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Pyrrolo[2,3-*d*]pyrimidines Containing an Extended 5-Substituent as Potent and Selective Inhibitors of lck I

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Abstract—Pyrrolo[2,3-*d*]pyrimidines containing a 5-(4-phenoxyphenyl) substituent are potent and selective inhibitors of lck in vitro; some compounds are selective for lck over src. Data are shown for two compounds demonstrating that they are potent and selective inhibitors of IL2 production in cells.

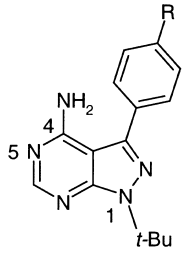
lck, an src-family tyrosine kinase expressed primarily in T lymphocytes, plays an essential role in the immune response.[1] A selective inhibitor of lck should specifically inhibit T-cell activation and have use in the treatment of autoimmune and inflammatory diseases and also in organ transplantation. The src-family kinases src, lck, lyn, hck, blk, fyn, fgr, yes, and yrk share a high level of homology at the amino acid level (>70% identical in the kinase domain) and have varying patterns of expression in the body. A significant challenge in the discovery of a lck inhibitor is in achieving selectivity within this family.

All src-family kinases have a similar domain structure,[2] comprised of an N_t unique domain, an SH3 domain, an SH2 domain, a tyrosine kinase catalytic domain and a short C_t tail. Activity is regulated by tyrosine phosphorylation at two sites. Phosphorylation of a tyrosine (Y505) in the C_t tail leads to down regulation by promoting an intramolecular interaction between the C_t tail and the SH2 domain. Phosphorylation of a tyrosine (Y394) in the activation loop segment of the kinase domain activates kinase activity.

Two crystal structures of the catalytic domain of lck in its active state (Y394 phosphorylated) have been published, one of the apoenzyme[3] and the other complexed[4] with AMP-PNP, staurosporine or PP2. The structure of two src-family kinases, in their inactive states (Y394 unphosphorylated) has also been determined; namely src[5] and hck[6] complexed with AMP-PNP and hck[7] with PP1.

Comparison of these structures suggests that there may be more structural difference between inactive forms of different src-family kinases thus affording an increased opportunity for obtaining selectivity.[7]

We screened inhibitors using two constructs of the human lck kinase, lck (64–509), and lckcd.[8] Using phosphopeptide mapping and MS we determined that lck (64–509) is in the inactive state (activation loop Y394 not phosphorylated) while lckcd is in the active state (activation loop Y394 is phosphorylated). Compounds inhibited the inactive form of lck more potently than the active form. The phosphorylation state of Y394 was determined to be solely responsible for these differences by studies using enzymatically dephosphorylated and phosphorylated versions of each of the lck protein constructs (data not shown).



1 R = Me, PP1
R = Cl, PP2

Compounds were also screened against src, however, the activation state of the src enzyme was not determined.

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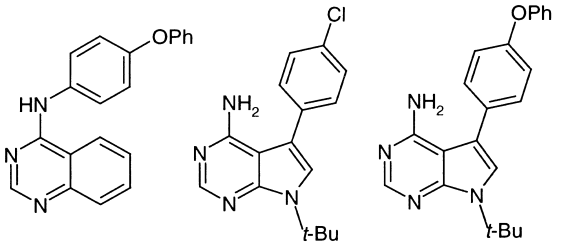
Nanomolar inhibitors of lck, e.g., PP1 (**1**) and PP2 had been described.[9] These compounds were reported as selective for src-family kinases over the non-receptor tyrosine kinase ZAP70 and the receptor tyrosine kinases JAK2 and EGFR.

This, and the following report, describe pyrrolo[2,3-*d*]pyrimidines containing an extended substituent in the 5-position which are potent inhibitors of lck, with some compounds being selective over src.[10]

Results and Discussion

From a library of 4-arylaminoquinazolines, the 4-phenoxphenylquinazoline **2** was identified as a potent inhibitor of lck (64–509) (Table 1). We reasoned that the phenoxyphenyl moiety of **2** occupies a hydrophobic pocket, present in a number of kinases, which is not accessed by ATP.[11] Also, we considered that PP1 and ATP may bind to lck in a similar manner (i.e., the 4-NH_2 group and N5 of PP1 form the H-donor/H-acceptor system) the *t*-butyl group occupies the sugar pocket and the 5-(4-tolyl) group is positioned in the hydrophobic pocket. Replacement of the methyl substituent in PP1 by a phenoxy group and conversion of the pyrazole into a pyrrole ring ought lead to a novel lck inhibitor **4**, with increased potency over PP1 by virtue of improved occupation of the hydrophobic pocket. Evaluation of **4** (Table 1) confirmed that it is a potent inhibitor of lck (64–509), being at least 75-fold more potent than PP1.



2 **3** **4**

Compared to PP1, **4** showed selectivity for lck (64-509) and lckcd (300- and 30-fold, respectively) over the closely related enzyme src and demonstrated at least 100-fold selectivity for lck over the human receptor tyrosine kinases kdr and tie-2. The 4-phenoxy group confers both improved potency for lck and selectivity over src. The pyrrolo[2,3-*d*]pyrimidine analogue of PP2, **3**, is equipotent to PP1, further supporting the crucial role of the phenoxy group in inhibition of lck.

Table 1. Inhibition of lck (64–509), lckcd, src, kdr and tie-2 for compounds **1**–**4** (IC_{50}s, 5 μM ATP)

Compound	lck (64–509)	lckcd	src	kdr	tie-2
1	0.151	0.25	0.17	1.6	7
2	< 0.008	NT	NT	2.72	17.87
3	0.22	NT	0.15	2.68	7
4	0.002	0.014	0.61	2.29	2.63

To explore structure–activity relationships (SARs) and to improve synthetic accessibility, N7 alkyl and cycloalkyl substituted analogues[12] of **3** were synthesised (Table 2). Compounds **4** to **9** in Table 2 are potent inhibitors of lck (64–509) with reduced potency against lckcd; they are selective for both forms of lck over kdr and tie-2. All these compounds demonstrate selectivity for lck (64–509) over src; however, only compounds **3**, **6**, **7**, **8**, and **9**, show greater than 30-fold selectivity for lckcd over src, which in view of the uncertain activation state of the src enzyme used is the most stringent selectivity measure. All the compounds are competitive with ATP.

The 3-hydroxycyclopentyl analogue **8** is the most potent inhibitor of lck of this set of compounds. The hydroxycyclopentyl analogues **7**, **8**, and **9** are mixtures of stereoisomers; no separation or resolution was attempted.[16]

Varying the position of the phenoxy substituent, as in compounds **10** and **11**, led to a large loss of potency against both forms of lck.

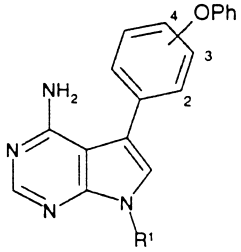
The activity of **1**, **6**, and **8** against a range of kinases and in cellular assays[13] is in Tables 3 and 4. They are selective for lck over a range of receptor, non-receptor tyrosine kinases and seronine/threonine kinases. The cellular profile of **6** and **8** compared with PP1 is in Table 4. The pyrrolopyrimidines **6** and **8** are potent inhibitors of IL2 production in Jurkat cells stimulated with anti-CD3 antibody, being at least 100-fold more potent than PP1. The increased potency of **8** in vitro is mirrored by its high potency in cells. Both **6** and **8** display remarkable cellular selectivity. Their potencies for inhibition of IL2 production are in the micromolar range when the cells are stimulated with phorbol 12-myristyl 13-acetate (PMA) in the presence of ionophore. Such stimulation bypasses lck indicating minimal inhibition of PKC or downstream kinases.

Modelling

A homology model of lck based on Kuriyan's inactive hck (PDB accession code 1AD5) structure[6] was constructed in Quanta using the program Modeler. The pyrrolopyrimidine ring of **6** (the structure of **6** was built in Quanta and minimized in Mopac) was overlaid with the adenine ring of AMP-PNP and the system minimised using Charmm. An excellent fit of **6** into the ATP binding site of lck was observed, the phenoxyphenyl group having near optimal occupation of the hydrophobic pocket. The 4-NH_2 group makes a hydrogen bond donor contact with the backbone carbonyl of Glu317 and the N-3 of the pyrimidine ring accepts a hydrogen bond from the backbone NH of Met319. The cyclopentyl ring is in the space occupied by the ribose ring of AMP-PNP.

Comparison of the crystal structures of active and inactive src family kinases suggest that the hydrophobic pocket accessed by the phenoxyphenyl moiety of **6** in our model is subject to dramatic changes in size and

Table 2. Inhibition of lck (64–509), lckCD, src, kdr and tie-2 for compounds **4**–**11** (IC_{50} values)



			lck(64-509)		lckcd		src	kdr	tie-2
Compound	R^1	PhO	5 μM ATP	1 mM ATP	5 μM ATP	1 mM ATP	5 μM ATP	5 μM ATP	5 μM ATP
4	*t*-Butyl	4	0.002	0.075	0.0138	NT	0.61	2.29	2.68
5	*i*-Propyl	4	<0.001	0.011	0.01	NT	0.048	0.43	0.43
6	Cyclopentyl	4	<0.001	0.016	0.002	1.07	0.07	1.57	1.98
7	2-OH-Cyclopentyl	4	<0.001	0.24	<0.008	1.32	0.04	1	0.6
8	3-OH-Cyclopentyl	4	<0.001	0.005	<0.001	0.544	0.088	0.085	0.0583
9	3,4-Di-OH-cyclopentyl	4	<0.002	0.023	0.005	0.317	0.084	0.27	0.15
10	Cyclopentyl	3	0.14	1.18	1.2	>10	>1	7.1	3.4
11	Cyclopentyl	2	0.39	NT	NT	NT	NT	>50	4.9

Table 3. Kinase activity of **6** and **8**

Compound	Zap70	EGFR	PKC	CDC2/CyB	lck (64–509)
6	>50	3.2	>33	>50	<0.001
8	>50	NT	>33	>50	<0.001

Table 4. Cellular activity of compounds **1**, **6**, and **8**

Compound	Jurkat cell antiCD3	Jurkat cell PMA	MLR
1	0.1–1.2	NT	0.3–0.4
6	<0.001–0.04	1.5	0.2–0.8
8	<0.001	3	0.028

character. Helix αC of phosphorylated lck (PDB accession code 3LCK) is rotated and drawn inward relative to the same structural element of unphosphorylated hck. This conformational change results in a binding pocket approximately 20% smaller in the phosphorylated enzyme. The reduced potency of compounds **6** to **9** for lckcd compared to lck (64–509) in Table 2 is particularly evident at 1 mM ATP and may be a result of the reduced volume of the hydrophobic pocket in the active form of lck. We propose that the increased potency of **8** against both forms of lck is due to a hydrogen bond between the 3-hydroxyl on the cyclopentyl ring of **8** with either Ser343 or Asp345; the 2-hydroxyl in **7** appears to be too far away to make a similar contact.

The proposed binding mode of **6** to lck (64–509) in our homology model (Fig. 1) is virtually identical to that described by Kuriyan[6] for PP1 bound to inactive hck (the 5-(4-tolyl) group of PP1 only partially filling the hydrophobic pocket) and to that described by Zhu[5] of PP2 bound to active lck. Currently we have no structural explanation for the apparent selectivity of **6** (or **4**, **7**, **8**, and **9**) for lck over src. The residues in the ribose binding pocket of src family kinases are identical (apart from blk, Ser323 to Cys). However, there are differences between lck and src in the hydrophobic binding pocket, the nature and structure of which are likely due to the relative orientation of the N and C lobes and the activation state of the protein. Rationalisation of selectivity awaits a crystal structure comparison with bound inhibitor.

Maintaining the H-bond donor/acceptor interactions for **10** and **11** with Glu317 and Met319, respectively, in the lck homology model prevents the phenoxyphenyl moiety from making optimum use of the hydrophobic pocket. Alternatively the 4-NH_2 and the pyrimidine N3 cannot make productive H-bond contacts with the protein if the phenoxyphenyl group is docked into the hydrophobic pocket.

Synthesis

Two routes were used to prepare the pyrrolopyrimidine compounds described in this paper. Scheme 1, starting

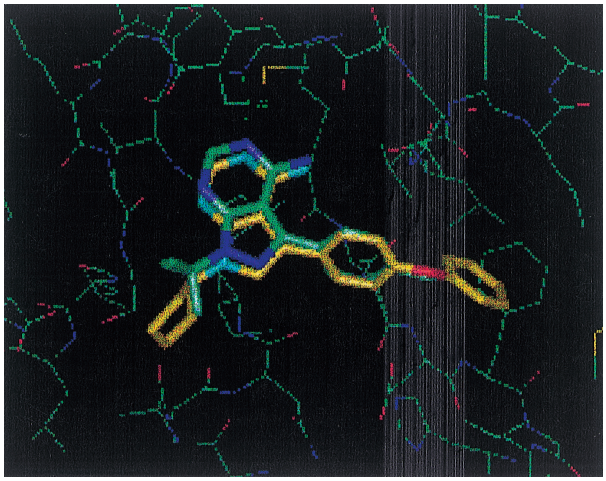
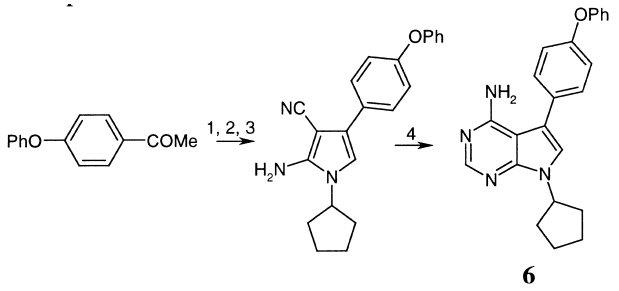
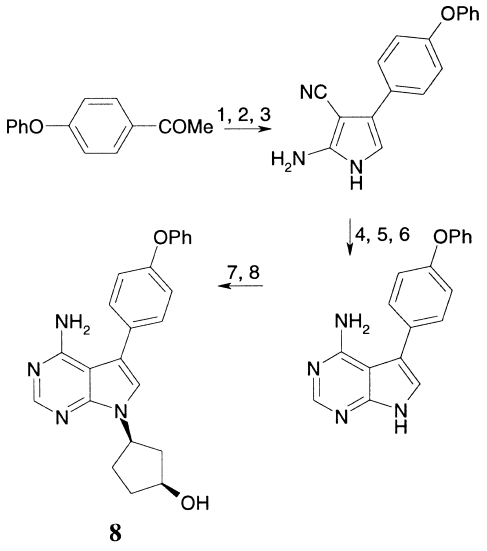


Figure 1. Model of **6** (yellow) and PP1 bound to lck (64–509).



Scheme 1. Reagents and conditions: (1) pyridinium perbromide, acetic acid; (2) cyclopropylamine, toluene, $<15\,^{\circ}C$ then rt, 20 h, 38%; (3) malononitrile, methanol, KOH, water, $<10\,^{\circ}C$ then $65\,^{\circ}C$, 1 h, 53%; (4) formamide, ammonia (gas), $200\,^{\circ}C$, 2.5 h, 31%.



Scheme 2. Reagents and conditions: (1) pyridinium perbromide, acetic acid; (2) potassium phthalimide, DMF, rt, 18 h; (3) malononitrile, NaOMe, $35\,^{\circ}C$ for 1 h; rt for 18 h, 47% overall; (4) triethyl orthoformate, $140\,^{\circ}C$, 1 h, 100% (5) ammonia (gas), methanol, $<30\,^{\circ}C$ then rt for 18 h, 68%; (6) NaOEt, ethanol, reflux, 1 h, 83%; (7) 3,4-epoxycyclopentene, $Pd(PPh_3)_4$, 0–$25\,^{\circ}C$ (exclude light), 65%; (8) H_2, Pd/C, EtOH (100%).

from 4-phenoxyacetophenone, is a four-step route, exemplified for **6**.

The more versatile route is shown in Scheme 2 for **8**.

In summary, novel pyrrolopyrimidine compounds containing a 5-(4-phenoxyphenyl) substituent are described, some of which are potent and selective inhibitors of active and inactive forms of lck in vitro, some compounds show selectivity over src. Compounds **6** and **8** are potent inhibitors of IL2 synthesis in cells and inhibit the MLR. They also show cellular selectivity by virtue of the lack of inhibition of IL2 release from Jurkat cells when stimulated with PMA in the presence of ionophore.

References and Notes

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16. **7** is a racemic mixture of (1*S*,2*R*)- and (1*R*,2*S*)-*trans*-2-(4-amino-5-(4-phenoxyphenyl)-7*H*-pyrrolo[3,2-*d*]pyrimidin-7-yl) cyclopentanol. **8** is a racemic mixture of (1*S*,3*R*)- and (*1R*,3*S*)-*cis*-3-(4-amino-5-(4-phenoxyphenyl)-7*H*-pyrrolo[3,2-*d*]pyrimidin-7-yl)cyclopentanol. **9** is a mixture of *cis* and *trans* stereoisomers, namely c-4 and t-4-(4-amino-5-(4-phenoxyphenyl)-7*H*-pyrrolo[3,2-*d*]pyrimidin-7-yl)cyclopentan-r-1,c-2-diol.