

The development of new isoxazalone based inhibitors of tumor necrosis factor-alpha (TNF- α) production

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Abstract—4-Aryl-3-pyridyl and 4-aryl-3-pyrimidinyl based tumor necrosis factor-alpha (TNF- α) inhibitors, which contain a novel isoxazalone five-membered heterocyclic core are described. Many showed sub-micromolar activity against lipopolysaccharide-induced TNF- α production.

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In our continuing efforts toward the development of disease modifying treatments for inflammatory diseases, we are targeting the development of novel mitogen-activated protein (MAP) kinase inhibitors. MAP kinases are part of a signal transduction pathway responsible for the relay of information from the cell surface to the nucleus. Some of these MAP kinase families, such as p38 and JNK (c-Jun amino terminal kinase), are activated in response to infection, cellular stress and the proinflammatory cytokines TNF- α (tumor necrosis factor) and IL-1 β (interleukin-1 β).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. It has been established that agents that inhibit p38 MAP kinase can decrease levels of these proinflammatory cytokines,¹ and thereby reduce inflammation and prevent further tissue destruction.

Previous work from our laboratories described the synthesis and SAR of a series of bicyclic pyrazolone TNF- α inhibitors, exemplified by compound **1**,⁴ that contain the prototypical vicinal bis-aryl pharmacophore found in many p38 inhibitors. The pyrazolone work has demonstrated that optimizing the TNF- α inhibitory activity,

by utilizing a cellular assay as a primary screening tool, can successfully lead to potent and efficacious cytokine inhibitors.⁴ Therefore, our primary assay entails screening analogues for inhibition of lipopolysaccharide (LPS)-stimulated TNF- α production in the THP-1 cellular assay.⁵ Herein, we report a new structural class of 4-aryl-3-pyrimidinyl (pyridinyl) based TNF- α inhibitors that contain a novel isoxazalone core. Our investigations have yielded potent TNF- α production inhibitors such as pyridinyl isoxazalone **2** and phenoxy-pyrimidineisoxazalone **3** (Fig. 1).

Synthesis of the pyridinyl isoxazalone **5** was accomplished in only three steps, from commercially available

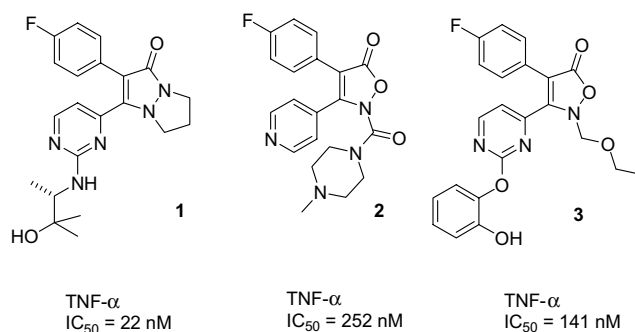
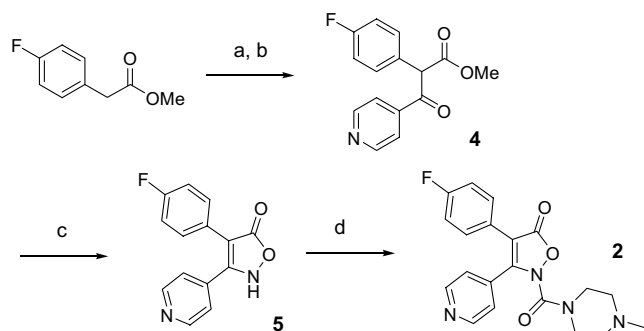


Figure 1. Vicinal bis-aryl TNF- α production inhibitors.

Keywords: p38 Inhibitor; Isoxazalone; TNF- α ; Cytokine inhibitor.

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Scheme 1. Reagents and conditions: (a) LDA, THF, 4-pyridine carboxaldehyde -78°C , 91%; (b) CrO_3 , pyridine, 43%; (c) hydroxylamine-HCl, pyridine, 90°C , 95%; (d) 4-methyl-1-piperazine carbonyl chloride, Et_3N , CH_2Cl_2 , 51%.

starting materials (Scheme 1). Treatment of 4-pyridine carboxaldehyde with the anion of 4-fluorophenylacetate, followed by chromium(VI) oxidation provided the known β -ketoester **4**.⁶ Cyclocondensation of hydroxylamine with β -ketoester **4** provided the core isoxazolone ring system **5**. While the unsubstituted parent isoxazolone **5** possessed only modest activity ($\text{TNF-}\alpha$, $\text{IC}_{50} = 5.8\text{ }\mu\text{M}$), numerous opportunities existed for further functionalization to optimize the potency of these molecules (Table 1). Acylation with the corresponding carbamoyl chlorides afforded compounds **2** and **9–10**. Alkylation of the ring nitrogen yielded compounds **11–17**. Alternatively, substituted isoxazolone analogues ($\text{R} = \text{methyl}$ (**7**) and $\text{R} = \text{cyclohexyl}$ (**8**)) can be accessed via a similar route with the appropriate *N*-alkylated hydroxylamine. This allowed for analogues to be made which were difficult to synthesize via the alkylation method shown in Scheme 1, due to the weak nucleophilic nature of the isoxazolone ring nitrogen.

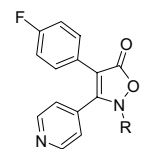
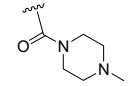
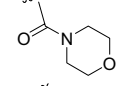
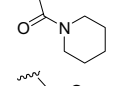
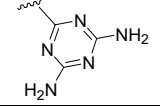
Examination of the results in Table 1 shows that both the *N*-methyl (**7**) and *N*-cyclohexyl (**8**) analogues showed little potency in the whole cell assay. One of the most promising compounds was the *N*-methylpiperazine urea derivative **2**. This compound was significantly more potent than the morpholine (**9**) and piperidine (**10**) analogues. Unfortunately, the urea derivatives showed significant metabolism in our *in vitro* rat hepatocyte metabolic stability assay.⁷ We then turned our efforts toward the alkyl derivatives. Alkylated derivatives (**11–16**) showed some modest activity. The most potent alkyl substituent ($2.5\text{ }\mu\text{M}$) was the ethoxymethyl compound **11**, which had shown a positive effect as a substituent on one of our previous $\text{TNF-}\alpha$ inhibitor scaffolds.⁸ The other ether containing substituents were not tolerated as well. The benzyl derivative (**16**) showed little potency ($>10\text{ }\mu\text{M}$) and other benzyl analogues (data not shown) were likewise inactive. Another promising alkylated product was the *N*-triazin-2-yl derivative (**17**) with an $\text{IC}_{50} = 200\text{ nM}$.

The related pyrimidinyl isoxazolone series, which was made analogously to the pyridinyl isoxazolones (Scheme 2), afforded a second site of diversity at the 2-position of the pyrimidine ring. Based on the survey of isoxazolone

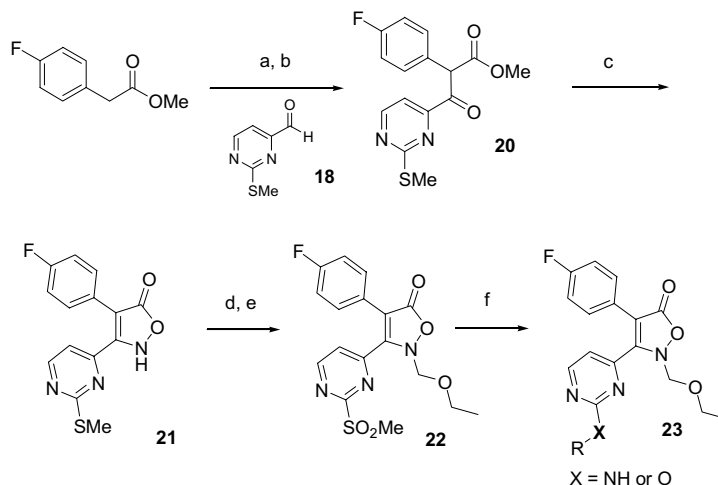
substituents performed on the pyridinyl series,⁹ the ethoxymethyl substituent was held constant while a series of substituents on the pyrimidine ring was examined. *N*-Alkylation of isoxazolone **21** with 2-chloromethyl ethyl ether was followed by oxidation of the thiomethyl group with Oxone[®]. Reaction of the resulting sulfone **22** with either an alcohol under basic conditions (NaH , THF) or neat amine at elevated temperature, respectively, yielded the desired substituted derivative **23**.

Table 2 summarizes a survey of phenoxyisoxazolone analogues, while Table 3 summarizes results for a series of alkylaminopyrimidine isoxazolone analogues. In general, both groups produced several compounds at or below $1\text{ }\mu\text{M}$ potency level. In the phenoxy series, both the phenoxy substituted **26** and the fluorinated phenoxy (**27–29**) yielded compounds in the $1\text{ }\mu\text{M}$ range. It is possible that the more potent phenoxy substituents (**24–26**) may be picking up

Table 1. $\text{TNF-}\alpha$ inhibition data for pyridyl isoxazolone derivatives

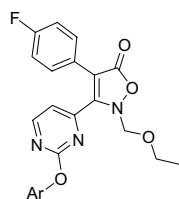
		
Compound	R	$\text{TNF-}\alpha\text{ IC}_{50}^a\text{ (}\mu\text{M)}$
5	H	5.9
7	CH ₃	>10
8	Cyclohexyl	>10
2		0.2
9		5.0
10		8.0
11	CH ₂ CH ₂ OC ₂ H ₅	2.5
12	CH ₂ CH ₂ OC ₆ H ₅	4.4
13	CH ₂ CH ₂ CH=CH ₂	6.9
14	CH ₂ CH ₂ OC ₂ H ₅	8.6
15	CH ₂ CH ₂ OC ₂ H ₅ CH ₂ OC ₂ H ₅	>10
16	CH ₂ CH ₂ OC ₆ H ₅	>10
17		0.2

^a IC_{50} of LPS-stimulated $\text{TNF-}\alpha$ production in human monocytic cells (THP-1). Standard deviation for assays typically $\pm 30\%$ of the mean or less.



Scheme 2. Reagents and conditions: (a) LDA, THF, -78°C , aldehyde **18** 85%; (b) CrO_3 , pyridine, 43%; (c) H_2NOH , pyridine, 90°C , 80%; (d) chloromethyl-ethyl ether, Et_3N , CH_2Cl_2 , 45%; (e) Oxone®, THF/MeOH/ H_2O ; (f) R-NH_2 , toluene 120°C or R-OH , NaH, THF.

Table 2. TNF- α inhibition values of the phenoxy-pyrimidine bicyclic pyrazolone analogues



Compound	O-Ar	TNF- α IC_{50}^a (μM)
3	2-Hydroxyphenoxy	0.14
24	3-Acetamidophenoxy	0.46
25	3-Hydroxyphenoxy	0.53
26	Phenoxy	1.00
27	4-Fluorophenoxy	1.00
28	2-Fluorophenoxy	1.01
29	2,6-Difluorophenoxy	1.08
30	3-Dimethylaminophenoxy	2.00
31	4-Methoxyphenoxy	6.30
32	2-Acetamidophenoxy	7.50
33	2,4-Dimethylphenoxy	>10
34	4-Ethylphenoxy	>10

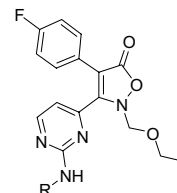
^a IC_{50} of LPS-stimulated TNF- α production in human monocytic cells (THP-1). Standard deviation for assays typically $\pm 30\%$ of the mean or less.

additional hydrogen bonding interactions with other side chains in the enzyme active site, such as Gly-110 (Figs. 2 and 3).

In the alkylamino series (Table 3) the pyrimidine substituents that possessed a secondary α -methyl amine showed greater potency than the corresponding primary amines. The three most potent analogues (**35–37**) contain substituents at the 2-position of the pyrimidine ring that have increased potency in the previously disclosed bicyclic pyrazolone series.⁴ Interestingly, the *S*-enantiomer of the α -methyl benzyl amine analogue **36** is five times more potent than the corresponding *R*-enantiomer **40**.

A compound of note from this series is isoxazolone **43**, which incorporates an *N*-acetaldehyde substituent on the isoxazolone ring nitrogen. This compound contains

Table 3. TNF- α inhibition values of the alkylamino-pyrimidine isoxazolone analogues



Compound	R-NH	TNF- α IC_{50}^a (μM)
35		0.18
36		0.20
37		0.21
38		0.94
39		1.00
40		1.08
41		7.40
42		>10

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

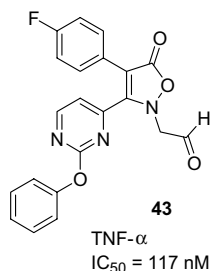


Figure 2. *N*-Acetaldehyde phenoxy-pyrimidine isoxazalone **43**.

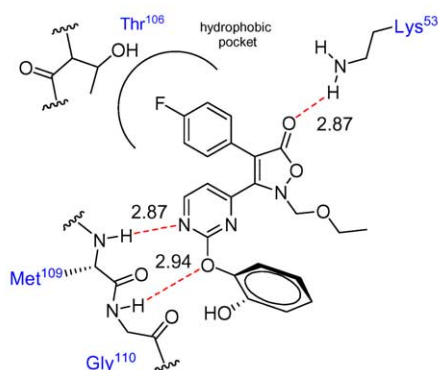
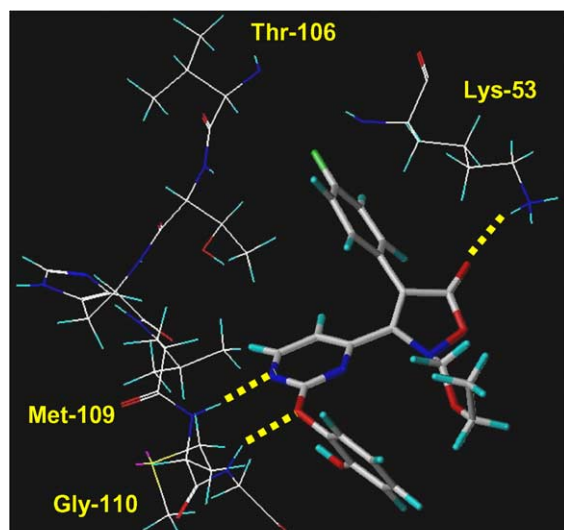


Figure 3. Isoxazalone **3** co-crystallized in mutated p38 active site.

a phenoxy-substituted pyrimidine and was made by alkylating the isoxazalone nitrogen with allyl bromide, then oxidatively cleaving the olefin with OsO₄/NaIO₄. The resulting isoxazalone proved to be our most potent compound in this series (IC₅₀ = 117 nM).

Compounds **3**, **25**, **36**, **40**, and **43** were tested in a human p38 α kinase assay (Table 4). Compounds **25** and **40** showed comparable values to the THP-1 whole cell data (520 and 1700 nM, respectively). Compounds **36** and **43** showed increased potency in the p38 α kinase assay (84 and 48 nM, respectively). Surprisingly, the 2-hydroxyphenoxy analogue (**3**) showed much weaker potency in the kinase assay (460 nM) than the cellular assay

Table 4. Comparison of IC₅₀ values/inhibition data for **3**, **25**, **36**, **40**, and **43**

Compound	3 (nM)	25 (nM)	36 (nM)	40 (nM)	43 (nM)
TNF- α ^a	140	530	200	1080	117
p38 α	460	520	84	1700	48

^a IC₅₀ of LPS-stimulated TNF- α production in human THP-1 cells. Standard deviation for assays typically $\pm$ 30% of the mean or less.

(140 nM). The possibility of isoxazalone **3** inhibiting TNF- α production via multiple mechanisms may explain this difference in potency.

In order to elucidate binding site interactions in the isoxazalone series, X-ray data was collected for the highly active isoxazalone inhibitor **3** co-crystallized with mutated p38 α ¹⁰ (Fig. 3). We observed the typical vicinal bis-aryl p38 inhibitor interactions, including binding of the fluorophenyl ring into the Thr-106 hydrophobic pocket and a hydrogen bonding interaction between the Met-109 amide NH and the nitrogen of the pyrimidine ring. We also saw a second hydrogen bonding interaction between the Gly-110 amide NH and the oxygen of the phenoxy group. One of our goals when designing the isoxazalone series was to pick up the important Lys-53 interaction with a strong hydrogen-bond acceptor, in this case the carbonyl oxygen. A strong hydrogen bonding interaction between the carbonyl oxygen of the isoxazalone ring and Lys-53 in the binding site is confirmed by the X-ray data.

In conclusion, we have reported a novel series of pyridinyl and pyrimidinyl isoxazalone scaffold that maintains the important binding motif of the vicinal bis-aryl ring systems in the p38 ATP binding site. Two of the pyridinyl isoxazolones showed sub-micromolar potency against LPS-stimulated human monocytic cells (THP-1). In general, the pyrimidinyl isoxazolones were more potent, yielding several compounds at or below the 1 μ M potency level.

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References and notes

- Recent reviews: (a) Adams, J. L.; Badger, A. M.; Kumar, S.; Lee, J. C. In *Progress in Medicinal Chemistry*; King, F. D., Oxford, A. W., Eds.; Elsevier: Amsterdam, 2001; Vol. 38, p 1; (b) Chen, Z.; Gibson, T. B.; Robinson, F.;

- Silvestro, L.; Pearson, G.; Xu, B.-e.; Wright, A.; Vanderbilt, C.; Cobb, M. H. *Chem. Rev.* **2001**, *101*, 2449.
2. Pargellis, C.; Regan, J. *Curr. Opin. Invest. Drugs* **2003**, *4*, 566.
 3. (a) Brennan, F. M.; Feldman, M. *Curr. Opin. Immunol.* **1996**, *8*, 872; (b) Camussi, G.; Lupia, E. *Drugs* **1998**, *55*, 613.
 4. Clark, M. P.; Laughlin, S. K.; Laufersweiler, M. J.; Bookland, R. B.; Brugel, T. A.; Golebiowski, A.; Sabat, M. P.; Townes, J. A.; VanRens, J. C.; Djung, J. F.; Natchus, M. G.; De, B.; Hsieh, L. C.; Xu, S. C.; Heitmeyer, S. A.; Brown, K. K.; Juergens, K.; Walter, R. L.; Mekel, M. J.; Taiwo, Y. O.; Janusz, M. J. *J. Med. Chem.* **2004**, *47*(12), 2724.
 5. 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)¹¹ 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 lipopolysaccharide (LPS, 1 μ g/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 incubation was measured using MTS assay¹² (Promega Co., Madison, WI). Standard deviation for THP-1 cellular assays were typically $\pm 30\%$ of the mean or less.
 6. deLaszlo, S. E.; Visco, D.; Agarwal, L.; Chang, L.; Chin, J.; Croft, G.; Forsyth, A.; Fletcher, D.; Frantz, B.; Hacker, C.; Hanlon, W.; Harper, C.; Kostura, M.; Li, B.; Luell, S.; MacCoss, M.; Mantlo, N.; O'Neill, E. A.; Orevillo, C.; Pang, M.; Parsons, J.; Rolando, A.; Sahly, Y.; Sidler, K.; Widmer, W. R.; O'Keefe, S. J. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2689.
 7. In vitro metabolism assay procedure. In vitro metabolic stability of analogues in plated rat hepatocytes (Sprague–Dawley) obtained from Cedra Corporation. Metabolic activity was determined in triplicate using a total volume of 0.2 mL containing 0.25 μ M 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 (post-incubation) into a multi-compound 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 over the internal standard response area. Percent loss was calculated by dividing the 2 and 4 h ratios by the 0 h ratio.
 8. Tullis, J. S.; VanRens, J. C.; Natchus, M. G.; Clark, M. P.; De, B.; Hsieh, L. C.; Janusz, M. J. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1665.
 9. Efforts to synthesize the corresponding *N*-triazinyl substituent on the pyrimidinyl isoxazolones were unsuccessful.
 10. The mutated p38 α herein described is an inactivated, double mutant (S180A, Y182F) of murine p38 α . For complete information on crystallization and data collection, see Ref. 4. In addition, the X-ray coordinates for the co-crystallization of isoxazalone **3** are deposited with the RCSB Protein Data Bank (PDB), deposition number 1YW2.
 11. Mohler, K. M.; Sleath, P. R.; Fitzner, J. N.; Cerretti, D. P.; Alderson, M.; Kerwar, S. S.; Torrance, D. S.; Otten-Evans, C.; Greenstreet, T.; Weerawarna, K.; Kronhelm, S. R.; Petersen, M.; Gerhart, M.; Kozlosky, C. J.; March, C. J.; Black, R. A. *Nature* **1994**, *370*, 218.
 12. Barltrop, J. A.; Owen, T. C.; Cory, A. H.; Cory, J. G. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 611.