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Discovery of thienopyridines as Src-family selective Lck inhibitors

Lily Abbott, Patrick Betschmann, Andrew Burchat, David J. Calderwood, Heather Davis, Peter Hrnciar, Gavin C. Hirst,* Biqin Li, Michael Morytko, Kelly Mullen and Bryant Yang

Abbott Bioresearch Center, 100 Research Drive, Worcester, MA 01605-5314, USA

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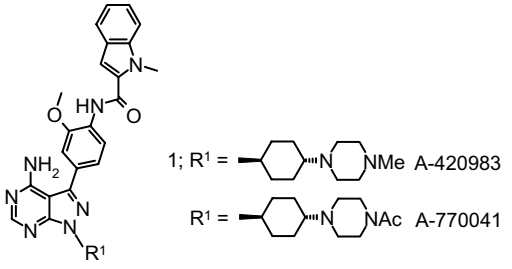
Abstract—We describe the identification, SAR, and in vivo pharmacology of a new series of Src-family selective Lck inhibitors. These thienopyridines were designed based on a desire to access the unique residues in the extended hinge region of Lck.

The Src-family of tyrosine kinases comprises eight highly homologous proteins that are primarily expressed in hematopoietic tissues, two of which, Lck and Fyn, are expressed in T cells. Lck plays a critical function during the initial steps of T-cell receptor signaling[1] resulting in a cascade of downstream signaling pathways leading to T-cell activation and the production of cytokines such as IL-2 and IFNγ.[2,3] A selective inhibitor of Lck should prevent T-cell activation and thus have broad application for the treatment of T-cell dependent processes such as autoimmune and inflammatory diseases as well as organ transplant rejection.



We previously reported the identification of a novel pyrazolopyrimidine A-420983, a potent Lck inhibitor that prevents TCR-mediated T-cell activation and prolongs survival of major histocompatibility mismatched allografts in models of solid organ transplant rejection after oral administration.[4] Although A-420983 provides a useful tool, it exhibits little selectivity within the Src-family. Herein we describe our efforts that result in the identification of a new class of Lck inhibitors with high selectivity within the Src-family.

The crystal structure of A-420983 in highly homologous Hck (residues 60–505, pY^{501}-EEI) as a Lck surrogate has been determined[4] and the binding mode is represented in Figure 1 (Lck residues in red). The 4-amino group makes a key H-bond donor contact to the backbone C=O of Glu317 (Lck numbering) whilst the N5 pyrimidine nitrogen contacts the backbone NH of Met319 in the hinge region. The indole extends into the hydrophobic pocket and is surrounded by residues Leu303, Ile314, Met292, and Leu385. The methoxy group makes a van der Waals contact with the side chain of Thr316 whilst the amide carbonyl contacts the backbone NH of Asp382 in the conserved DFG-motif. The *trans*-cyclohexyl piperazine moiety extends into the solvent exposed region where the terminal piperazine nitrogen makes a charge-reinforced hydrogen-bond to the side chain of the conserved Asp326. As previously demonstrated with A-770041[5], making this contact with basic amines was detrimental for selectivity, hence our current approach was to focus on breaking this latter interaction and probe alternative extended hinge interactions. In particular, we felt that making productive contacts with the side chains of Tyr318 and the unique Glu320 in the extended hinge region of Lck would drive selectivity within the Src-family.

Inhibitors were screened against a non-phosphorylated construct of human Lck, Lck (64–509) in HTRF format

Keywords: Lck inhibitors; Src-family; Kinase inhibitors; Thienopyridines.
* Corresponding author at present address: SGX Pharmaceuticals, 10505 Roselle St., San Diego, CA 92121, USA. Tel.: +1 858 228 1630; fax: +1 858 558 0642; e-mail: gavin_hirst@sgxpharma.com

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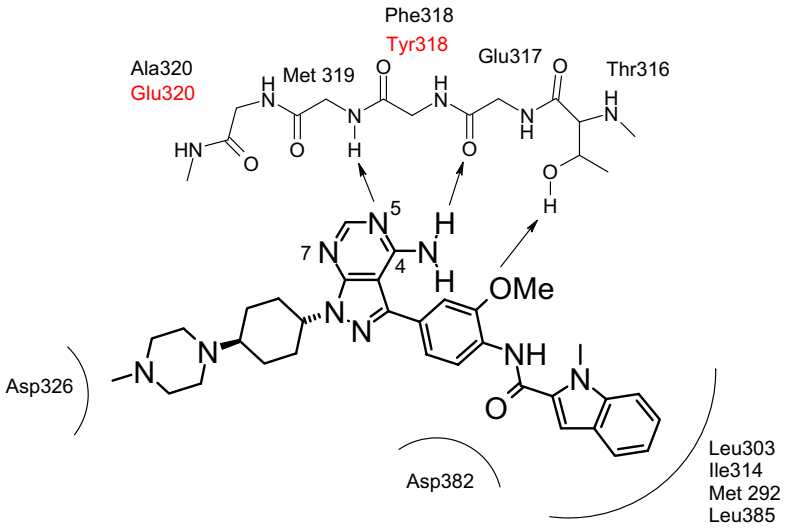
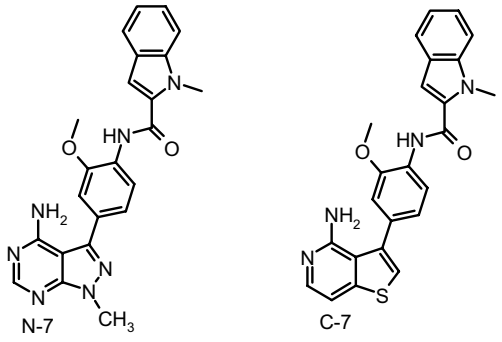


Figure 1. Binding mode of A-420983 in Hck (Black). Lck residues in red.

at physiologically relevant 1 mM ATP concentration and biotinylated Lck peptide as substrate.[6] The closely related Src-family kinase Hck served as our initial selectivity counterscreen. This was predicated on the fact that Hck differentiates itself from Lck at the residues of current focus, Tyr318 and Glu320, as shown in Figure 1. Selected compounds were progressed for wider kinase profiling and for activity in cellular settings and ultimately in vivo. Data are presented here for the inhibition of anti-CD3 mAb induced IL-2 production in human whole blood[6] and for an acute in vivo assay of the inhibition of TCR stimulated (anti-CD3 mAb) IL-2 production in mice after oral dosing. Resulting IL-2 levels were measured by an Enzyme Linked Immunosorbent Assay (ELISA) method.



2 Lck IC_{50} = 0.031 μM

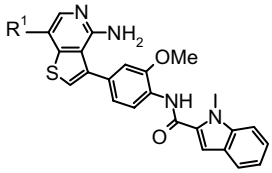
3 Lck IC_{50} = 5.2 μM

We were very encouraged by the fact that the truncated analog **2**, despite being devoid of the cyclohexyl piperazine, was equipotent to A-420983. In order to access the extended hinge region, our analysis of the crystal structure of A-420983 suggested branching at N-7 was desirable, a site excluded by valency requirements. We therefore elected to probe thienopyridines as potential Lck inhibitors, an approach that would allow branching at C-7. The drop in potency of the thienopyridine benchmark compound **3**, a 5.2 μM Lck inhibitor, suggests a significant reduction in hinge binding efficiency in this core compared to the pyrimidine counterpart **2**. Our initial investigation of the impact of branching at C-7 is shown in Table 1.

It is clear that substitution at C-7 can provide a significant potency increase. Comparison of the Lck potency of the two regioisomeric amides **5** (20.8 μM) and **11** (0.24 μM) suggests a requirement for an Sp^2 carbon at the C-7 branch point. This observation is supported by data for the vinyl amide **12** and by the improved potency of the allylic alcohol **14** compared to its saturated counterpart **15**. Cyclization of amide **9** to the piperazine amide **10** results in greater than 100-fold reduction in potency. Despite increases in potency, the only compound in Table 1 that exhibited more than 14-fold selectivity against Hck was the allylic amine **16**. Although definitive proof awaits the identification of Lck: ligand cocrystal structures, these data are consistent with the allylic amine making a productive interaction with the distinguishing Tyr318 in Lck and that the carboxamides such as **9** and **11** are unable to access this side chain.

Having established that our initial area of opportunity to identify compounds with improved Src-family selectivity resided in the allylic amines, we investigated this in more detail. These results are shown in Table 2. Initially noteworthy is the decreased Lck potency of the *cis*-allylic amine **17** compared to the trans diastereomer **18**. In addition, we observed an increased selectivity for Lck against Hck going from the amide **13** (10-fold) to the corresponding amine **25** (220-fold) primarily due to an 18-fold reduction in Hck potency. This suggests a detrimental impact of either a basic residue or increased entropy for Hck but not for Lck. This observation mirrored the analogous selectivity found in Table 1 for the amide **12** (4-fold) and amine **16** (38-fold). Non-basic sulfonamide, urea and amide termini represented by **19**, **20**, and **21**, respectively, lost selectivity and/or potency compared to the amine parent **16**. Further extending the distance from C-7 to the basic terminus as seen in **22**, **25**,

Table 1. Inhibition of Lck and Hck (IC_{50}, μM) by compounds **4**–**16**



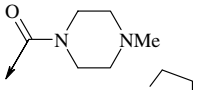
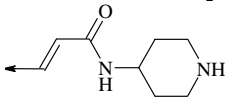
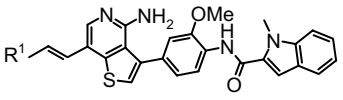
R^1	Compound	Lck	Hck
$-NH_2$	**4**	9.65	>50
$-NHCOCH_2CH_2N$	**5**	20.8	45.29
$-NHCONHCH_2CH_2N$	**6**	6.85	>50
–COOH	**7**	>50	>50
$-CONH_2$	**8**	0.38	1.41
$-CONHCH_2CH_2NMe_2$	**9**	0.14	1.97
	**10**	17.6	>50
$-CONHCH_2CH_2N$	**11**	0.24	2.55
trans-$CH{=}CHCONH_2$	**12**	0.541	2.26
	**13**	0.13	1.29
trans-$CH{=}CHCH_2OH$	**14**	0.77	2.36
$-CH_2CH_2\ CH_2OH$	**15**	4.81	>50
trans-$CH{=}CHCH_2NH_2$	**16**	0.23	8.8

Table 2. Inhibition of Lck and Hck (IC_{50}, μM) by compounds **16**–**26**



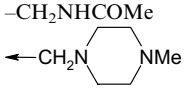
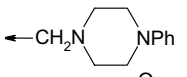
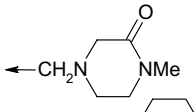
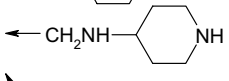
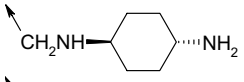
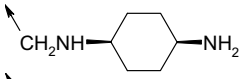
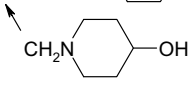
R^1	Compound	Lck	Hck
cis-CH_2NEt_2	**17**	4.47	>50
$-CH_2NEt_2$	**18**	0.29	13..6
$-CH_2NHSO_2Me$	**19**	1.37	4.3
$-CH_2NHCONHPh$	**20**	6.7	21.9
$-CH_2NHCOMe$	**21**	0.72	2.36
	**22**	0.29	5.97
	**23**	2.95	>50
	**24**	1.52	12.9
	**25**	0.19	24.2
	**26**	0.11	24.1
	**27**	0.48	20.1
	**28**	0.21	10.0

Table 3. Kinase inhibition (IC_{50} μM) by compounds **18**, **25**–**28**

Compound	Lck	Src	Fyn	Fgr	Hck	Lyn	Tie-2	Kdr
18	0.29	9.05	44.1	14.1	13.6	1.18	>50	>50
25	0.19	14.4	26.2	26.5	24.2	3.7	3.7	>50
26	0.11	19.7	25.9	17.9	24.1	18.5	1.9	18.5
27	0.48	27.1	26.3	21.7	20.1	12.9	0.57	>50
28	0.21	33.9	35.1	37.9	10.0	5.3	10.1	>50

26, and **27** maintains both Lck potency and selectivity against Hck. Of note, the trans amine **26** is the more potent of the two diastereomers. We selected a subset of compounds for wider kinase profiling both within the Src-family and against two receptor tyrosine kinases, Kdr and Tie-2. These data are shown in Table 3.

Although many of these analogs portrayed very high selectivity across all members of the Src-family, the amino alcohol **28** was the only example with >20-fold selectivity against all the kinases. It is of note that this selectivity profile is increased compared to the previously published pyrazolopyrimidine A-770041.[5] In particular, A-770041 exhibited only 8-fold selectivity against Hck and Lyn.

In accord with our previous publications[4,5] all compounds profiled in the human whole blood assay (data not shown) showed alignment with the Lck in vitro potency suggesting that the compounds achieved effective cellular penetration and that plasma protein binding was not effecting potency. In mice, compound **28** inhibited TCR stimulated (anti-CD3 mAb) IL-2 production in a dose-responsive manner with an ED_{50} of 5 mg/kg after oral administration. The pharmacokinetic profile in rats is shown in Table 4.

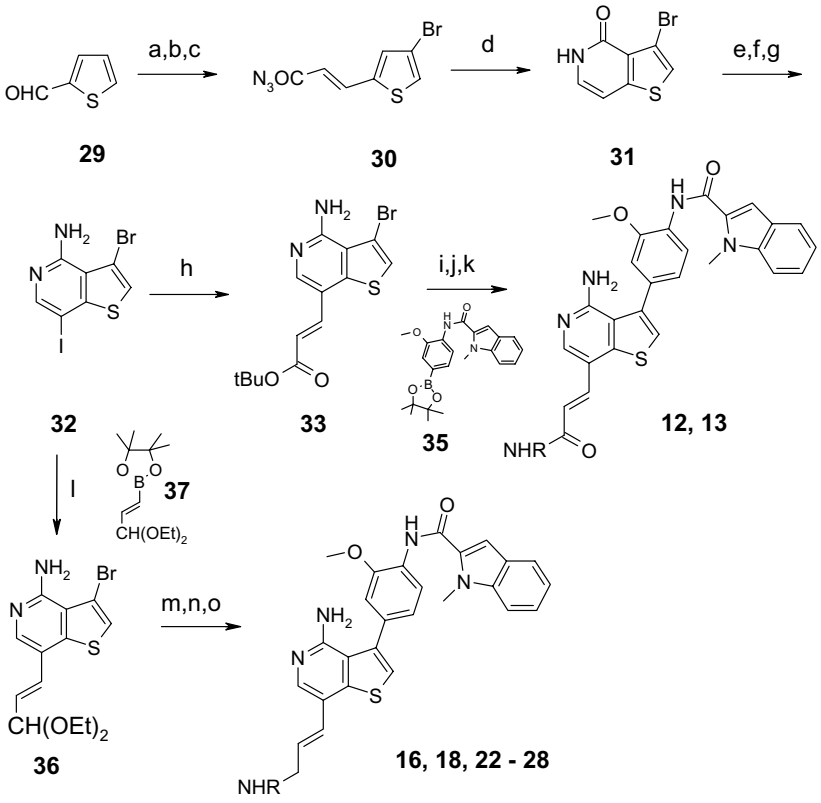
Despite exhibiting a long half-life, compound **28** had a pharmacokinetic profile that excluded it from advancement into chronic disease models. In particular, the high clearance and moderate oral bioavailability were areas of concern. Efforts to marry the unprecedented Src-family selectivity of this series of compounds with improved pharmacokinetic profile are ongoing.

Access to the compounds in Tables 1 and 2 was achieved from the orthogonally functionalized thienopyridine **32**. Many examples are captured in Schemes 1 and 2. Electrophilic bromination of thiophene **29** followed by a Knoevenagel reaction with malonic acid and acyl azide formation gave the cyclization precursor **30** in 86% yield over three steps. Curtius rearrangement followed by intramolecular cyclization at high temperature gave the pyridone **31** in 90% yield. Chlorination and subsequent displacement followed by regioselective iodination gave the key building

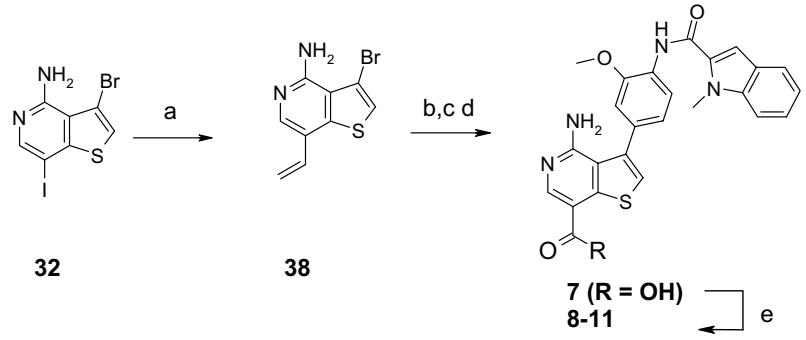
Table 4. Rat pharmacokinetic parameters for compound **28**

T_{max} (h)	Clp (l/h/kg)	$T_{1/2}$ (h)	F (%)
5.3	6.3	8.1	21

5 mg/kg iv, 10 mg/kg po.



Scheme 1. Reagents and conditions: (a) Br_2, $AlCl_3$, CH_2Cl_2, 90%; (b) $CH_2(COOH)_2$, piperidine(cat) pyridine, 100 °C, 22 h, 96%; (c) i—$(COCl)_2$, DMF (cat), 14 h, rt; ii—NaN_3, rt, 3 h, 100%; (d) Bu_3N, PhOPh, 225 °C, 45 min, 86%; (e) $POCl_3$, 135 °C, 2.5 h, 95%; (f) NH_4OH, dioxane, 150 °C, 18 h, 94%; (g) NIS, DMF, rt, 75%; (h) *tert*-butyl acrylate, $Pd(OAc)_2$, PPh_3, Na_2CO_3, DMF, 80 °C, 68%; (i)—**35**, DME/H_2O, Na_2CO_3, $Pd(PPh_3)_4$, reflux, 75%; (j) TFA, CH_2Cl_2, 100%; (k) HBTU, HOBT, DIEA, DMF, amine, 55–76%; (l) **37**, DME/H_2O, Na_2CO_3, $Pd(PPh_3)_4$, reflux, 75%; (m) **35**, DME/H_2O, Na_2CO_3, $Pd(PPh_3)_4$, reflux, 76%; (n) p-TSA, acetone, water, 100%; (o) amine, $NaB(OAc)_3$, DCE, 67–88%.



Scheme 2. Reagents and conditions: (a) $Bu_3SnCH{=}CH_2$, KF/toluene, $Pd(PPh_3)_4$, reflux, 90%; (b) **35**, DME/H_2O, Na_2CO_3, $Pd(PPh_3)_4$, reflux, 76%; (c) $OsO_4/NaIO_4$, dioxane/water, 22%; (d) TPAP, 49%; (e) HOBt/HBTU, DMF, RNH_2, 85%.

block **32**. Heck reaction with the corresponding acrylic ester presented ester **33**. Suzuki coupling with the previously published boronate ester **35** installed the northern domain which gave the α–β unsaturated amides **12** and **13** after standard deprotection and coupling protocols.

Alternatively, regioselective Suzuki reaction of **32** with the vinyl boronate **37** gave acetal **36**. A second Suzuki reaction with boronate **35** and unmasking to the α–β unsaturated aldehyde followed by reductive amination gave amines **16**, **18**, and **22**–**28** after appropriate deprotection where required.

Scheme 2 portrays our route to the amides **8**–**11**. Stille coupling with vinyl-tributyl tin gave **38** which was functionalized to the aforementioned amides through standard oxidative and coupling procedures.

In summary, by designing molecules to probe the unique residues in the hinge region of Lck, we have identified a series of potent and highly selective Lck inhibitors that

exhibit oral activity in acute in vivo models of T-cell activation. This represents an advancement in the understanding of features important for selectivity within the Src-family of tyrosine kinases.

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6. Mean of two experiments performed with seven concentrations of test compound.