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New pyrazolo[1,5*a*]pyrimidines as orally active inhibitors of Lck

Nina Gommermann *, Peter Buehlmayer, Anette von Matt, Werner Breitenstein, Keiichi Masuya, Bernard Pirard, Pascal Furet, Sandra W. Cowan-Jacob, Gisbert Weckbecker

Novartis Institute of Biomedical Research, Forum 1, Novartis Campus, 4056 Basel, Switzerland

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ABSTRACT

A novel series of pyrazolo[1,5*a*]pyrimidines was optimized to target lymphocyte-specific kinase (Lck). An efficient synthetic route was developed and SAR studies toward activity and selectivity are described, leading to Lck inhibitors with enzymatic, cellular and in vivo potency.

Lymphocyte-specific protein tyrosine kinase (Lck) is a member of the Src-family of non-receptor protein tyrosine kinases with preferential expression in T-cells and natural killer (NK) cells.[1–3] Lck plays a critical role in the initial steps of T-cell receptor (TCR) signaling.[4] Activation of TCR signaling by Lck triggers a cascade of downstream signaling pathways, leading to the production of cytokines such as interleukin-2 (IL-2) and interferon-γ, and ultimately T-cell activation and proliferation.[5–7] Genetically modified mice deficient in Lck exhibit defects in T-cell maturation and signaling.[8] These findings suggest that Lck inhibitors should inhibit T-cell activation and therefore are potentially useful in the treatment of T-cell mediated autoimmune diseases and organ transplant rejection.[9] Because of the restricted expression pattern of Lck, selective Lck inhibitors would be expected to have an improved safety profile over current immunosuppressive agents possessing non-lymphocyte-related toxicities.

A selective Lck inhibitor has been shown to prolong graft survival in preclinical animal models of solid organ transplantation.[10] In this paper, we discuss the design, synthesis and structure–activity relationships (SAR) of a novel class of potent Lck inhibitors based on pyrazolo[1,5*a*]pyrimidines.

The development of this new class of Lck inhibitors was based on an initial screening hit. We carried out a focused screen of in-house kinase scaffolds likely to inhibit Lck enzyme activity, selected on the basis of structural information. Using a Lck lance assay,[11] we found that the pyrazolo[1,5*a*]pyrimidine **1a** (Fig. 1, X=O, R=H, *N*-methylpiperazine in *meta*-position) potently inhibits Lck and shows interesting selectivity against the anti-targets Hck, cSrc and KDR. The pyrazolo[1,5*a*]pyrimidine scaffold was initially designed by molecular modeling as an ATP-mimic for tyrosine kinase projects.[12,13]

Figure 2 shows the X-ray structure of **1a** in complex with the kinase domain of Lck, which confirms the design principle. The compound binds in an ATP-competitive binding mode to an active conformation of Lck (DFG-in, C-helix-in), with the pyrazolo-nitrogen and the NH_2-group acting as hinge binders. Furthermore, the NH_2 donates an H-bond to the Thr gate keeper. The phenol is point-

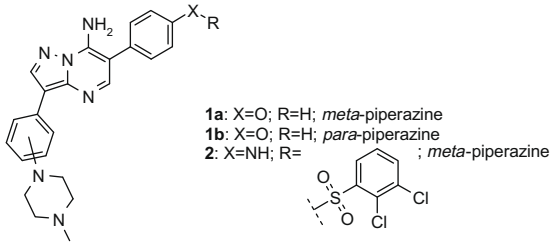

Figure 1. General structure of Lck inhibitors based on pyrazolopyrimidines.

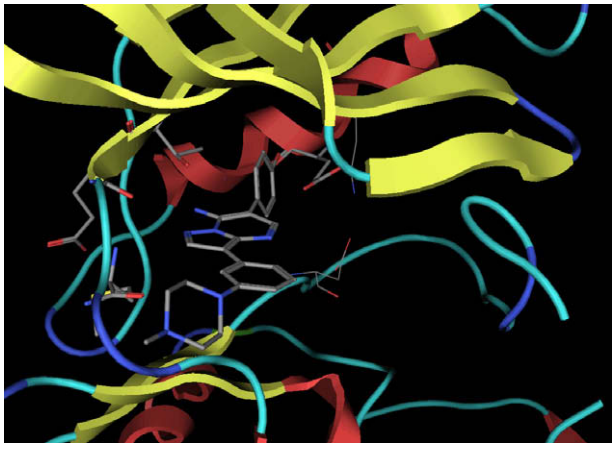

Figure 2. X-ray structure of **1a** in complex with Lck. Amino acids involved in H-bonds are displayed as sticks.

* Corresponding author. Tel.: +41 613248560.
E-mail address: nina.gommermann@novartis.com (N. Gommermann).

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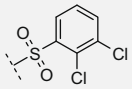
Table 1
Inhibition of Lck activity and selectivity of initial screening hits **1**–**2** in biochemical assays (all IC_{50} values in nM).

No.	X	R	Piperazine	Lck lance	cSrc lance	Hck lance	KDR
1a	O	H	*meta*	30	442	342	470
1b	O	H	*para*	36	396	85	1300
2	NH		*meta*	28	>1000	>1000	5400

ing toward the back pocket, and donates an H-bond to the side chain of Glu288 (helix C). The methylpiperazine is solvent exposed and makes no direct contact to the protein, the closest potential interaction being the main-chain carbonyl of Glu320.[14]

SAR by screening of selected in-house compounds[15] revealed that the methylpiperazine-moiety can also be shifted from the *meta*- to the *para*-position as exemplified by **1b**, and the phenol can be replaced by sulfonamides like **2** with no significant loss in potency (Fig. 1 and Table 1). *meta*-Piperazine **1a** showed superior selectivity against Hck over *para*-substituted isomer **1b**, whereas **1b** was more selective against KDR compared to **1a**. Sulfonamide **2** showed improved selectivity against cSrc, Hck and KDR compared to phenols **1a–b** and maintained good Lck inhibition.

Unfortunately, physicochemical properties of sulfonamides, such as **2**, were less favorable, for example, low aqueous solubility and permeability as well as high log *P* values. We therefore decided to explore other aniline substituents in combination with both the *meta*- and *para*-piperazine moiety. Compound **1a** matches the recently described type I1/2 pharmacophore for DFG-in kinases.[16] We used the crystal structure of **1a** to rationalize the structure–activity relationships (SAR) for Lck activity and to guide the selection of substituents binding to the back pocket (X—R in Fig. 1) and to the hydrophobic channel.[17]

The synthetic route toward compounds **3a–g** is shown in Figure 3, regioisomers **4a–g** were synthesized in a similar manner.[13,15] Palladium-catalyzed amination of 3-bromobenzylcyanide **5** with *N*-methylpiperazine **6** led to 3-(*N*-methyl)piperazinylbenzylcyanide **7**. Condensation with ethylformate in the presence of NaOMe as a base furnished **8**, which was cyclized with hydrazine without purification to give the aminopyrazole **9**. Condensation of 4-nitrobenzylcyanide **10** with DMF–DMA resulted in the formation of **11**. Under acidic conditions, **9** and **11** were cyclized to give the pyrazolopyrimidine **12**. Subsequent reduction of the nitro-group under hydrogenation conditions finally gave key intermediate **13**. The building block **13** was then reacted with acid chlorides and chloroformates to give the target compounds **3a–g**.

Table 2
Inhibition of Lck activity and selectivity in biochemical assays and cellular potency (all IC_{50} values in nM) of compounds **3**,**4a**–**g**.

No.	Lck lance	cSrc lance	Hck lance	KDR	FACS
3a	4	135	37	690	33
3b	23	>1000	295	2300	323
3c	24	1706	594	5350	63
3d	16	688	193	4100	140
3e	8	1000	172	nd	31
3f	6	227	70	130	16
3g	8	554	110	6000	333
4a	4	142	14	1015	76
4b	75	>1000	256	8300	106
4c	22	662	181	4233	107
4d	14	394	88	1000	37
4e	25	>1000	215	nd	92
4f	5	143	21	nd	13
4g	13	497	178	435	128

* nd, not determined.

All analogues were tested for their ability to inhibit Lck enzyme and for their selectivity against cSrc, Hck and KDR in a biochemical assay (Table 2). Compounds that potently inhibited Lck in the

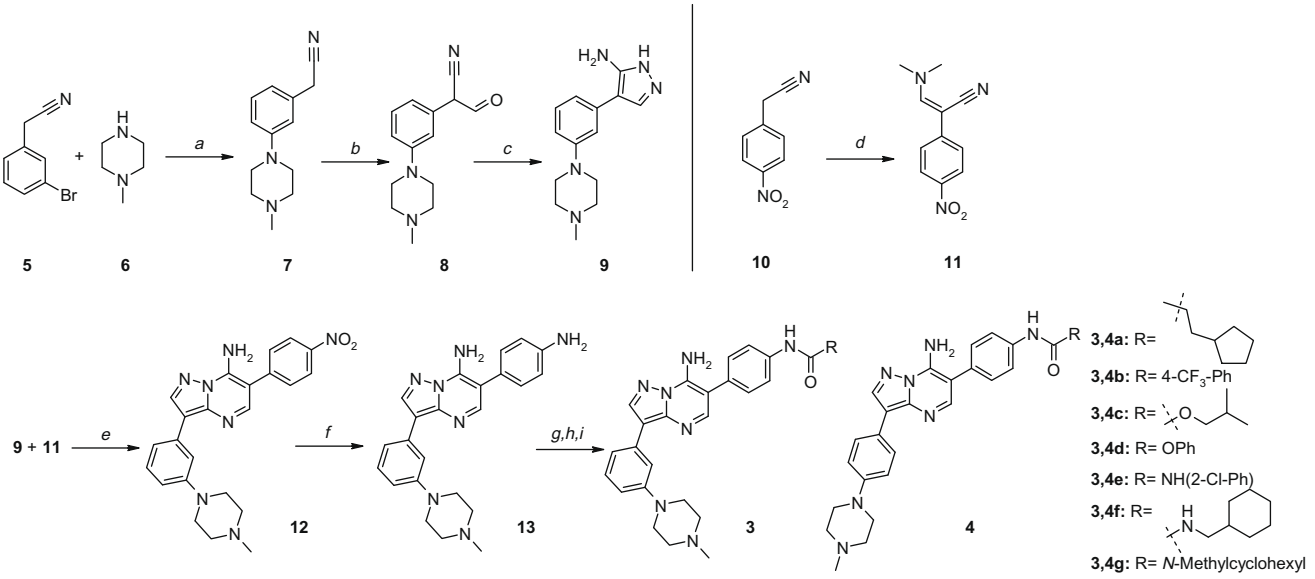


Figure 3. Synthesis of compounds **3**. Reagents and conditions: (a) $Pd(OAc)_2$, 2-(di-*t*-butylphosphino)biphenyl, K_3PO_4, DME, 85 °C, 20 h, 67%; (b) NaOMe, Ethyl formate, toluene, 40 °C, 2 h, quant.; (c) hydrazine-hydrate, toluene, reflux, 4 h, 59% over 2 steps; (d) DMF–DMA, toluene, 120 °C, 1.5 h, 96%; (e) **9** + **11**, 1.25 M HCl in EtOH, AcOH, 130 °C, 36 h, 72%; (f) H_2, Pd–C, MeOH, rt, 18 h, 67%; (g) **3a**,**b**: RCOCl, NMP, 5 °C, 1 h, 64–71%; (h) **3c**,**d**: ROCOCl, pyridine, DCM, rt 1 h, 50 °C, 1.5 h, 25–36%; (i) **3e**–**g**: 1—*p*-NO_2Ph-OCOCl, NMP, 5 °C, 30 min, 2—RNH_2, NMP, 120 °C, 1–3 h, 31–60%.

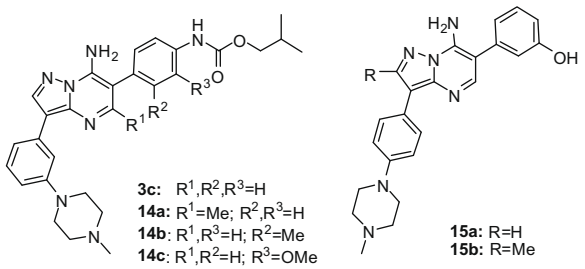


Figure 4. Core-substituted pyrazolopyrimidines **14**–**15**.

Table 3
Inhibition of Lck activity and selectivity in biochemical assays (all IC_{50} values in nM) of compounds **14**–**15**.

No.	Lck lance	cSrc lance	Hck lance	KDR
3c	24	1706	594	5350
14a	116	>1000	>1000	>10,000
14b	23	462	456	3400
14c	546	>1000	>1000	9200
15a	8	2	8	96
15b	>1000	>1000	>1000	500

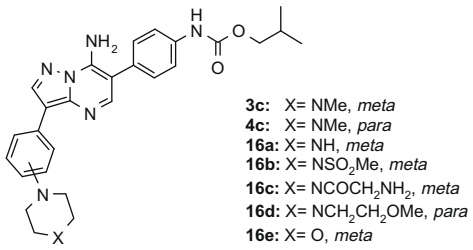


Figure 5. Variations of the solubilizing group.

enzymatic assay were also tested in T cells by measuring ZAP70 phosphorylation as a function of Lck activity by Phosflow FACS technology.[18] Interestingly, amides, carbamates and ureas were tolerated as substituents in the enzymatic Lck assay and showed good selectivity over KDR, the selectivity over cSrc and especially Hck depends on the substitution pattern. Aliphatic amides **3a** and **4a** demonstrated potent inhibition of Lck in the enzymatic assay and cellular system but weak selectivity over Hck. The aromatic amides **3b** and **4b** showed inferior Lck potency compared to the aliphatic amides. In the carbamate series, both aliphatic (**3c** and **4c**) and aromatic carbamates (**3d** and **4d**) were tolerated. These compounds displayed weaker Lck activity compared to amides **3a** and **4a**, but improved selectivity, especially over Hck. Aromatic urea **3e** was highly active in the enzymatic assay and showed similar inhibition to amide **3a** in the cellular FACS assay, in addition it displayed an increased selectivity over cSrc and Hck. Aliphatic ureas (**3f** and **4f**) were superior to aromatic ureas (**3e** and **4e**) in the cellular assay, but less selective. Interestingly, methylation of the urea NH (**3g** and **4g)** was tolerated in the enzymatic system and found beneficial for selectivity, but did not translate into cellular potency.

Analysis of the carbamate series by docking into the active site of Lck showed that the carbamate NH donates an H-bond to the side chain of Glu288 (helix C), while the sp^2 oxygen of the carbamate accepts an H-bond from the backbone NH of Asp382 (DFG motif). Compound **3c** showed the most promising selectivity profile combined with only a small potency shift from the enzymatic

Table 4
Inhibition of Lck activity, selectivity (all IC_{50} values in nM) and microsomal stability of compounds **16**.

No.	Lck lance	cSrc lance	Hck lance	KDR	Rat Cl (μL/min)	$t_{1/2}$ (min)
3c	24	1706	594	5350	612.5	2.3
4c	22	662	181	8300	161.2	8.6
16a	7	642	310	2100	29	48
16b	209	>1000	676	>10,000	58.4	23.7
16c	7	762	270	1900	11.2	123.6
16d	37	1000	349	2200	nd	nd
16e	699	>1000	>1000	7350	nd	nd

Table 5
Kinase selectivity profile of **4c**.

Kinase	IC_{50} (μM)	Kinase	IC_{50} (μM)
Lck	0.026	Ins1R	>10.000
cSrc	5.700	Jak1	>10.000
Hck	9.050	Jak2	>10.000
KDR	4.233	Jak3	>10.000
Tie-2	1.000	cKit	0.089
cAbl	0.040	cMet	>10.000
cAblT315	3.250	PDGFRa	0.074
Alk	>10.000	PDK1	>10.000
Cdk2A	>10.000	PKA	>10.000
FGFR3K	>10.000	PKBa	>10.000
Her1	>10.000	Ret	4.700
Her2	>10.000	Syk	>10.000
IGF1R	>10.000	Zap70	>10.000

to the cellular assay, we therefore decided to retain the *i*-butyl carbamate and *meta*-piperazine moiety for further SAR elaboration.

We investigated the influence of substituents on the pyrazolopyrimidine core and the aniline ring (Fig. 4 and Table 3). Introduction of a methyl group (**14a**) to the pyrimidine ring led to a decreased Lck inhibitor potency. Introduction of a methyl group to the aniline ring, *ortho* to the pyrazolopyrimidine (**14b**), retained potency but its selectivity profile was inferior to the unsubstituted analogue **3c**. The introduction of a methoxy-group to the aniline ring, *meta* to the pyrazolopyrimidine (**14c**) was not tolerated and led to loss in activity. The introduction of a methyl-group to the pyrazole ring of the core was initially investigated in the phenol-subseries (**15a** vs **15b**). This did not warrant further investigation as it led to a complete loss in activity.

Finally, we turned our attention to optimization of the solubilizing group. The *N*-methylpiperazine was found to be a useful substituent to achieve good inhibitor potency, but we observed high to very high clearance in rat liver microsomes when this group was present. In silico prediction of the clearance mechanisms using *MetaSite*[19] revealed *N*-demethylation as a major pathway. In order to overcome this route of metabolism, we tested several other solubilizing groups for both their effect on Lck activity and their microsomal stability (Fig. 5 and Table 4).

The des-methyl-compound **16a** was found to be a very potent inhibitor of Lck and displayed very good selectivity against cSrc, Hck and KDR. The clearance in rat liver microsomes could be substantially reduced by switching from *N*-methylpiperazine to piperazine, increasing the half-life from 2.3 min (**3c**) to 48 min (**16a**). This suggests that the computational prediction of *N*-demethylation as a major pathway for metabolism was correct. The introduction of a sulfonamide on the piperazine (**16b**) was not tolerated by the enzyme, but acylation with glycine (**16c**) led to a highly active compound that demonstrated a low clearance and long half-life, but only marginal aqueous solubility. Alkylation with a methoxyethyl-group led to **16d** with only mediocre activity on the enzyme. Complete removal of the basic nitrogen (morpholino-compound

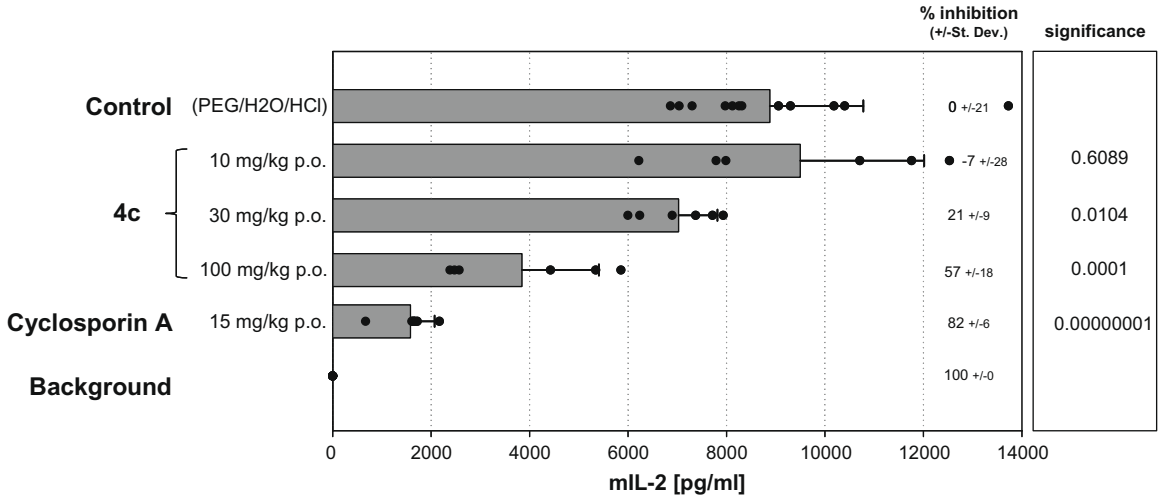


Figure 6. Effect of **4c** on *anti*-CD3-induced IL-2 response in BALB/c mice.

16e) destroyed Lck activity. Although the solubilizing group is solvent exposed and forms only limited contacts with the protein, we reasoned that the lower Lck activity of **16b** and **16e** is due to repulsive electrostatic interactions between the sulfonamide group (**16b**) or the morpholine (**16e**) and acidic residues located on the surface of Lck (Glu249, Glu320 and Asp326).

The NH-piperazine **16a** showed increased metabolic stability over its *N*-Me analogue **4c**, but a substantial decrease in permeability was also observed. Compound **4c** was selected for further profiling since it displayed promising PK-properties in rat (V_D = 6421 mL/kg; Cl = 1928 mL/h/kg; $t_{1/2}$ = 5.7 h; BAV 95%). The broader selectivity profile of **4c** (Table 5) showed good selectivity against the major anti-targets cSrc, Hck, KDR and Tie-2, and showed crossreactivity on only 3 out of 26 kinases tested, namely cAbl, cKit and PDGFRa.

Lck inhibitors have been previously reported to inhibit the *anti*-CD3-mediated IL-2 production in whole blood as well as the concanavalin A-induced IL-2-release in vivo.[20] Compound **4c** was therefore tested in vivo for the inhibition of T-cell activation, the *anti*-CD3 antibody-induced IL-2 response in BALB/c mice.[21] A dose-dependent inhibition of IL-2 production was observed, with 57% (±18%) inhibition at 100 mg/kg po of **4c** (Fig. 6).

In summary, a promising new structural class of potent Lck inhibitors was discovered. Selective Lck inhibitors were obtained through structure-based drug design and SAR-guided optimization. It was demonstrated that pyrazolopyrimidine Lck inhibitors show an effect in an in vivo model of T-cell activation.

Acknowledgments

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