizes a broad survey of alkylamino bicyclic pyrazolone analogues. Examination of the more potent inhibitors in Table 2 demonstrates a greater tolerance for diverse substituents in the alkylamino series not seen in the aniline appendages in Table 1. In general, the most potent analogues possessed an α-methyl group on the amino substituent, as exemplified by comparison of **2** and **11**−**17** relative to **23**. One of the most active compounds (**2**) was 10-fold more potent than its corresponding (*R*)-methylbenzylamino[10] enantiomer **17**. Comparison of the [5,5]- and [5,6]-bicyclic pyrazolone scaffolds shows more than a 10-fold decrease

Table 3. Solubility, Metabolism, and Rat Oral Bioavailability of Aminopyrimidine Bicyclic Pyrazolone Analogues

compd	TNF-α IC_{50} (nM)[a]	solubility (mg/mL)	metabolism[b] (%)	% *F* (rat)
2	3	0.20	50	29
11	2	0.46	60	10
14	22	1.85	29	25
21	50	0.84	<5	63
23	74	0.10	46	18

[a] IC_{50} of LPS-stimulated TNF-α production in human monocytic cells (THP-1). [b] Metabolism[11] measured as percent loss at 4 h in rat hepatocytes.

Table 4. Comparison of IC_{50} Values/Inhibition Data for **2**, **14**, and **21**

	IC_{50} (nM)			
compd	TNF-α THP-1	TNF-α PBMC[a]	IL-1β PBMC[b]	p38α
2	3	0.4	0.8	8
14	22	8	42	54
21	50	16	39	27

[a] IC_{50} of LPS-stimulated TNF-α production in human PBMC. [b] IC_{50} of LPS-stimulated IL-1β production in human PBMC.

in potency in the [5,6]-analogues (**2**, **11**, **14**, and **21** relative to **13**, **12**, **15** and **22**).

An early goal was to improve the aqueous solubility of our initial, more potent alkylaminopyrimidine bicyclic pyrazolone leads. Both **2** and **23** suffered from poor solubility[11] and moderate instability in our in vitro rat hepatocyte metabolic stability assay[12] (Table 3). Not surprisingly, substitution of the methylbenzylamine with smaller alkylamines provided more soluble compounds such as pyrazolone **11**. Unfortunately, **11**, while it is our most active compound to date, still suffered from metabolic instability. Analysis of the major metabolites led to the conclusion that the butyl side chain of the amine was being oxidized, most likely in the terminal position. To circumvent this oxidation, we chose to make **21**. The addition of the methoxy group in the terminal position dramatically decreased metabolism to less than 5% while increasing the solubility to 840 μg/mL. These two factors likely contributed to the large increase in bioavailability (63%) in the rat model. The most solubilizing amino substituent, the 2-hydroxy-1,2-dimethylpropylamine (**14**), showed a significant increase in solubility while still retaining good potency. Compounds **2** and **14** have been screeened in the rat iodoacetate model, and both showed positive oral efficacy (15−25 mg/kg).[13]

Compounds **2**, **14**, and **21** were tested in a human p38α kinase assay,[14] and the observed values closely corresponded with the THP-1 whole-cell inhibition data (Table 4). Compound **2** was the most potent p38 inhibitor at 8 nM, while **14** and **21** also showed excellent potency at 54 and 27 nM, respectively. All three amino-substituted pyrimidine bicyclic pyrazolone derivatives showed excellent inhibition in peripheral blood mononuclear cells (PBMC)[15] of both cytokines TNF-α and interleukin-1β (IL-1β).

A kinase profiling study on **2**, **14**, and **21** was then conducted (Table 5). Compound **14** shows the greatest selectivity versus the JNK kinases while only significantly inhibiting ERK1 kinase. The increased selectivity against JNK-3 and good selectivity versus JNK-1 and JNK-2 make pyrazolone **14** currently the most selective of the aminopyrimidine bicyclic pyrazolones.

Table 5. Kinase Profiling Data[a]

kinase	**2**[b]	**14**[b]	**21**[b]
CRAF	61	91	55
MEK1	99	95	102
ERK1	82	9	96
ERK2/MAPK2	80	108	88
MKK4/SKK1	166	117	90
JNK1α1	52	105	65
JNK2α2	8	73	8
JNK3	4	36	5
GSK3β	101	99	72
Lck	161	141	-
PKBocAkt1	98	119	89
CDK2/cyclin A	117	101	108
CDK1/cyclin B	95	99	96
MAPK1/IKKB		111	
MAPKAP-K2	91	112	113

[a] All assays done in triplicate, values at 1 μM compound concentration with ATP at the K_m. [b] Kinase activity expressed as a percentage of that in control incubations. Standard deviation for assays is typically ±10%.

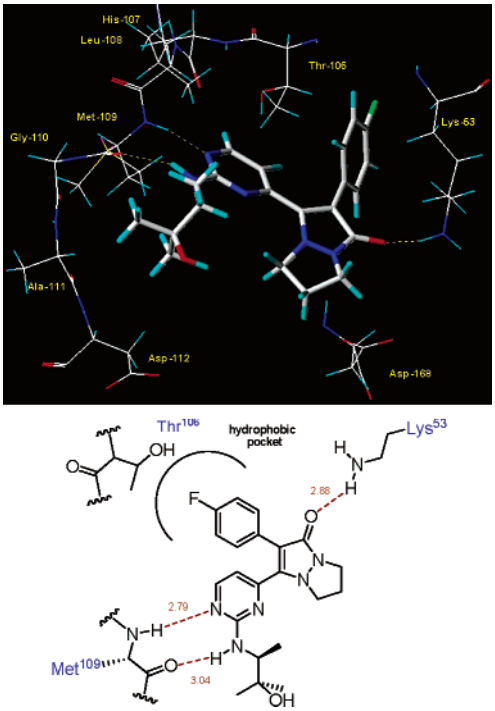


Figure 2. Pyrazolone **14** cocrystallized in mutated p38 active site.

After achieving good selectivity, solubility, and oral efficacy in the rat IA model with **14**, we then cocrystallized **14** with mutated p38α[16] (Figure 2). We observed the typical vicinal bis-aryl p38 inhibitor interactions, including binding of the fluorophenyl ring into the Thr-106 hydrophobic pocket and two hydrogen-bonding interactions between the Met-109 amide NH and the nitrogen of the pyrimidine ring and between the Met-109 carbonyl oxygen and the nitrogen of the secondary amine. We also observed a strong hydrogen-bonding interaction between the carbonyl oxygen of the pyrazolone ring and Lys-53.

In conclusion, we have developed a novel bicyclic pyrazolone scaffold that maintains the important binding motif of the vicinal 4-fluorophenyl and pyrimidinyl ring systems in the p38 ATP binding site. In general,

the alkylaminopyrimidine bicyclic pyrazolones tended to be more potent than the aminophenylpyrimidine derivatives against LPS-stimulated human monocytic cells (THP-1). Compounds **2**, **14**, and **21** are effective inhibitors of TNF-α and IL-1β cytokine release from PBMC. These three compounds show excellent potency against human p38, good selectivity in the kinase panel, and positive oral efficacy (15−25 mg/kg) in the rat iodoacetate in vivo model. Further optimization of this novel scaffold will be reported in future publications.

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Supporting Information Available: Experimental procedures for all final compounds and intermediates, including characterization data for final compounds **1**, **2**, **7**−**29**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) Procedure for determining IC_{50} values of LPS-stimulated TNF-α production in human monocytic cells (THP-1): Duplicate cultures of human monocytic cells (THP-1)[17] cells (2.0×10^5/well) were incubated for 15 min in the presence or absence of various concentrations of inhibitor before the stimulation of cytokine release by the addition of LPS (1 μg/mL). The amount of TNF-α released was measured 4 h later using an ELISA (R&D Systems, Minneapolis, MN). The viability of the cells after the 4 h of incubation was measured using MTS assay[18] (Promega Co., Madison, WI). Standard deviation for THP-1 cellular assays were typically ±30% of the mean or less.

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(11) Solubility assay procedure. Solubility of analogues was measured in 50 mM phosphate buffer, pH 7.4, ionic strength 0.15 M. The solubilities were determined by the shake flask method after 24 h of equilibration. Concentrations in aqueous solutions were determined for the supernatant of centrifuged and filtered samples by UV−vis spectrophotometry. Calibration solutions were prepared in acetonitrile/buffer in a 1:1 volume ratio. The samples were diluted with acetonitrile to obtain identical media with calibration standards.

(12) In vitro metabolism assay procedure. The in vitro metabolic stability of analogues in plated rat hepatocytes (Sprague-Dawley) obtained from Cedra Corporation was determined in triplicate using a total volume of 0.2 mL containing 0.25 μM NCE incubated in rat hepatocyte and matrigel blank microtiter plates. The plates were maintained at 37 °C throughout the study. Samples were removed from wells at 0, 2, and 4 h, and NCE samples were analyzed by HPLC/MS/MS with reverse-phase chromatography. To improve analytical efficiency, compounds were grouped together (postincubation) into a multicompound assay. Samples from like time points containing the different compounds were combined, and an internal standard (1.1 ng/mL stock) was added. Results for each compound were expressed as the ratio of the compound response area to the internal standard response area. Percent loss was calculated by dividing the 2 and 4 h ratios by the 0 h ratio.

(13) See Supporting Information for details of the procedure for the rat iodoacetate in vivo model.

(14) See Supporting Information for details of the p38α kinase assay.

(15) See Supporting Information for details of measuring the inhibition of TNF-α and IL-1β in PBMC.

(16) The mutated p38α herein described is an inactivated, double mutant (S180A, Y182F) of murine p38α. See Supporting Information for more details on crystallization and data collection.

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