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Pyrazolo[3,4-*d*]pyrimidines Containing an Extended 3-Substituent as Potent Inhibitors of Lck — a Selectivity Insight

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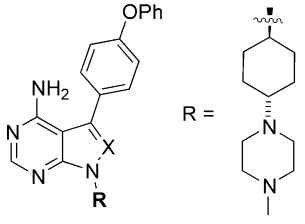
Abstract—A series of *para*-substituted 3-phenyl pyrazolopyrimidines was synthesized and evaluated as inhibitors of lck. The nature of the substitution affected enzyme selectivity and potency for lck, src, kdr, and tie-2. The *para*-phenoxyphenyl analogue **2** is an orally active lck inhibitor with a bioavailability of 69% and exhibits an extended duration of action in animal models of T cell inhibition.

The src family tyrosine kinase lck expressed primarily in T lymphocytes,[1,2] plays an essential role in the immune response.[3] Antigen-induced T cell activation is characterized by the appearance of a lck-driven hyperphosphorylated TcR ζ-chain, a critical event necessary for recruitment of the syk family kinase ZAP-70 to the signaling complex.[4] Genetic data validate lck as an immunosuppressive molecular target as a SCID-like phenotype was observed in mice rendered lck deficient by targeted gene disruption.[5] Lck −/− mice are incapable of rejecting either MHC-incompatible or minor incompatible skin grafts despite the presence of peripheral T cells.[6] A selective inhibitor of lck should inhibit T-cell activation and have broad application for the treatment of autoimmune and inflammatory diseases and also organ transplant rejection.[7]



1 X = CH
2 X = N

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The preceding publication in this journal[8] described SAR of a series of pyrrolo[2,3-*d*]pyrimidines containing substituents at N-7. Compound **1** was identified as an orally active lck inhibitor that inhibits T-cell receptor (TCR) stimulated (α-CD3 mAb) IL-2 production in mice after oral administration with an $ED_{50} = 2.5$ mg/kg.

Although compound **1** demonstrated sub-optimal pharmacokinetic characteristics such as a very high volume of distribution and high plasma clearance, it represented a key compound for further optimization. We also showed that occupying the lipophilic pocket in lck with the phenoxyphenyl moiety resulted in increased potency versus lck.[9] Our results also indicated that an appended solubilizing heterocycle in the ribose pocket, such as the *N*-methyl piperazine in **1**, facilitated oral dosing.[8]

This paper describes our studies towards modifying the pyrrolopyrimidine core and the phenoxyphenyl motif of **1** to optimize the in vivo profile. In order to reduce the volume of distribution of **1** by lowering its lipophilicity, we elected to probe the corresponding pyrazolo[3,4-*d*]pyrimidine, compound **2**.[10] These compounds have measured LogD values at pH 7.4 of 4.5 and 3.04, respectively.

We screened inhibitors against a non-phosphorylated construct of human lck kinase,[9] in an HTRF format[11,12] at a physiologically relevant[13] ATP concentration of 1 mM. The closely related kinase src, and two receptor tyrosine kinases (RTKs), kdr and tie-2, served as

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counterscreens. Inhibitors were progressed for profiling in cellular settings and ultimately in vivo. Data are shown for the inhibition of α-CD3 mAb induced IL-2 production in human whole blood and for inhibition of TCR stimulated (α-CD3 mAb) IL-2 production in mice after oral dosing. In lieu of extensive pharmacokinetic profiling we probed the pharmacodynamic duration of action by measurement of the inhibition of IL-2 production 8 h and 18 h after oral dosing. This approach was designed to identify those compounds with the most advantageous pharmacodynamic coverage as a harbinger of good pharmacokinetics.

The pyrazolopyrimidine **2** and its pyrrolopyrimidine congener **1** in Table 1 exhibited comparable in vitro potency for lck showing the functionally silent nature of the CH to N interchange and suggested no major binding contribution from N2 in compound **2**.

This observation supports our working model that the key beneficial protein interactions of compound **1** are N3 and the 4-NH_2 making contacts with Glu317 and Met319 of lck. A critical hallmark is also that the lipophilic phenoxyphenyl group accessed the hydrophobic cavity which is not exploited by ATP.[9]

As shown in Table 2, compound **2** was a potent inhibitor of IL-2 production in vivo with an ED_{50} of 1.5 mg/kg and greater than 18 h duration of action when dosed at ED_{90}.

The data for compound **2** showed that there was value in prosecuting the pyrazolopyrimidine series further. To explore potential selectivity enhancements we studied the role of the atom linking the two phenyl groups. Atomic substitution in the 'linker' is forgiving as the aniline **3** is indistinguishable from the ether **2** in our kinase panel. This enabled utilization of a parallel chemistry approach for exploration of the role of the tether. In addition, molecular modeling studies suggested that a productive contact with the side-chain hydroxyl group of Thr316 could be achieved by installation of a methoxy residue juxtaposed to the linker atom (shown as R^1). This latter modification also simplified our synthetic approaches vide infra. Combination of these two concepts led to the benzamide **5** and the carbamate **6**, both of which showed increases in selectivity against the two RTKs when compared to the benchmark of compound **2**. In the first instance, this was primarily due to a reduction in potency for kdr and tie-2 and in the latter, because of an enhancement in potency for lck.

In accord with the close catalytic site homology,[14] src inhibition closely mirrored potency against lck. These results showed that the oxygen bridge in **2** could be substituted by linkers of varying lengths and differing functionalities resulting in enhancement of both potency for lck and selectivity against the receptor tyrosine kinases. Further modification of the linker utilizing aniline **21** in Scheme 1 as our building block allowed for rapid exploration of the generality of this observation. These analogues are exemplified in Tables 3 and 4. The most striking results were obtained for the amide **5**, the carbamate **6**, the urea **7** and the sulfonamide **8** in Table 3 as these highlight remarkable selectivity switches across the kinases on linker modification. Amide **5**, and in particular carbamate **6**, exhibited high selectivity for lck over kdr and tie-2. Conversely, the urea linker in **7** enhanced kdr potency to such an extent that this resulted in a pluripotent compound. Also surprising was the identification of the tie-2 selective sulfonamide **8**, a 137 nM inhibitor of tie-2 with greater than 20- and 300-fold selectivity over the src family kinases and kdr, respectively. Although a fuller understanding of the structural basis of these observations awaits the identification of ligand:protein co-crystal structures, these two com-

Table 1. Inhibition of lck, src, kdr, and tie-2 for **1** and **2** (IC_{50} μM)[a]

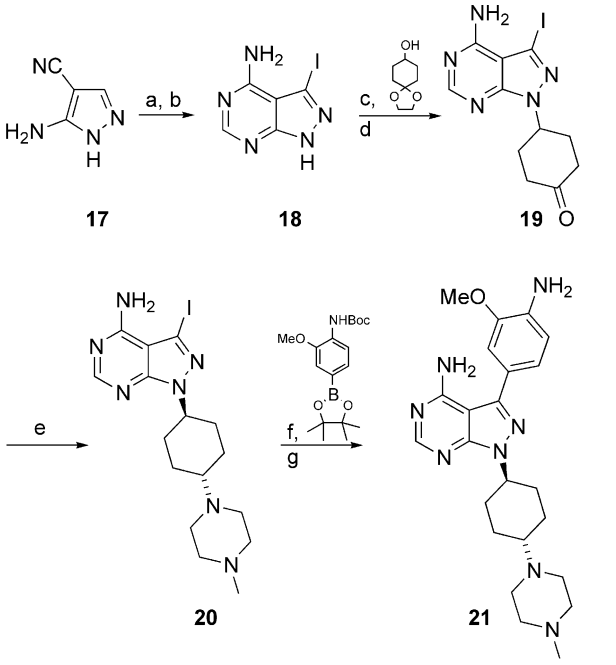
	lck	src	kdr	tie-2
1	0.015	0.042	1.19	0.25
2	0.040	0.035	5.32	0.75

[a]Mean of two experiments performed with seven concentrations of test compound.

Table 2. Mouse in vivo data for **2**

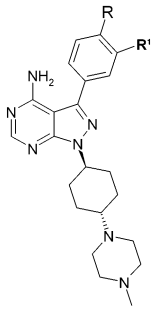
ED_{50}[a] (mg/kg)	ED_{90}[a] (mg/kg)	Inhibition (%)[b]	
		8 h	18 h
1.5	6	84	49

[a]Oral dosing, measured 2.5 h. after dosing.
[b]After dosing at ED_{90}.



Scheme 1. Reagents and conditions: (a) formamide, 180 °C; (b) NIS, DMF, 50 °C; 84% two steps; (c) Ph_3P, DEAD, THF; (d) acetone, 5 N HCl (aq), 50%; (e) *N*-methyl piperazine, $Na(OAc)_3BH$, DCE, AcOH, 17%; (f) $Pd(PPh_3)_4$, Na_2CO_3, DME, H_20, 80 °C, 77%; (g) TFA, DCM 74%.

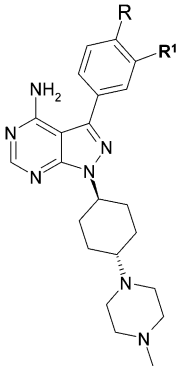
Table 3. Inhibition of lck, src, kdr and tie-2 for **3–8** (IC_{50} μM)[a]

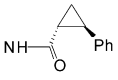


R	R^1		lck	src	kdr	tie-2	kdr/lck	tie-2/lck
NHPh	H	**3**	0.040	0.065	3.81	0.606	95	15
COPh	H	**4**	0.110	0.427	>50	5.73	>450	52
NHCOPh	OMe	**5**	0.093	0.189	>50	4.68	>500	50
$NHCO_2Bn$	OMe	**6**	0.004	0.0125	34.2	0.521	**8550**	**130**
NHCONHPh	OMe	**7**	0.209	0.310	0.263	0.933	**1.25**	4.4
$NHSO_2Ph$	OMe	**8**	3.120	3.910	>50	0.137	>16	**0.044**

[a]Mean of two experiments performed with seven concentrations of test compound.

Table 4. Inhibition of lck, src, kdr, and tie-2 for **9–16** (IC_{50} μM)[a]



R	R^1		lck	src	kdr	tie-2
$NHSO_2NHPh$	OMe	**9**	0.167	0.463	NT	0.888
$NHCH_2Ph$	OMe	**10**	0.055	0.068	>50	1.13
$NHCOCH_2CH_2Ph$	OMe	**11**	0.028	0.155	>50	2.45
$NHCOCMe_2CH_2Ph$	OMe	**12**	0.092	0.46	>50	1.44
$NHCOCH_2CMe_2Ph$	OMe	**13**	0.031	0.115	34.6	0.91
	OMe	**14**	0.019	0.027	>50	0.275
NHCO(benzfuran-2-yl)	OMe	**15**	0.010	0.043	>50	1.02
$NHCH_2$(indol-2-yl)	H	**16**	0.050	0.526	0.435	0.203

[a]Mean of two experiments performed with seven concentrations of test compound.

pounds suggest a possible starting point for the design of selective kdr and tie-2 inhibitors.

Emphasis was then placed on exploration of the extended amides exemplified by the phenethyl amide **11**. The increased degrees of freedom compared to the benzamide **5** do not translate into a reduction in lck potency as **11** is 3-fold more potent than **5**.

Table 5. Human whole blood and murine in vivo data

Whole blood	Mouse in vivo data			
IC_{50} (μM)	[a]ED_{50} (mg/kg)	[a]ED_{90} (mg/kg)	Inhibition[b] 8 h	18 h
3 0.135	2.5	10.7	95	<10
5 0.050	9	23	37	<10
6 0.002	3	14	51	14
11 0.038	5	30	63	<10
13 0.008	2	12.5	98	22
15 0.006	8.5	12.5	48	<10

[a]Oral dosing measured 2.5 h after dosing.
[b]After dosing at ED_{90}.

Table 6. Pharmacokinetic parameters for **2**

C_{max} (μmol/L)	T_{max} (h)	V_d (l/kg)	Cl_p (l/kg)	$T_{1/2}$ (h)	F (%)
0.62	1.5	9.6	1.2	5.2	69

As a strategy to further increase potency in the extended amide **11**, we elected to reduce the entropic options available to the linker. This tactic led to the synthesis of the two regioisomeric geminal dimethyl analogues **12** and **13** in addition to the two amides incorporating cyclized extended motifs, exemplified in **14** and **15**. In striking contrast to the unadorned aminomethyl analogue **10**, that showed no inhibition of kdr at 50 μM, it is interesting to note the increased potency of the aminomethyl indole **16** for kdr.

Table 5 shows the human whole blood potency and in vivo data for a selection of these compounds. The alignment of the in vitro lck potency with the whole blood data suggested that the compounds achieved effective cellular penetration and that plasma protein binding was not effecting potency. All the compounds were potent inhibitors of IL-2 production in vivo, possessing an ED_{50} of less than 10 mg/kg after oral dosing. Although two of the compounds exhibited greater than 90% inhibition of IL-2 production at 8 h, they portrayed minimal activity at 18 h. As these compounds showed efficacy at 2.5 h, we ascribed their low efficacy at 18 h to be a function of poor pharmacokinetics. In contrast, compound **2** had sufficient activity at 18 h to warrant full pharmacokinetic profiling.

The pharmacokinetic parameters for compound **2** after a single (5 mg/kg) oral and intravenous dose to male Sprague–Dawley rats are detailed in Table 6.

The 5 mg/kg dose generated C_{max} plasma levels 23-fold higher than the whole blood IC_{50} for IL-2 inhibition (data not shown). This, in combination with the high bioavailability and acceptable elimination half-life, account for the extended pharmacodynamic efficacy observed. Compound **2** showed a 14-fold reduction in volume of distribution and a 4-fold drop in plasma clearance compared to compound **1**.

Utilizing an analogous modeling approach to our earlier paper[9] Figure 1 depicts an array of germane hydrogen

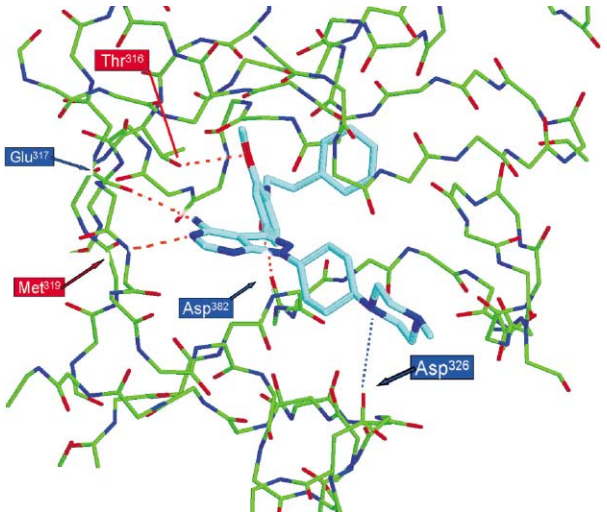


Figure 1. Model of **11** bound to lck.

bond interactions that orient compound **11** within the protein. The donor–acceptor pair of the 4-NH_2 and N5 make contact with the main chain carbonyl of Glu317 and Met319 of lck, respectively. The methoxy group is within hydrogen bond donating distance of Thr316 and N2 has no interactions with the protein. The solvent exposed region is characterized by contacts between the internal piperazine nitrogen and the conserved side-chain acid of Asp326. Productive contacts to the aspartic acid 382 in the DFG motif of lck are established from the amide linker. An X-ray crystallographic analysis of a representative set of these inhibitors bound to lck will be the subject of future publications.

Cyclization of the amino-pyrazole **17** with formamide followed by iodination at C3 gave the pyrazolopyrimidine **18**. Mitsunobu coupling with the ketal-alcohol followed by ketone unmasking gave **20** as a key intermediate. Reductive amination with *N*-methyl piperazine gave a 2.5:1 ratio of the *cis*/*trans* diastereomers, the latter possessed the desired[8] configuration. Suzuki coupling with the appropriate boronate ester followed by deprotection of the carbamate furnished the pivotal amine **21** allowing for further functionalization. Reaction with benzoyl chloride gave the amide **5** and comparable chemistry gave access to the other amides in Tables 3 and 4. The absence of the central methoxy group rendered the aniline less nucleophilic as supported by the isolation of products as a result of reaction at the 4-NH_2 position in addition to the desired aniline (data not shown). Reductive amination of aniline **21** with benzaldehyde generated amine **10**. Compound **2** was accessed by Suzuki coupling of iodide **20** with 4-phenoxyphenylboronic acid. Other derivatives were made by analogous chemistry.

In summary, the nature of the Ph-**X**-Ph linker in these tyrosine kinase inhibitors defines the potency and, perhaps more interestingly, the selectivity for lck, src, kdr and tie-2. A sulfonamide linker as in **8** drives tie-2 potency, whereas a urea, such as **7** introduces kdr activity. Ethers, ketones, carbamates and amides engender lck and src activity. In line with its lower LogD compared to compound **1**, the ether-linked pyrazolopyrimidine **2** exhibits an enhanced pharmacokinetic profile and also portrays prolonged activity in animal models of T cell activation after oral administration.

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References and Notes

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