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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 4360–4365

The development of novel C-2, C-8, and N-9 trisubstituted purines as inhibitors of TNF-α production

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Received 10 April 2006; revised 15 May 2006; accepted 16 May 2006
Available online 5 June 2006

Abstract—A series of C-2, C-8, and N-9 trisubstituted purine based inhibitors of TNF-α production are described. The most potent analogs showed low nanomolar activity against LPS-induced TNF-α production in a THP-1 cell based assay. The SAR of the series was optimized with the aid of X-ray co-crystal structures of these inhibitors bound with mutated p38 (mp38).
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In our efforts toward the discovery of therapeutics for the treatment of inflammatory conditions we are developing novel small molecule inhibitors of TNF-α (tumor necrosis factor-α) via modulation of the MAPK (mitogen-activated protein kinase) cascade.[1] The p38 protein is part of a larger MAP kinase family involved in signal transduction that also includes ERK (extracellular regulated kinase) and JNK (c-Jun amino terminal kinase).[1a,2] The p38 and JNK kinase families are activated in response to infection, cellular stress, and cytokines.[2] Additionally, p38 is centrally involved in the regulation of many proinflammatory cytokines most particularly TNF-α (tumor necrosis factor-α) and IL-1β (interleukin-1β).[2] The overexpression of these cytokines has been implicated in the pathogenesis of rheumatoid arthritis (RA), osteoarthritis (OA), and Crohn's disease.[3,4] Thus, agents that inhibit p38 MAPK can decrease levels of TNF-α and the related IL-1β, and thereby reduce inflammation and halt tissue destruction.[4]

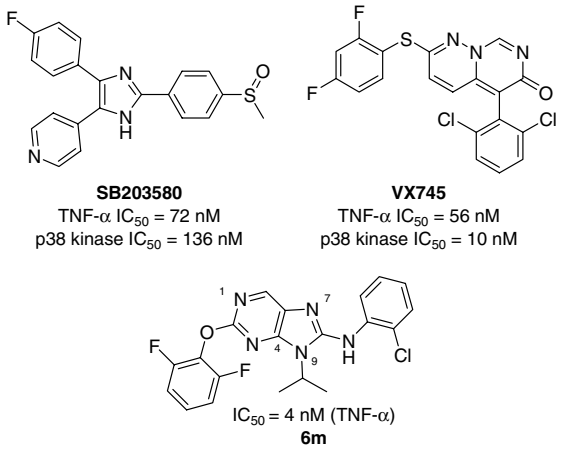

Figure 1. Inhibitors of p38 MAPK.

The prototypical p38 inhibitors (e.g. SB-203580, Fig. 1)[5] contained a 4-aryl-5-pyridinyl motif and were found to interact competitively with the p38 ATP binding site. To date, numerous chemical scaffolds (e.g. imidazoles, pyrroles, pyrimidines, pyridines, pyrimidones, indoles, heteroindoles, ureas, and various fused bicyclic heterocycles) containing a variety of functionality have been reported to inhibit TNF-α production.[2a,6] Of the non-vicinal diaryl scaffolds VX745 has been shown to be a potent and selective inhibitor of p38.[2a,2d,5a,6a,7]

Keywords: Purines; TNF-α; MAP kinase; p38; Mutated p38; THP-1; THP-1 cell.
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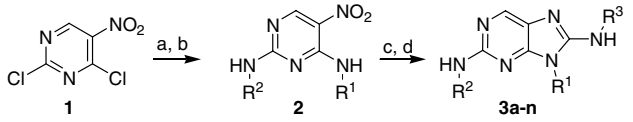
doi:10.1016/j.bmcl.2006.05.050

The purine scaffold has also been exploited for designing biologically relevant molecules including inhibitors of kinase signaling.[8] Utilizing structure based drug design in conjunction with X-ray co-crystallography we achieved the synthesis of a series of novel inhibitors of TNF-α containing a C-2, C-8, and N-9 trisubstituted purine scaffold. This communication details the synthesis, biological activity, and the mode of binding for this class of compounds in mutated p38.

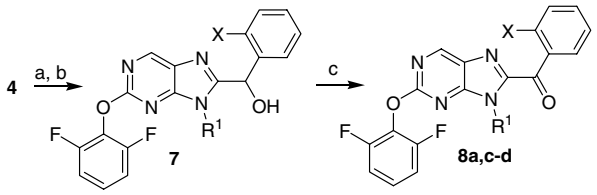
The trisubstituted purines were prepared from readily available 2,4-dichloro-5-nitro-pyrimidine **1**[9] using general procedures depicted in Schemes 1–3. Early lead molecules which contain C-2 anilino- or alkylamino-groups were accessed via a 4-step sequence (Scheme 1). Initial displacement of the 4-chloro of **1** with various alkyl amines was performed at 0 °C in THF. Subsequent substitution of the 2-chloro with various amines occurred readily to give **2**.[10] These compounds were then hydrogenated with Pd on carbon and the resultant amines were treated with a series of isothiocyanates to provide purines **3a**–**n** (Table 1).[11]

Dichloropyrimidine **1** was also used to generate purines containing a C-2 phenoxy group (Schemes 2 and 3). While displacement of amines at the 2-chloro position occurred readily at rt, substitution with phenols required reflux for 3–6 h in DIPEA. Catalytic reduction of **4** followed by reaction with 1,1′-carbonyldiimidazole and treatment with $POCl_3$afforded **5**. The 8-chloro of compound **5** was then displaced with various anilines, 2-chlorophenol and 2-chlorobenzenethiol, to give **6a**–**m**. Compound **4** was also treated with 2-(2-halo-phenyl)-2-hydroxy-acetimidic acid ethyl ester.[12] The resulting benzylic alcohol **7** was subsequently oxidized with MnO_2 in DCM to furnish **8a**, **c**, **d** (Scheme 3).

Tables 1 and 2 summarize the screening results for a variety of trisubstituted purines on the inhibition of



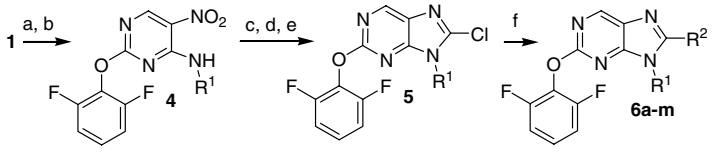
Scheme 1. General procedure for the preparation of compounds **3a**–**n**. Reagents and conditions: (a) alkylamine, THF, 0 °C, 10 min, 42–94%; (b) alkylamine or aniline, rt, 3–6 h, 24–72%; (c) Pd/C, H_2, EtOH, rt, 1.5 h, 81–98%; (d) various isothiocyanates, DCM, DCC, DIPEA, rt, 15 min then reflux 2 h, 7–64%.



Scheme 3. General procedure for preparation of **7** and **8a**, **c**, **d**. Reagents and conditions: (a) Pd/C, H_2, EtOH, 45 °C, 2 h, 79%; (b) 2-(2-halo-phenyl)-2-hydroxy-acetimidic acid ethyl ester, EtOH, reflux, 18 h, 24–38%; (c) MnO_2, DCM, 1 h, 74–85%.

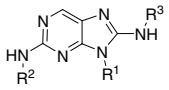
TNF-α production in LPS (lipopolysaccharide) stimulated human monocytic cells (THP-1).[13–15] The initially synthesized compound **3a** (Table 1) was a weak inhibitor of TNF-α release (IC_{50} = 6.6 μM). Exchange of the 4-aminotetrahydropyran moiety in analog **3a** for an alkyl group slightly improved potency (**3b**; IC_{50} = 3.2 μM). Maintaining R2 and insertion of a methylene or ethylene spacer group into the chlorophenyl group (**3c** and **3d**) resulted in a loss of activity (IC_{50} > 10 μM). Similar results were obtained for compound **3e** which contained a 2-chlorobenzamide group at position C-8. Installation of a 2,6-difluoroaniline group at C-2 gave the most potent analog **3f** (IC_{50} ~ 1.5 μM). We then examined the effect of incorporating various alkyl groups at position N-9 (**3g**–**k**). Purines with an ethyl (**3g**; IC_{50} = 542 nM) or isopropyl (**3h**; IC_{50} = 630 nM) group at N-9 displayed increased activity, whereas larger functionality and the cyclopropyl group at this position attenuated potency (**3i**–**k**). Having explored N-9 substitution we next attempted to vary the ring substituents on R2. Replacement of the 2,6-difluoroaniline group present in **3f** and **3g** with a 2-fluoro-, 4-fluoro- or 2, 4-difluoroaniline group at C-2 gave analogs with poor activity (**3l**–**n**).

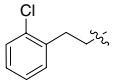
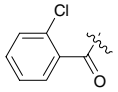
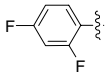
To facilitate the SAR and delineate the binding mode of these molecules an X-ray crystallographic structure[16–18] of **3g** with mutated p38 (mp38) was obtained (Fig. 2). The mutated p38α herein described was a double mutant (S180A, Y182F) of murine p38α.[18] Inhibitor **3g** orients itself in the mp38 enzyme (PDB accession code 2GTM) such that the 2-chloroaniline ring (at C-8) lies in the hydrophobic (specificity) pocket adjacent to Thr_{106}. There was a hydrogen bond between N-1 of the pyrimidine ring and the Met_{109} N–H. Additionally, the hydroxyl of Thr_{106} formed a hydrogen bond with N-7 of the inhibitor. A water molecule (removed for clarity in Fig. 2) was observed to bridge Lys_{53} and the N–H of position C-8 on the purine ring. The N-9 methyl



Scheme 2. General procedure for preparation of **6a**–**m**. Reagents and conditions: (a) ethylamine or isopropylamine, THF, 0 °C, 10 min, 79% (R^1 = ethyl or isopropyl); (b) 2,6 difluorophenol, DIPEA, reflux, 3–6 h, 32–48%; (c) Pd/C, H_2, EtOH, 45 °C, 2 h, 87–98%; (d) 1,1′-carbonyldiimidazole, THF, 21 °C, 1 h, 94–97%; (e) $POCl_3$, DCM, reflux, 24 h, 29–40%; (f) various phenols and thiophenols, DIPEA, 120 °C, 6 h, 4–32%.

Table 1. IC_{50} values for purine derivatives **3a**–**n**



Compound	R^1	R^2	R^3	TNF-α IC_{50}[a] (nM)
3a	Me-			6579
3b	Me-			3171
3c	Me-			>10,000
3d	Me-			>10,000
3e	Me-			>10,000
3f	Me-			1541
3g	Et-			542
3h	Isopropyl-			630
3i				>1000
3j	*n*-Butyl-			798
3k	*t*-Butyl-			>1000
3l	Et-			2110
3m	Me-			10,000
3n	Me-			6556

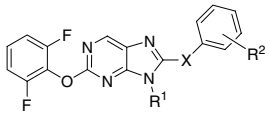
[a] IC_{50} of LPS-stimulated TNF-α production in human monocytic cells (THP-1).

group was positioned toward Leu_{171}, however this substituent appeared too short for maximal interaction with Leu_{171}.

In the Met_{109}-Gly_{110} region of the protein a peptide flip was observed (the carboxyl of Met_{109} turned away from the inhibitor and the amide N–H of Gly_{110} turned in toward the inhibitor) (Fig. 2). This orientation induced a repulsive interaction between the N–H of the 2,6-difluorophenylanalino-hydrogen at C-2 and the N–H of Gly_{110}.

Using insights from the co-crystal structure of mp38 with **3g** we synthesized compound **6a** (Table 2) incorporating a 2,6-difluorophenoxy group at C-2 and an ethyl group at N-9. These changes corrected the deficiencies observed in the X-ray co-crystal structure (O at C-2 H-bonds the amide N–H of Gly_{110} and the ethyl at N-9 is better positioned toward Leu_{171}) thus providing a significant increase in potency (IC_{50} = 69 nM) relative to **3g**. The use of an O linker (**6b**) resulted in a moderate decrease in activity (IC_{50} = 86 nM). The corresponding S linked analog **6c** was better tolerated (IC_{50} = 14 nM).

Table 2. IC_{50} values of the amine derivatives of **6a–m**, **7**, and **8a–d**



Compound	R^1	X	R^2	TNF-α IC_{50}[a] (nM)
6a	Et-	NH	2-Chloro	69
6b	Et-	O	2-Chloro	86
6c	Et-	S	2-Chloro	14
6d	Et-	NH	2-Methyl	48
6e	Et-	NH	2-Fluoro	35
6f	Et-	NH	2-Methoxy	2050
6g	Et-	NH	2-Hydroxy	906
6h	Et-	NH	2-Amino	473
6i	Et-	NH	2-Chloro-4-fluoro	237
6j	Et-	NH	2-Chloro-4-methyl	273
6k	Et-	NH	2,3-Dichloro	300
6l	Et-	NH	2,6-Dichloro	100
6m	Isopropyl-	NH	2-Fluoro	4
7	Et-	CHOH	2-Chloro	169
8a	Et-	C=O	2-Chloro	4
8b[b]	Et-	S=O	2-Chloro	>1000
8c	Et-	C=O	2-Fluoro	207
8d	Isopropyl-	C=O	2-Chloro	650

[a] IC_{50} of LPS-stimulated TNF-α production in human monocytic cells (THP-1).
[b] Analog **8b** was synthesized from **6c** with 1 eq. Oxone®.

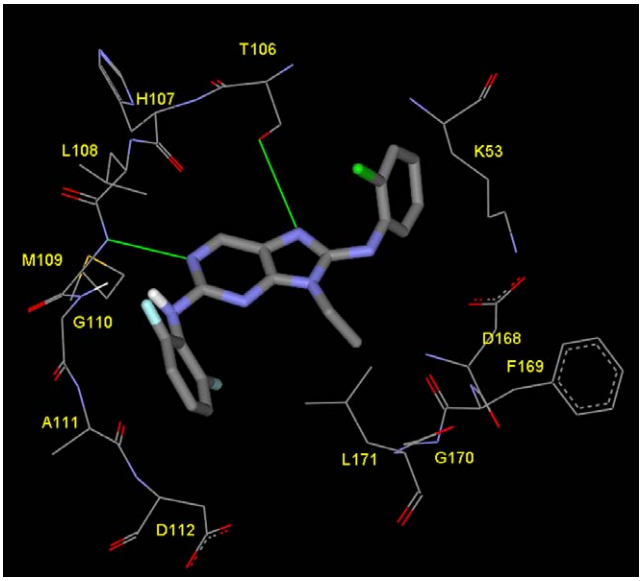


Figure 2. Key hydrogen bonds between **3g** and mp38 with key mismatch at Gly110 backbone N–H.

This was attributed to a more favorable orientation of the 2-chloro phenyl ring in the hydrophobic pocket. Replacement of the 2-chloro with a methyl **6d** or fluorine **6e** increased activity (IC_{50} = 48 nM; IC_{50} = 35 nM). Further changes at the 2 position of the 8-phenoxy ring: methoxy-, hydroxy-, and amino- (**6f–h**) led to a drop in activity.

Attempts to improve activity by disubstitution (**6i–l**) on the 8-phenoxy ring with: 2-chloro-4-fluoro (**6i**; IC_{50} = 237 nM), 2-chloro-5-methyl (**6j**; IC_{50} = 273 nM), 2,3-dichloro (**6k**; IC_{50} = 300 nM) or the 2,6-dichloro (**6l**; IC_{50} = 100 nM) reduced potency. Finally, replacement of the N-9 ethyl on compound **6a** with an isopropyl group gave the most potent purine **6m** in this series (IC_{50} = 4 nM) (Table 2 and Fig. 3). This inhibitor showed all expected binding interactions with mp38 (PDB accession code 2GTN) including the torsional flip at Met_{109}-Gly_{110} reported to confer p38 selectivity.[7b]

Additional SAR included replacement of the linker heteroatom at C-8 with an alcohol moiety to afford **7**

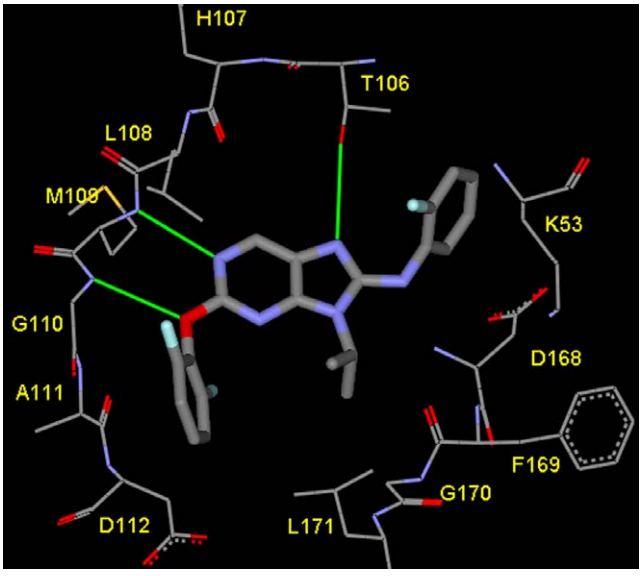


Figure 3. Key hydrogen bonds between **6m** and mp38 including the torsional flip at Met_{109}-Gly_{110}.

(IC_{50} = 169 nM), oxidation of this group with MnO_2 affords the potent keto analog **8a** (IC_{50} = 4 nM). Exchange of the ketone linker in **8a** with a sulfoxide group muted activity (**8b**; IC_{50} > 1000 nM). Substitution of the 2-chloro atom with 2-fluoro **8c** or the exchange of the N-9 ethyl for isopropyl **8d** was also deleterious and underscores the sensitivity of the binding pocket to these inhibitors.

The most potent analogs (**6c**–**e**, **6m**, and **8a**) were screened for in vitro metabolism.[20] Compounds **6d**, **6e**, and **6m** were moderately metabolized, while analogs **6c** and **8a** suffered high loss (56, 65, 61, 75, and 82% loss, respectively). Inhibitors with the greatest metabolic stability (**6d** and **6m**) were subsequently evaluated in a rat PK study to determine bioavailability and half-life. Both molecules displayed low BA (~9%) and possessed a short $T_{1/2}$ (1.5 h).

In summary we have described a series of novel trisubstituted purines which inhibit the production of TNF-α. The design, synthesis, and mode of binding (to mutated p38) of these compounds was detailed. We hope to report further progress in development of this series in the near future.

Acknowledgments

We are grateful to A. L. Roe, C. A. Cruze, W. E. Schwecke, C. R. Dietsch for pharmacokinetic studies, M. Buchalova for chemical stability and solubility studies, and M. Mekel for X-ray co-crystallization studies. We would like to acknowledge that X-ray data were collected at beamline 17-BM in the facilities of the Industrial Macromolecular Crystallography Association Collaborative Access Team (IMCA-CAT) at the Advanced Photon Source, Argonne National Laboratory. Supporting institutions may be found at www.ser-cat.org/members.html. Use of the Advanced Photon Source was supported by the US Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. W-31-109-Eng-38.

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20. (a) Measured as percent loss at 4 h in rat hepatocytes; (b) In vitro metabolism assay procedure: In vitro metabolic stability of analogs 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 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 HP-LC/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.