Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 14 (2004) 2613–2616
Bioorganic & Medicinal Chemistry Letters

A-420983: a potent, orally active inhibitor of lck with efficacy in a model of transplant rejection

David W. Borhani, David J. Calderwood,* Michael M. Friedman, Gavin C. Hirst,* Biqin Li, Adelaine K. W. Leung,† Brad McRae, Sheldon Ratnofsky, Kurt Ritter‡ and Wendy Waegell

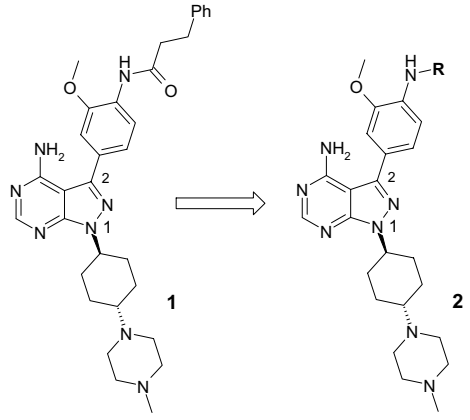
Abbott Bioresearch Center, 100 Research Drive, Worcester, MA 01605-5314, USA

Received 6 February 2004; accepted 17 February 2004

Abstract—We have identified the pyrazolo[3,4-*d*]pyrimidine A-420983 (compound **7**) as a potent inhibitor of lck. A-420983 exhibits oral efficacy in animal models of delayed-type hypersensitivity and organ transplant rejection.

Lck, a src family tyrosine kinase expressed primarily in T lymphocytes, provides a critical function during the initial steps of T-cell receptor (TCR) signaling.[1] A cascade of downstream signaling pathways ultimately leads to T-cell activation and the production of cytokines such as interleukin-2 (IL-2) and IFNγ.[2,3] A selective inhibitor of lck should prevent T-cell activation and thus has broad application for the treatment of T-cell dependent processes such as autoimmune and inflammatory diseases as well as allogeneic organ transplant rejection. Of importance to transplant rejection, $lck^{-/-}$ mice are incapable of rejecting either major or minor MHC-incompatible skin grafts despite the presence of peripheral T-cells.[4]

Early work from our laboratories described the synthesis and SAR of a series of pyrazolo[3,4-*d*]pyrimidines as lck inhibitors, exemplified by compound **1**.[5] This molecule accesses the lipophilic pocket in lck, a feature desirable for binding and therefore potency. This communication describes the exploration of this region with the goal of retaining potency versus lck whilst improving the in vivo properties and pharmacokinetic profile of the class. Compounds represented by general structure **2** (R = COHet, CH_2Ar) were synthesized using acylation or reductive amination protocols starting from amine **2** (R = H). Other chemistries accessible from **2** (R = H) have been reported elsewhere.[6]

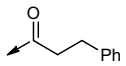
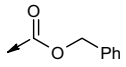
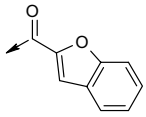
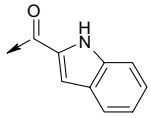
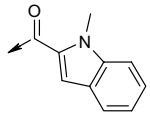
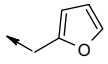


Inhibitors were screened against a nonphosphorylated construct of human lck, lck (64-509) in HTRF format using 1 mM ATP and biotinylated lck peptide as substrates.[7] The closely related kinase src and two receptor tyrosine kinases, kdr and tie-2, served as counterscreens. Potent compounds were progressed for profiling in cellular settings and ultimately in vivo. Data are presented here for the inhibition of anti-CD3 mAb induced IL-2

* Corresponding authors. Tel.: +1-508-688-8003; fax: +1-508-688-8100 (D.J.C.); tel.: +1-508-688-8016; fax: +1-508-688-8100 (G.C.H.); e-mail addresses: david.calderwood@abbott.com; gavin.hirst@abbott.com
† Present address: MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK.
‡ Present address: Aventis Pharma Deutschland GmbH, Frankfurt am Main, Germany.

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.02.101

Table 1. Inhibition of lck, src, kdr, tie-2 enzymatic activity, and whole blood IL-2 production (IC_{50} μM) by compounds **1** and **3**–**8**

R		Lck	Src	Kdr	Tie-2	Whole blood
	**1**	0.028	0.155	>50	2.450	0.038
	**3**	0.005	0.013	34.0	0.521	0.002
	**4**	0.093	0.189	>50	4.680	0.050
	**5**	0.024	0.057	>50	1.130	0.007
	**6**	0.020	0.056	ND	0.520	0.006
	**7**	0.037	0.070	>50	1.484	0.008
	**8**	0.064	0.097	ND	1.020	0.010

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. Resulting IL-2 levels were measured by an enzyme linked immunosorbent assay (ELISA) method.

Table 1 highlights selected compounds derived from this in vivo optimization strategy. All compounds were potent inhibitors of lck with >500-fold selectivity over kdr and >25-fold selectivity over tie-2. In accord with the close catalytic site homology within the src family, no selectivity was observed versus the closely related family member src. The potency observed against the enzyme translated into comparable inhibition of IL-2 production in the whole blood setting. This alignment presumably reflects good cell permeability as well as inconsequential plasma protein binding. Compounds **3**, **5**, **6**, and **7**, which displayed suitable receptor tyrosine kinase selectivity and whole blood activity,[8] were further progressed into the acute in vivo assay.

Table 2 shows in vivo data for these compounds (dosed as maleate salts) after oral dosing in mice. Initial potency data were generated 2.5 h after compound dosing. In this setting, all compounds were effective at suppressing IL-2 production, exhibiting ED_{50} values <10 mg/kg. Although both indole amides **6** and **7** displayed >75% inhibition of IL-2 production at 8 h, the *N*-methylindole amide **7** (hereafter A-420983) distinguished itself, as it showed >90% inhibition at 18 h when dosed at ED_{90}.

The extended pharmacodynamic effect of A-420983 compared to the other amides prompted us to explore the pharmacokinetic profile of A-420983 in detail. These

Table 2. Mouse in vivo data (IL-2 production) for **3**, **5**, **6**, and **7**

	Inhibition (%)[a]		ED_{50}[b] (mg/kg)	ED_{90}[b] (mg/kg)
	8 h	18 h		
3	51	14	3	14
5	48	<10	8.5	12.5
6	79	33	7	12.5
7 (A-420983)	>90	>90	1.5	6

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

parameters for A-420983 in both mouse and dog are shown in Table 3.

A-420983 has an oral bioavailability of ~30% in mice and dogs with a long elimination half-life. Plasma clearance is ~1/2 of liver blood flow in the mouse. Accordingly, in human liver microsomes, A-420983 exhibited high metabolic stability (>85% parent remaining at 1 h). In addition, the cytochrome P450 enzymes (Cyp) 3A4, 1A2, 2C9, 2C19, or 2E1 were not inhibited by A-420983 at concentrations below 100 μM. In cultured human hepatocytes, no induction of Cyp3A4 was seen using 10 μM A-420983. This is

Table 3. Pharmacokinetic parameters for A-420983

	C_{max} (ng/mL)	T_{max} (h)	Vd (L/kg)	Clp (L/h/kg)	$T_{1/2}$ (h)	*F* (%)
Mouse[a]	51	8	25	1.7	10	30
Dog[b]	74	24	25	0.7	45	32

[a] Mouse 5 mg/kg iv, 6 mg/kg po.
[b] Dog 1 mg/kg iv, 5 mg/kg po.

particularly relevant to the synergistic efficacy of A-420983 and cyclosporine (CsA) in the cardiac allograft model described below. Peak plasma levels were 10-fold in excess of the whole blood IC_{50}.

To assess further the potential of lck inhibitors in T-cell mediated disease models, A-420983 was advanced into a $CD4^+$ T-cell dependent delayed type hypersensitivity (DTH) model.[9] DTH reactions are characterized by recruitment of antigen-specific $CD4^+$ T-cells to the skin followed by release of cytokines and vasoactive peptides resulting in edema. T-cell inhibitors such as CsA can suppress DTH when dosed daily from the time of priming through challenge. CsA is also effective when dosed at the challenge (7 days post-priming).

C57Bl/6J mice were immunized (id) with methylated bovine serum albumin (mBSA) emulsified with complete Freund's adjuvant. At day 7, all mice were challenged with a sc injection of mBSA in phosphate buffered saline (PBS) in the right footpad; the left footpad was injected with PBS alone. A-420983 was dosed orally at the time of challenge. Twenty-four hours post-challenge footpad swelling was measured using calipers.

Figure 1 shows that A-420983 is an orally active inhibitor of DTH when dosed once daily at 18 mg/kg at the time of challenge; the inhibitor also displayed a suitable dose–response. Comparable data for CsA at 100 mg/kg are also shown.

The PK profile and efficacy in the DTH model suggested that further study of A-420983 in disease models was warranted. Clinical studies with immunosuppressants indicate that solid organ transplant (SOT) rejection is highly T-cell dependent. Studies in $lck^{-/-}$ mice also support the importance of lck in SOT as these mice are incapable of rejecting either MHC-incompatible or minor incompatible skin grafts despite the presence of peripheral T-cells.[4] We have used a murine cardiac transplantation model to assess the potential utility of lck inhibitors in SOT.[10,11] Transplantation of neonatal cardiac grafts from BALB/c (allografts) or C57BL/6 (isografts) mice into a subcutaneous pocket in the ear pinnae of adult C57BL/6 host results in acute rejection of the allograft within 7–10 days. As seen in Figure 2, A-420983 dosed at 6 or 12 mg/kg (q.d.) starting at day 1 protected cardiac allografts from rejection, showing 50% and 100% survival, respectively. Less than 10% survival was seen at 3 mg/kg (q.d.) for A-420983 and also for 20 or 40 mg/kg of CsA. Percent survival at day 14 was determined from the EKG trace.

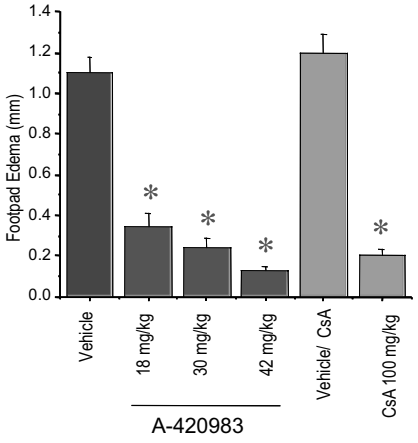


Figure 1. Delayed type hypersensitivity data for A-420983 and CsA.

This dose–response mirrored the compound plasma concentrations observed at the termination of the study as compound levels at 3, 6, and 12 mg/kg were 22, 48, and 118 nM, respectively. As expected, sub-therapeutic doses of CsA (20 or 40 mg/kg, q.d.) were not effective, leading to rejection of all allografts. Combination of sub-therapeutic doses of A-420983 (3 mg/kg) and CsA (20 or 40 mg/kg), however, led to synergistic inhibition of allograft rejection, resulting in greater than 80% survival. Importantly, dose–linear pharmacokinetics of A-420983 and CsA in the presence of the other was observed (data not shown), supporting our belief that a drug–drug interaction was not elevating plasma levels thereby resulting in immunosuppression.

To understand the structural features of compounds that impact binding to lck, we have utilized structural biology as a key part of our discovery effort. Specifically, we have been successful in generating a protein:ligand crystal structure of compound **6** with lck (unphosphorylated lck residues 237-501, D364N mutant). Figure 3 highlights key amino acid contacts of compound **6** with lck. The 4-amino group makes a key H-bond donor contact to the backbone C=O of Glu317 whilst the N5 pyrimidine nitrogen contacts the backbone NH of Met319.

The *trans* cyclohexylpiperazine moiety extends into the solvent exposed region where the terminal piperazine nitrogen makes a charge-reinforced H-bond to the sidechain of Asp326. The 3-aryl moiety extends into the hydrophobic pocket (unoccupied by ATP). The hydroxyl group in the side chain of the gatekeeper residue Thr316 provides a H-bond interaction with the methoxy group whilst the amide carbonyl of the ligand makes contact with the backbone NH of Asp382 in the highly conserved DFG-motif. The indole moiety pushes deep into the hydrophobic pocket and is surrounded by residues Leu303, Ile314, Met292, and Leu385.

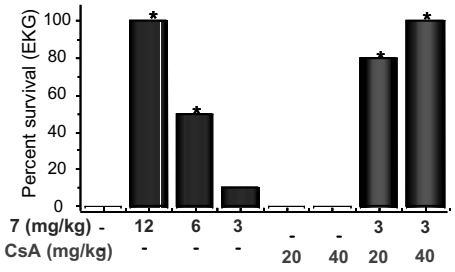


Figure 2. Cardiac allograft data for A-420983 and CsA alone and in combination.

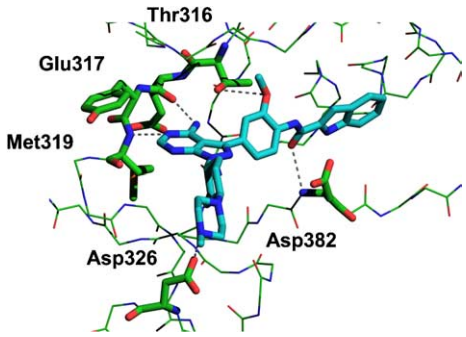


Figure 3. X-ray structure of **6** bound to lck.

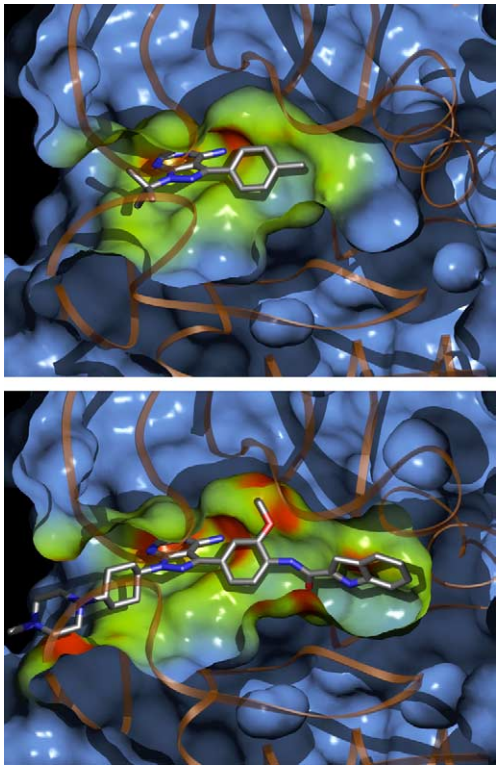


Figure 4. View of lck hydrophobic pocket—PP1 versus **6**.

Compound **6** (20 nM) is 310-fold more potent than PP1[12] (6200 nM vs lck at 1 mM ATP). The additional lck contacts made by **6**, compared to PP1, provide a rational explanation for this potency improvement. The combination of contacts provided by Asp326, Thr316, Asp382, and the interactions of the amino acids surrounding the indole moiety of **6** significantly increase the solvent-accessible surface area buried upon its binding to lck. Binding of **6** to lck leads to burial of 1.215 Å^2 of surface area (740 Å^2 lost from **6** itself, 475 Å^2 lost from lck). In contrast, binding of PP1 leads to burial of only 722 Å^2 (PP1: 486 Å^2 ; lck: 286 Å^2).

Figure 4 provides an alternative view of the lck active site that shows the aryl–amide–indole moiety of compound **6** occupying the hydrophobic pocket in the binding site, highlighting the differences between **6** and PP1. Crystal structures of a variety of ligands bound to lck and other src family members will be the subject of future publications.

In summary, we have identified A-420983, an orally active inhibitor of lck that is active in T-cell mediated animal disease models. Our findings establish that an orally available lck inhibitor produces sufficient immune suppression to prevent organ transplant rejection in preclinical models. Immunosuppression, as demonstrated here with A-420983 and in earlier publications represents an advance in the pharmacologic arsenal for the treatment of transplant rejection and possibly other immunological conditions.[13]

Acknowledgements

We are grateful to Brian Bettencourt, Dean Hickman, and Edit Tarcsa for generating the in vitro metabolism and in vivo PK data on compound **7** and to the Southern Research Institute (Birmingham, Alabama) for providing research facilities that allowed the protein crystallography work to occur.

References and notes

1. Straus, D. B.; Weiss, A. *Cell* **1992**, *70*, 585.
2. Weil, R.; Veillette, A. *Curr. Top. Microbiol. Immunol.* **1996**, *205*, 63.
3. Van Oers, N. S.; Kileen, N.; Weiss, A. *J. Exp. Med.* **1996**, *183*, 1053.
4. Yamada, H.; Kong, Y.-Y.; Kishihara, K.; Mak, T. W.; Nomoto, K. *Immunology* **1997**, *92*, 33.
5. Burchat, A. F.; Calderwood, D. J.; Friedman, M. M.; Hirst, G. C.; Li, B.; Rafferty, P.; Ritter, K.; Skinner, B. S. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1687.
6. Burchat, A. F.; Calderwood, D. J.; Deng, B.; Friedman, M.; Hirst, G. C.; Li, B.; Ritter, K.; Skinner, B. *Abstracts of Papers*, 224th ACS National Meeting, Boston, MA, United States, Aug 18–22, 2002.
7. Kolb, A. J.; Kaplita, P. V.; Hayes, D. J.; Park, Y.-W.; Pernell, C.; Major, J. S.; Mathis, G. *Drug Discovery Today* **1998**, *3*, 333.
8. The reason for the slightly increased potency in whole blood assay compared to enzyme assay is currently unclear but may reflect the fact that the local concentration of ATP in an activated T-cell is actually lower than 1 mM.
9. Magram, J.; Turek, C. W.; Killeen, N. *Immunity* **1996**, *4*, 471.
10. Judd, K. P.; Trebtin, J. J. *Transplantation* **1971**, *11*, 298.
11. Fey, T. A.; Krause, R. A.; Hsieh, G. C. *J. Pharmacol. Toxicol. Methods* **1998**, *39*, 9.
12. Hanke, J. H.; Gardner, J. P.; Dow, R. L.; Changelian, P. S.; Brissette, W. H.; Weringer, E. J.; Pollok, B. A.; Connelly, P. A. *J. Biol. Chem.* **1996**, *271*, 695.
13. Waegell, W.; Babineau, M.; Hart, M.; Dixon, K.; McRae, B.; Wallace, C.; Leach, M.; Ratnofsky, S.; Belanger, A.; Hirst, G. C.; Rossini, A.; Appel, M.; Mordes, J.; Greiner, D.; Banerjee, S. *Transplant. Proc.* **2002**, *34*, 1411.