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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 3646–3650

Development of *N*-4,6-pyrimidine-*N*-alkyl-*N*′-phenyl ureas as orally active inhibitors of lymphocyte specific tyrosine kinase

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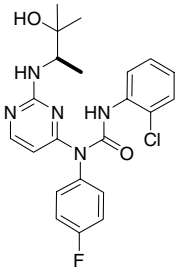
Received 28 March 2006; revised 21 April 2006; accepted 24 April 2006
Available online 8 May 2006

Abstract—A new class of lymphocyte specific tyrosine kinase (lck) inhibitors based on an *N*-4,6-pyrimidine-*N*-alkyl-*N*′-phenyl urea scaffold is described. Many of these compounds showed low-nanomolar inhibition of lck kinase activity as well as IL-2 synthesis from Jurkat cells. One of these analogs, **7i**, was shown to be orally efficacious by in vivo testing in a rat adjuvant-induced arthritis study.

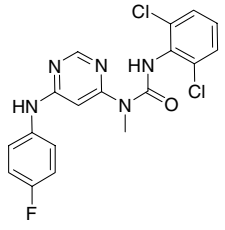
Lymphocyte specific protein tyrosine kinase (Lck), one of the eight members of the Src family of tyrosine kinases, is expressed on T-lymphocytes and natural killer cells. Lck plays a critical role in T-cell antigen receptor (TCR) signal transduction. Upon presentation of antigens associated with the MHC complex to the T-cell expressed CD4/CD8 co-receptors, auto-phosphorylation of Lck occurs initiating additional kinase activity.[1,2] The immunoreceptor tyrosine activation motif (ITAM) of the ζ chain of the CD3 subunit is phosphorylated by Lck, leading to binding of ZAP-70 through its SH2 domain. This in turn leads to phosphorylation of ZAP-70 by Lck.[3] Following activation of additional signaling molecules within the T-cell, Ca^{2+} is released inducing calcineurin activation and the ultimate production of interleukin-2 (IL-2) and interferon-γ (INF-γ) cytokines.[4] Expression of these cytokines leads to further activation and proliferation of T-lymphocytes characterized as the immune response.

The over-expression of pro-inflammatory cytokines has been implicated in a number of autoimmune disorders such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD), psoriasis, systemic lupus erythematosus (SLE), and organ graft rejection. Lck −/− mice have been shown to lack proper T-cell development and activation.[5] Therefore, selective inhibition of Lck by small molecule inhibitors would provide an orally bioavailable therapy for suppressing cytokine production and ultimately treating autoimmune disorders.

We have previously investigated a series of trisubstituted ureas for the inhibition of TNF-α production. Using X-ray co-crystallization studies, the potent analog **1** (Fig. 1) was shown to bind to p38α MAP kinase.[6] The compound adopted a pseudo-bicyclic conformation through internal hydrogen bonding between the urea NH and one of the pyrimidine nitrogens. Compounds from this class containing the 2,4-pyrimidine substitution were not active when screened against lck



1
TNF-a IC_{50} = 122 nM
Lck IC_{50} = >10 μM



2
Lck IC_{50} = 3.86 μM

Figure 1. Urea inhibitors of TNF-α and lck kinase.

Keywords: Lck kinase; IL-2 cytokine; Rheumatoid arthritis; Trisubstituted ureas.
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0960-894X/$ - see front matter
doi:10.1016/j.bmcl.2006.04.072

kinase. However, the closely related analog **2** (Fig. 1), containing the 4,6-pyrimidine substitution, was shown to have moderate potency toward inhibition of lck kinase activity with an IC_{50} of 3.86 μM.

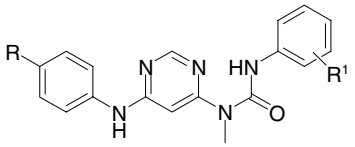
Recently, a patent application from Novartis disclosed their efforts toward the development of a similar series of trisubstituted ureas as protein kinase inhibitors.[7] In this manuscript, we would like to report the results of our own independent efforts in the development of trisubstituted ureas as lck inhibitors.

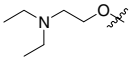
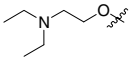
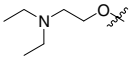
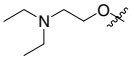
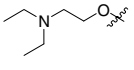
Synthesis of the 4,6-pyrimidine urea class of lck inhibitors is exemplified in Scheme 1. Boc protection of 4-morpholinoaniline (**3**) gave Boc-aniline **4**. Addition of **4** to 4,6-dichloropyrimidine gave the anilino-pyrimidine **5**. A second displacement with methyl amine using microwave irradiation (250 W, CEM Discover™ microwave synthesizer) led to the formation of diamino pyrimidine **6**. Urea formation was effected by reaction of **5** with 2,6-dichlorophenylisocyanate. Finally, Boc deprotection gave the desired urea **7c**. The remaining analogs in this report were synthesized using a modified version of this route.

All compounds in this study were tested for inhibition of lck kinase activity.[8] When the *N*-2,6-dichlorophenyl group was replaced with 2-chlorophenyl (**7a**), the lck inhibition improved marginally (Table 1). Further replacement of the 4-fluoroaniline with a 4-diethylaminoaniline gave compound **7b** which showed comparable lck inhibition to **2**. Morpholinyl analogs **7c** and **7d** containing an *N*′-2,6-disubstituted phenyl substituent showed greatly improved lck potency. However, the corresponding *N*′-2 substituted phenyl **7e** showed much reduced activity. A similar trend was seen with the corresponding *N*-methyl piperazine analogs **7f**–**7h**, although the compounds were relatively more potent overall. We also observed sub-100 nM lck inhibition for the 4-(2-diethylamino)-ethylether analogs **7i**–**7k**. An approximate 5-fold loss in potency was seen when substituting an *N*′-2,6-difluorophenyl for the *N*′-2,6-dichlorophenyl analog (**7l** vs **7i**). As with the *N*′-2-chlorophenyl analogs, a significant loss of activity was seen with the *N*′-2-methoxyphenyl analog **7m**.

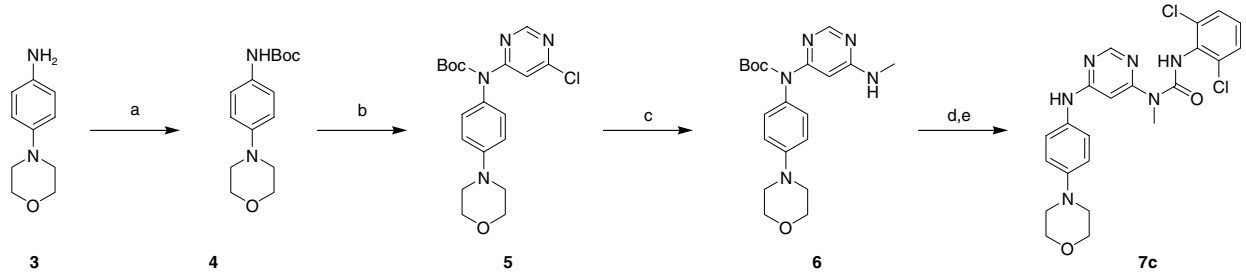
Having explored some general SAR about the 6-pyrimidyl and urea N′-positions, we next turned our attention to the urea N-position. We replaced the *N*-methyl group with various alkyl and aryl substituents as summarized in Table 2. The *N*-benzyl analog **8a** containing an *N*′-disubstituted phenyl group showed modest activity. However, other *N*-benzyl analogs containing an *N*′-chlorophenyl group (**8b** and **8c**) lost all inhibitory kinase activity. When the *N*-dimethylaminopropyl

Table 1. In vitro activity for *N*-methyl ureas



Compound	R	R^1	Lck IC_{50}[a] (μM)
2	F	2,6-di-Cl	3.86
7a	F	2-Cl	1.82
7b	Et_2N	2-Cl	3.13
7c		2,6-di-Cl	0.301
7d		2-Cl-6-Me	0.190
7e		2-Cl	4.29
7f		2,6-di-Cl	0.006
7g		2-Cl-6-Me	0.009
7h		2-Cl	0.708
7i		2,6-di-Cl	0.087
7j		2-Cl-6-Me	0.028
7k		2-Cl,6-CF_3	0.096
7l		2,6-di-F	0.418
7m		2-MeO	>10

[a] Standard deviation for the assay was ±15% or less.

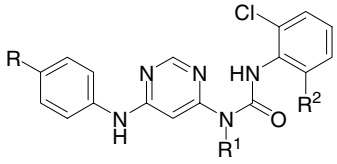


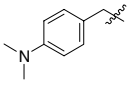
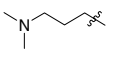
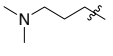
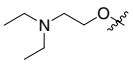
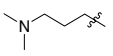
Scheme 1. Synthesis of 4,6-pyrimidine urea **7c**. Reagents and conditions: (a) Boc_2O, toluene, 110 °C; (b) 4,6-dichloropyrimidine, NaH, THF, 65 °C, 80%; (c) $MeNH_2$, dioxane, microwave, 125 °C, 15 min, 79%; (d) 2,6-dichlorophenylisocyanate, toluene, 110 °C, 80%; (e) TFA, CH_2Cl_2, rt.

group was incorporated (**8d**–**8g**), kinase inhibition was regained. The most potent analog, **8g**, contained the 4-morpholinoaniline group. When the dimethylaminopropyl group was replaced with a methylpiperazine (**8h**), only minimal attenuation of activity was observed. Substitution with a 4-pyridyl group (**8i**) conversely caused a complete loss of activity.

We also investigated various alkylamino substitutions at the 6-pyrimidyl position to replace the anilines used to this point (Table 3). The methylamine analog **9a**, a urea regioisomer of **7i**, was inactive toward lck inhibition. The *N*-dimethylaminopropyl analog **9b** showed some modest activity, but still much reduced compared to the corresponding 6-pyrimidyl aniline derivatives. While maintaining the *N*-methyl-*N*′-2,6-dichlorophenyl urea functionality constant, we looked at various 6-alkylamino pyrimidine analogs (**9c**–**9f**). Several combinations of linear and cyclic alkyl groups of various tether lengths were tested, but none of these analogs showed any appreciable activity. These results indicate the importance of having an anilino substituent off the 6-position of the pyrimidine in order to produce lck inhibition.

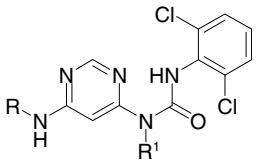
Table 2. Urea *N*-alkyl and phenyl SAR

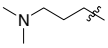
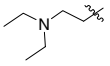
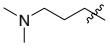
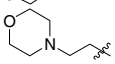


Compound	R	R^1	R^2	Lck IC_{50}[a] (μM)
8a	F	CH_2Ph	Me	1.45
8b	NEt_2	CH_2Ph	H	>10
8c	NEt_2		H	>10
8d	NEt_2		H	4.49
8e	NEt_2		Me	0.971
8f			Cl	0.229
8g			Cl	0.155
8h			Cl	0.230
8i			Cl	>10

[a] Standard deviation for the assay was ±15% or less.

Table 3. 6-Pyrimidine substitution SAR

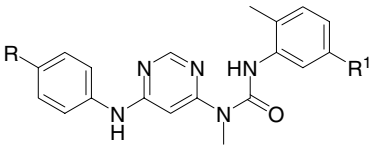


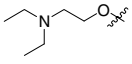
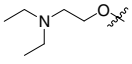
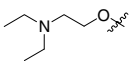
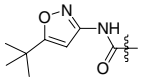
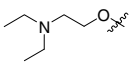
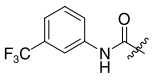
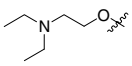
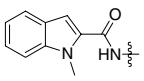
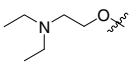
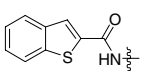
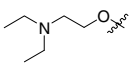
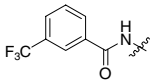
Compound	R	R^1	Lck IC_{50}[a] (μM)
9a	Me	4-Et_2N–$(CH_2)_2OPh$	>10
9b	Me		2.35
9c		Me	>10
9d		Me	5.99
9e		Me	>10
9f		Me	>10

[a] Standard deviation for the assay was ±15% or less.

Next, we investigated an alternative substitution pattern about the *N*′-phenyl group of the urea. An initial lead in the *N*′-2,5-disubstituted phenyl analogs was the 5-methoxy-2-methylphenyl derivative **10a** (Table 4). We discovered that demethylation of the methoxy group in **10a**

Table 4. *N*′-2,5-Disubstituted phenyl urea SAR



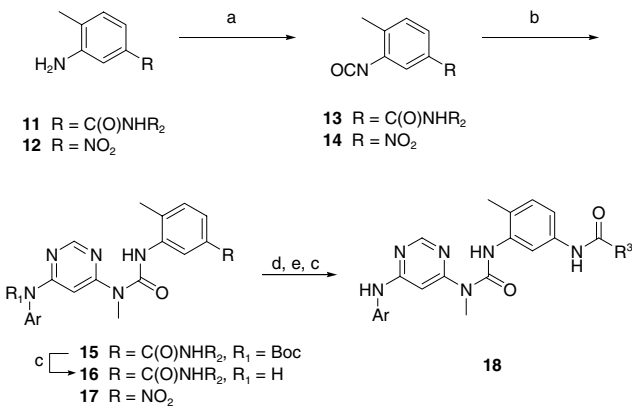
Compound	R	R^1	Lck IC_{50}[a] (μM)
10a		MeO	2.58
10b		OH	0.003
10c		OH	0.004
16a			0.004
16b			0.032
18a			0.149
18b			0.052
18c			0.003

[a] Standard deviation for the assay was ±15% or less.

(BBr_3, CH_2Cl_2, −78 °C to rt) gave 5-hydroxy-2-methyl derivative **10b** which showed excellent activity in the kinase assay. The substitution of the pyrimidyl 4-(2-diethylamino)-ethoxyaniline with a 4-morpholinyl aniline (**10c**) did not result in any reduction in activity.

Based on the success of the *N*′-5-hydroxy phenyl analogs **10b** and **10c**, we decided to extend the substitution at the 5-position of the *N*′-phenyl group to include amide functionality. Scheme 2 shows the synthesis of amides **16** and **18**. Starting with anilines **11**[9] or **12**, formation of the corresponding isocyanates was effected by heating the hydrochloride salts of the amides in the presence of phosgene in a sealed tube to give **13** and **14**. The Boc-protected ureas **15** and **17** were formed using standard conditions (see Scheme 1). Deprotection of the Boc group in **15** gave the desired amide urea **16**. Nitro analog **17** was reduced (H_2, Pd/C) and the resulting aniline acylated and Boc-deprotected to give the reverse amide **18**. Table 4 shows the results of kinase inhibition experiments for five of these analogs. Activities in the 3–149 nM range were observed for the various derivatives, indicating that even larger, more extended ureas were well tolerated in the assay. Perhaps most interesting was the 10-fold difference in potency between the two amide isomers **16b** and **18c**.

Compounds from above that showed promising lck inhibition were progressed into further in vitro and PK studies (Table 5). All compounds were tested for inhibition of IL-2 production in a Jurkat cellular assay.[10] Morpholinyl and piperazinyl analogs **7c** and **7g** showed fair to good IL-2 inhibitory activity (2.90 and 0.749 μM). However, both of these compounds showed poor aqueous solubility contributing to the reported poor bioavailability for **7c**. When the 4-(2-diethylamino-ethoxy)-aniline was used (**7i** and **7j**), the resulting compounds again showed good to fair IL-2 activity, but both compounds showed significantly improved aqueous solubility and good in vitro metabolism. Additionally, **7i** proved to be well absorbed orally in a rat PK study (F% = 66%). The highly potent lck inhibitor **10b** showed equally strong potency in the IL-2 assay. The compound also possessed good aqueous solubility. However, metabolism studies showed a high intrinsic clearance (CL_{int} = 108 mL/min/kg)[11] resulting in very poor oral bioavailability. We then transitioned to the equally potent amides **16a** and **18c**, which showed even greater relative inhibition of IL-2 production, but low solubility prohibited further progression of these compounds into PK studies.



Scheme 2. Reagents and conditions: (a) (i)—1 M HCl, Et_2O; (ii)—phosgene, toluene, 100 °C; (b) 4,6-diaminopyrimidine, CH_2Cl_2; (c) TFA, CH_2Cl_2; (d) **17**, H_2, Pd/C, MeOH; (e) NaOH (aq), CH_2Cl_2, $R^3C(O)Cl$.

Table 5. IL-2 inhibition and pharmacokinetic data for select ureas

Compound	IL-2 IC_{50}[a] (μM)	Solubility (μg/mL)	CL_{int}[b] (mL/min/kg)	C_{max} (ng/mL)	F (%)
7c	2.90	3	48	9	0
7g	0.749	2	—	—	—
7i	0.862	38	40	394	66
7j	3.10	34	25	—	—
10b	0.064	41	108	3	1
16a	<0.010	1	—	—	—
18c	<0.010	3	—	—	—

[a] IL-2 synthesis inhibition measured from Jurkat cells.
[b] Intrinsic clearance after 1 h for compounds (1 μM concentration) tested in cryopreserved rat hepatocyte suspensions.

Urea **7i** was progressed further into an in vivo rat adjuvant-induced arthritis study to test for oral efficacy. We observed that **7i** significantly ($p < 0.01$) inhibited hind paw swelling when administered orally twice daily at 25 mg/kg for 7 days by 63% compared to vehicle-treated control animals.[14]

In summary, we have reported a class of trisubstituted ureas that have shown nanomolar activity for inhibiting lck kinase activity. Six of the most potent analogs were further tested for inhibition of IL-2 cytokine production, showing a wide range of potencies. The urea analog that showed the best profile from an in vitro activity and pharmacokinetic stand point was tested for in vivo efficacy. Urea **7i** showed significant reduction in hind paw swelling when tested in a rat adjuvant-induced arthritis study. Therefore, this class of lck inhibitors shows promise as an orally bioavailable treatment for inflammatory disorders.

Acknowledgments

We thank M. M. Belkin for solubility and formulation studies, J. A. Troutman, C. A. Cruze, and W. P. Schwecke for metabolism and PK studies, C. M. Dunaway, M. Martin, and P. H. Zoutendam for analytical chemistry support, and J. T. Roesgen for kinase assay support.

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8. The ability of compounds to inhibit human Lck enzyme (IC_{50}) was determined using the commercially available ProFlour Src-family Kinase Assay (Promega Corporation, Madison, WI; cat. #1271). The assay was performed according to manufacturer's instructions, with 2 nM recombinant human active Lck (Upstate Cell Signaling Solutions, Charlottesville, VA; cat. #14-442) at an ATP concentration of 10 μM. Experiments were conducted in triplicate with an $n = 2$.
9. Aniline **11** was synthesized by amidation and subsequent hydrogenation of the commercially available 4-methyl-3-nitro-benzoyl chloride.
10. IL-2 release was measured by stimulating the Jurkat E6-1 T cell line (human acute T cell leukemia, ATCC, Manassas, VA) with monoclonal anti-human CD3ε antibody and phorbol myristate acetate (PMA) (Sigma, St. Louis, MO). Flat-bottommed 96-well plates were pre-coated with 400 ng/well of anti-human CD3ε mouse antibody UCHT1 (R&D systems, Minneapolis, MN) and incubated for 2 h at 37 °C. Jurkat cells maintained in RPMI-1640 containing 10% fetal bovine serum and 1% antibiotics in the log growth phase (2×10^5 to 1×10^6 cells/ml) were harvested and incubated in triplicate in 96-well plates for 30 min at 37 °C in the presence or absence of various concentrations of Lck inhibitors. The cell–inhibitor mixture was then transferred into the wells of the anti CD3ε-coated 96-well plates, and PMA was added to the wells at a final concentration of 10 ng/ml (1 ng/well). The plates were incubated overnight at 37 °C. The amount of IL-2 released into the culture media was measured by ELISA (R&D Systems) and the viability of the cells was determined using the MTS assay (Promega, Madison, WI).
11. Metabolic stability was assessed based on the extent of test article depletion over time. In vitro intrinsic clearance (CL_{int}) was calculated from the first-order depletion rate constant and appropriate biological scaling factors.[12,13]
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14. Adjuvant-induced arthritis was induced by a single subcutaneous injection of a 5 mg/kg dose of a suspension of *Mycobacterium butyricum* (Difco, Detroit, MI) in light mineral oil into the base of the tail of male Lewis rats (weights 190–220 g). Arthritis was assessed by the swelling in the hind paws by water displacement using a plethysmometer (Stoelting Co, Wood Dale, IL). Dosing of compound or vehicle (0.5% carboxymethyl cellulose with 1% Tween 80) was started once joint swelling was readily apparent (therapeutic protocol) which was about 10 days after injection of adjuvant. Compound **7i** was administered orally twice daily at 10 or 25 mg/kg for 1 week (day 10–17). The percent inhibition of paw swelling for compound **7i**-treated animals was calculated relative to the vehicle controls and statistical significance was determined using an analysis of covariance.