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# Discovery of A-770041, a src-family selective orally active lck inhibitor that prevents organ allograft rejection

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**Abstract**—We describe the identification, SAR, and pharmacology of the src-family selective lck inhibitor A-770041 that prolongs the survival of major histocompatibility mismatched allografts in models of solid organ transplant rejection for greater than 65 days.


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 37 nM 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 is less than 10-fold selective over fyn (fyn = 330 nM) and thus hindered the study of TCR signaling and interpretation of in vivo data due to the compensatory role of fyn in T-cell activation.[5] Herein we describe our efforts that result in the identification of lck inhibitors with high selectivity within the src-family, and in particular with greater than 250-fold selectivity against fyn. We focused our efforts on two historical compounds, the pyrazolopyrimidine **1**, (lck = 0.141 μM) and the pyrrolopyrimidine **2** (lck = 0.196 μM), both of which exhibited >15-fold selectivity for lck versus fyn and also src. It was noteworthy that the *o*-F, *p*-$CF_3$ benzamide in **1** and the piperidine in **2** were widely represented in clusters of compounds that exhibited similar kinase selectivity profiles suggesting that these pharmacophores were important for selectivity. With this in mind, we assembled iterative arrays to probe the southern ($R^1$) and northern ($R^2$) domains that captured these common features as represented by the pyrazolopyrimidine in Figure 1.

Inhibitors were screened against a non-phosphorylated construct of human lck, lck (64–509) in HTRF format using 1 mM ATP and biotinylated lck peptide as substrates. Fyn served as our counterscreen. Potent compounds were progressed for profiling 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 and for an acute in vivo assay of the inhibition of TCR stimulated (anti-CD3 mAb) IL-2 production in mice after oral dosing.

Table 1 shows initial $R^1$ modifications in the context of the *o*-F, *p*-$CF_3$ phenyl northern domain.

Incorporation of distal non-basic $R^1$ groups generated compounds with greater than 450-fold selectivity versus fyn. Particularly illustrative are the data for the *N*-methyl piperidine **7** and the *N*-acetyl analog **8**. The former is 45-fold selective for fyn whereas the latter, devoid of the basic nitrogen, is greater than 450-fold selective. This

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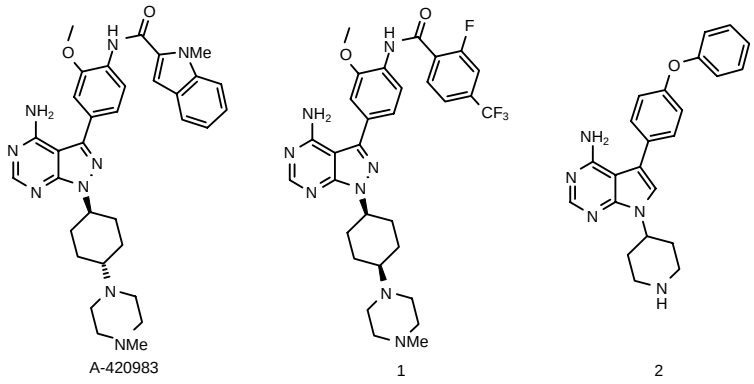


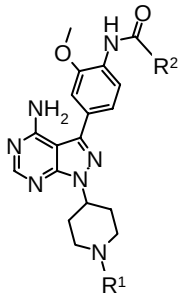
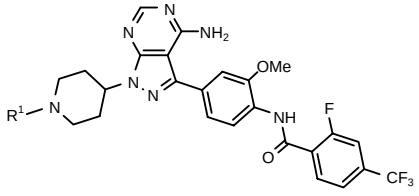
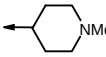
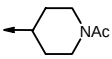



**Figure 1.** Northern and southern domain array assembly.

**Table 1.** Inhibition of lck, fyn whole blood IL-2 production ($IC_{50}$ μM) and in vivo potency ($ED_{50}$ mg/kg) by compounds **3**–**8**



| $R^1$ | Compound | lck | fyn | Whole blood | $ED_{50}$[a] (mg/kg) |
|---|---|---|---|---|---|
| H | **3** | 0.080 | >50 | 0.05 | 27.0 |
| Me | **4** | 0.106 | >50 | 0.03 | 17.8 |
| Bn | **5** | 0.341 | >50 | 0.54 | ND |
|  | **6** | 0.102 | >50 | 0.04 | 7.0 |
|  | **7** | 0.023 | 1.07 | 0.02 | ND |
|  | **8** | 0.093 | >50 | 0.03 | 9.2 |

[a] Oral dosing, measured 2.5 h after dosing.

observation is also seen for src itself, with compounds **7** and **8** being 0.15 and 15.8 μM inhibitors, respectively. Selected compounds that displayed suitable kinase selectivity and whole blood activity were further progressed into the acute in vivo assay. Initial potency data for the selective compounds after oral dosing in mice were generated 2.5 h after compound dosing. In this setting, compound **6** was the most potent at suppressing IL-2 production. Given these findings, evaluation of the northern domain ($R^2$) was subsequently conducted in the context of the most selective and potent tetrahydropyranyl southern domain ($R^1$) seen in **6**. These data are given in Table 2.

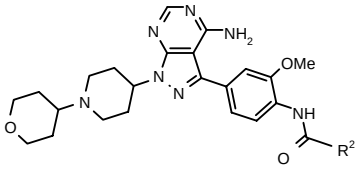
The tetrahydropyranyl indole amide **14** (hereafter A-641359) distinguished itself with an $ED_{50}$ of 1.8 mg/kg. A-641359 is a potent and selective lck inhibitor that inhibits IL-2 production in vivo. In mice, it has an oral bioavailability of 93% and an iv half-life of 3.1 h. In contrast to A-420983 which inhibits IL-2 production >90% at 18 h when dosed orally at $ED_{90}$, A-641359 exhibited a statistically significant inhibition for only 6 h when dosed at $ED_{90}$ (15.5 mg/kg).

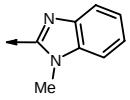
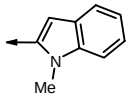
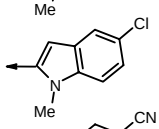
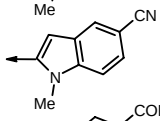
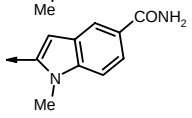
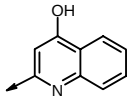
In an effort to enhance in vivo duration of action and take advantage of the selectivity profile of A-641359, we probed a further set of non-basic alternatives for the tetrahydropyranyl group, focusing on oxygen replacements.

The *N*-acetyl derivative **19** shown in Figure 2 emerged as the most potent and selective analog (lck = 0.074 μM, src = 17.15 μM, fyn = $\geqslant$50 μM) with comparable in vivo potency to A-641359 ($ED_{50}$ = 3.6 mg/kg, $ED_{90}$ = 19 mg/kg). Compound **19** exhibited an in vivo duration of action similar to A-641359 (data not shown), falling short of our goal. In contrast, analogous incorporation of the *N*-acetyl group into A-420983 gave compound **20** (A-770041) that is a src-family selective lck inhibitor with greater than 90% inhibition of IL-2 in vivo at 8 h when dosed at $ED_{90}$ in mice and rats. As can be seen in Table 3, it is noteworthy that A-770041 is greater than 300-fold selective against fyn, the other src-family kinase involved in T-cell signaling. A-770041 exhibits >60-fold selectivity versus the src-family members src and fgr, and >8-fold versus lyn and hck. Selectivity against the receptor tyrosine kinases tie-2 and kdr was >340-fold. This was also the case for downstream kinases such as ZAP-70, ITK, and PKC (data not shown).[7]

In the anti-CD3 stimulated human whole blood assay, A-770041 inhibited IL-2 production with an $IC_{50}$ of 0.08 μM. In rats, a correlation of concanavalin A-induced serum IL-2 levels to A-770041 concentrations was observed. Inhibition of IL-2 production was shown to be dependent upon in vivo plasma concentration of A-770041 (data not shown) with an in vivo $EC_{50}$ of

**Table 2.** Inhibition of lck, fyn, and whole blood IL-2 production ($IC_{50}$ μM) and in vivo potency ($ED_{50}$ mg/kg) by compounds **9**–**18**



| $R^2$ | Compound | lck | fyn | Whole blood | $ED_{50}$[a] (mg/kg) |
|---|---|---|---|---|---|
| 4-$NMe_2$-Ph | **9** | 0.106 | >50 | 0.035 | 17.9 |
| 4-Me-Ph | **10** | 8.96 | >50 | ND | ND |
| 4-$OCF_3$-Ph | **11** | 2.35 | >50 | ND | ND |
| 3-OH-Ph | **12** | 23.2 | >50 | ND | ND |
|  | **13** | 0.318 | >50 | 0.054 | 16%[b] |
|  | **14** | 0.107 | >50 | 0.020 | 1.8 |
|  | **15** | 0.054 | 13.6 | 0.013 | 54%[b] |
|  | **16** | 3.48 | 32.5 | ND | ND |
|  | **17** | >50 | >50 | ND | ND |
|  | **18** | 9.18 | >50 | ND | ND |

[a] Oral dosing, measured 2.5 h after dosing.
[b] Percent inhibition at 6 h after 12.5 mg/kg oral dose.

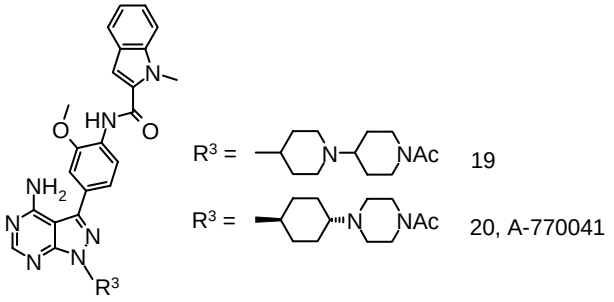



**Figure 2.**

**Table 3.** Kinase inhibition ($IC_{50}$ μM) by compound **20**

| lck | src | fyn | fgr | hck | lyn | tie-2 | kdr |
|---|---|---|---|---|---|---|---|
| 0.147 | 9.05 | 44.1 | 14.1 | 1.22 | 1.18 | >50 | >50 |

0.078 μM. The extended pharmacodynamic effect of A-770041 prompted us to explore the pharmacokinetic profile in rats and mice (Table 4).

A-770041 has a 7-fold lower clearance and a 2-fold higher bioavailability in the rat compared to the mouse

**Table 4.** Pharmacokinetic parameters for A-770041

| | $C_{max}$ (ng/ml) | $T_{max}$ (h) | $V_d$ (L/kg) | Clp (L/h/kg) | $T_{1/2}$ (h) | $F$ (%) |
|---|---|---|---|---|---|---|
| Mouse | 387 | 1.0 | 10.3 | 1.5 | 4.7 | 27 |
| Rat | 2079 | 4.7 | 1.2 | 0.2 | 4.1 | 52 |

enabling a wider exploration of chronic efficacy in rats rather than mice.

To establish further the potential of lck inhibitors in T-cell mediated diseases, A-770041 was advanced into a rat heterotopic transplant model of solid organ transplantation and data are shown in Figure 3. Hearts were transplanted from Brown Norway into Lewis rats by end to side anastamoses of the graft aorta to the recipient abdominal aorta and the graft pulmonary artery to the vena cava.[6] The abdomen was palpated daily to determine the graft survival. Recipient animals were treated for 14 days beginning on day 1 with vehicle control or 2.5-10 mg/kg/day of A-770041 dosed orally in equally divided doses 12 h apart. Hearts transplanted into animals receiving the vehicle control ceased beating between days 6 and 7. 2.5 mg/kg/day of A-770041 did

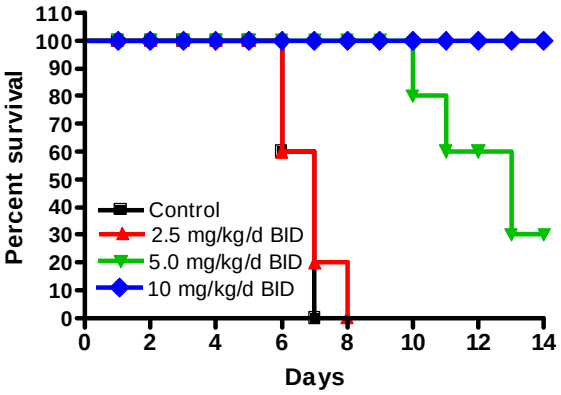



**Figure 3.** Effect of A-770041 on 14 day survival of heterotopically transplanted heart allografts.

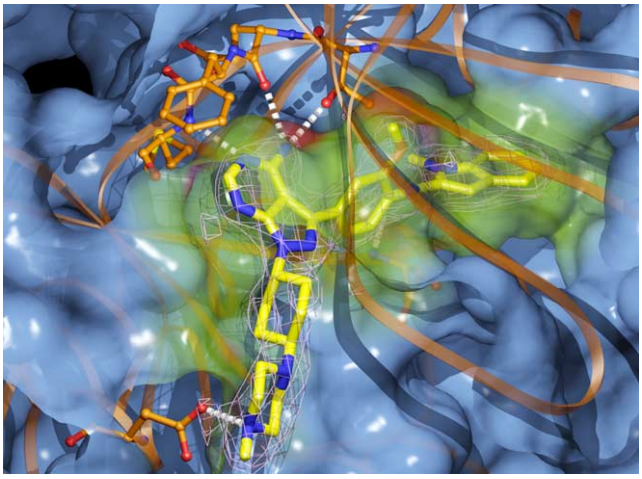


**Figure 4.** The crystal structure of A-420983 bound to human hck (residues 60–505, pTyr501).

not lengthen graft survival time. There was a dose-dependent increase in survival of transplanted hearts with doses of 5 and 10 mg/kg/day. At 10 mg/kg/day (bid) of A-770041, 100% of transplanted grafts were still beating at 14 days. This dose–response mirrored the compound plasma concentrations observed at the termination of the study as compound levels at 2.5, 5, and 10 mg/kg/day were 0.35, 1.06, and 2.9 μM, respectively. A subsequent study showed that all grafts in animals receiving 10 mg/kg/day (bid) A-770041 or 10 mg/kg/day cyclosporin A survived to 65 days after transplantation and will be the subject of a detailed future publication.[8]

The ability of A-770041 to provide sufficient immune suppression to prolong the survival of transplants for at least 65 days in this setting underscores the central role lck plays in T-cell activation. The fact that a compound selective for lck versus fyn achieves this supports the notion that fyn activation plays a minor, if any, role in acute rejection in concert with its documented partial contribution to T-cell activation.[5]

To understand the structural features of compounds that impact selectivity, we utilized protein:ligand:crystal-

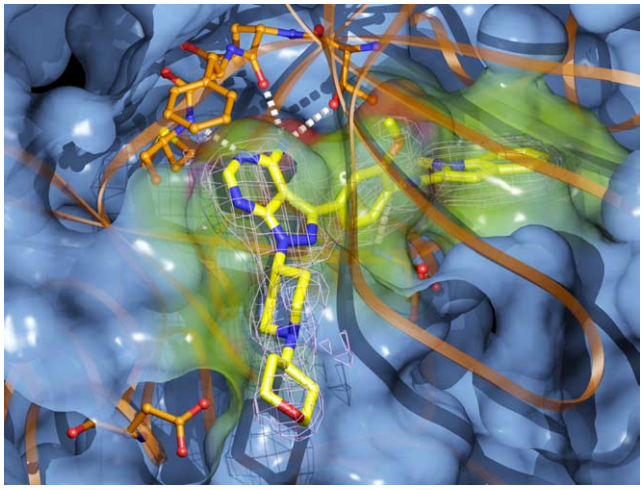


**Figure 5.** The crystal structure of A-641359 bound to human hck (residues 60–505, pTyr501).

lographic analysis. The crystal structures of A-420983 and A-641359 bound to human hck determined at 2.15- and 2.3-Å resolution are shown in Figures 4 and 5, respectively.[9] The cyclohexylpiperazine moiety of A-420983 makes a charge-reinforced hydrogen bond to the side chain of Asp326. The tetrahydropyran of A-641359 is unable to make this contact, resulting in the loss of fyn potency and greater than 220-fold lck selectivity. Although lck retains this conserved Asp, the differential rigidity of lck versus fyn in response to this hydrogen bond appears to drive selectivity. A discussion of this aspect will be the subject of a subsequent publication. 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 .

In summary, to our knowledge, A-770041 represents the most selective lck inhibitor described to date and represents an advance not only in the understanding of structural features important for src-family selectivity, but also in the potential arsenal for the treatment of immunological conditions, in particular transplant rejection.

## Acknowledgments

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## References and notes

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9. The X-ray coordinates have been deposited with the Protein Data Bank, www.rcsb.org, as entries 2c0i (HCK/A-420983), 2c0t (HCK/A-641359). Use of the IMCA-CAT beamline 17-ID & 17-BM at the Advanced Photon Source was supported by the companies of the Industrial Macromolecular Crystallography Association; use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. W-31-109-Eng-38.