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The development of novel 1,2-dihydro-pyrimido[4,5-*c*]pyridazine based inhibitors of lymphocyte specific kinase (Lck)

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Abstract—This communication details the synthesis, biological activity, and proposed binding mode of a novel class of tri-cyclic derivatives of 1,2-dihydro-pyrimido[4,5-*c*]pyridazines **1** and **2**. The most potent analogs disclosed showed low nanomolar activity for the inhibition of Lck kinase.

Lck is a 56-kDa Src family protein tyrosine kinase (PTK) that plays a critical role in the development and activation of T cells including T-cell antigen receptor (TCR) phosphorylation (an event necessary for signal transduction in the T-cell signaling cascade of the T-cell receptor).[1a,1b] Activation of this cascade ultimately results in the production of cytokines such as interleukin-2 (IL-2) and IFNγ.[1b,1c,1d] Unlike the widespread expression of some other Src family PTKs, Lck expression is restricted to T-cells and natural killer (NK) cells.[1d] As such the inhibition of Lck has been proposed as a potential treatment for a number of autoimmune diseases where T-cells are thought to play an important role in diseases such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD), psoriasis, systemic lupus erythematosus (SLE), and organ graft rejection.[1e]

Screening efforts in our laboratories identified compound **1** as a moderate Lck inhibitor (Fig. 1). The related analog **2** containing a single chloro-substituent on the C-3 phenyl ring was also screened and found to be devoid of biological activity. Initial efforts directed at optimization of this novel compound **1** were focused on the development of a synthetic methodology to the core structure. Molecular modeling was used to understand the binding mode of **1** in the active site of Lck.

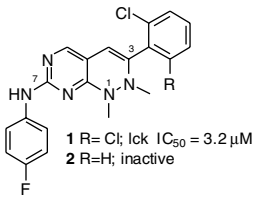

Figure 1. Initial 1,2-dihydro-pyrimido[4,5-*c*]pyridazine lead.

This communication details the synthesis, biological activity, and proposed binding mode of a novel class of tri-cyclic derivatives of 1,2-dihydro-pyrimido[4,5-*c*]pyridazine **1**.

A binding model for compound **1** in the active site of Lck was generated using literature coordinates for activated Lck co-crystallized with the inhibitor ANP (phosphoaminophosphonic acid-adenylate ester).[2] The pyrimido[4,5-*c*]pyridazine **1** forms two principal hydrogen bonds with Met319 (Fig. 2); namely, between N-1 of the pyrimidine ring and the protein backbone N–H, and from the aniline NH to the carbonyl group of this amino acid residue. The 2,6-dichlorophenyl group appears oriented toward the hydrophobic pocket not occupied by ATP. The *N*,*N*-dimethyl hydrazine portion of the molecule makes no contacts with the enzyme and appears to detract from molecular planarity. We decided to probe the SAR of **1** by constraining the two methyl groups by way of a pyrazolidinone ring. This structural change would potentially make the core structure more

Keywords: Lck; Lymphocyte specific kinase; T-cell; Pyridazines.
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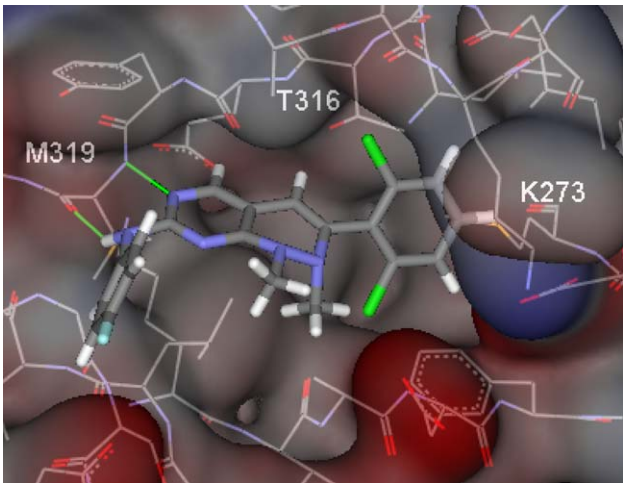
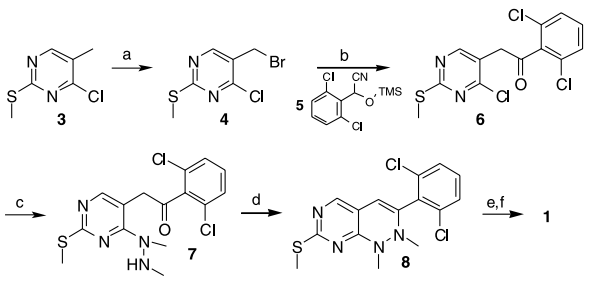


Figure 2. Key hydrogen bonds between **1** and Lck.

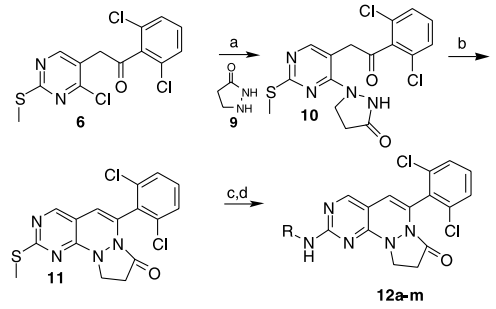
rigid and additionally position a hydrogen bond acceptor toward Lys273.

Syntheses of the heterocyclic core found in the lead molecules have been rarely described.[3a,3b] No literature reference was found detailing derivatives which contain a 3-aryl and 7-anilino group attached to the central ring system. Our methodology to access this novel heterocycle began with 4-chloro-5-methyl-2-methylsulfanyl-pyrimidine (**3**, Scheme 1).[4] Treatment of this material with NBS in the presence of benzoyl peroxide afforded the α-bromo analog **4**.[5] This compound was subsequently treated with the anion of cyanohydrin **5** (generated from the corresponding aldehyde with TMSCN and ZnI_2).[6] The resulting intermediate **6** was further reacted with *N*,*N*-dimethylhydrazine in the presence of DIPEA, to give adduct **7**. This material was cyclized under either basic (*n*-BuLi in THF) or acidic reaction conditions (catalytic HCL in THF) to give **8**. Oxidization of the thiomethyl group in compound **8** with *m*-CPBA gave a mixture of the corresponding sulfoxide/sulfone.[7] Displacement of this leaving group with 4-fluoroaniline afforded the desired product in low to moderate yield.

Common intermediate **6** was also used to generate tricyclic analogs **12a**–**m** (Scheme 2 and Table 1). Nucleophilic



Scheme 1. Procedure for preparation of compound **1**. Reagents and conditions: (a) NBS, benzoyl peroxide, DCE, 80 °C, 66%; (b) **5**, LDA, THF, 0 °C, 39%; (c) *N*,*N*-dimethylhydrazine·2HCl, DIPEA, THF, reflux, 82%; (d) *n*-BuLi, THF, 0 °C, 50% or HCl, THF, quantitative; (e) *m*-CPBA, DCM, 96%; (f) 4-fluoroaniline, NMP, MW 150 °C, 32%.



Scheme 2. Procedure for preparation of compound **12a**–**m**. Reagents and conditions: (a) pyrazolidinone **9**,[8] DIPEA, THF, 68 °C, 87%; (b) *p*-TSOH, toluene, Dean–Stark, reflux, 95%; (c) Oxone® THF, H_2O, 54%; (d) various anilines or CH_3NH_2, NMP, MW 130–180 °C, 15–45 min.

addition of pyrazolidinone **9**[8] gave intermediate **10** which was cyclized with TsOH[9] resulting in tricyclic compound **11**. Oxidation of this material followed by displacement of the resultant sulfone/sulfoxide mixture generated the final products **12a**–**r**.

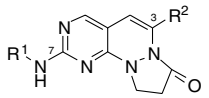
Table 1 summarizes the screening results for a variety of C-7 and C-3 substituted tricyclic analogs **12a**–**q**. Results are given for the inhibition of Lck kinase.[10] The initially synthesized compound **12a** was a dramatically more potent inhibitor (Lck IC_{50} = 124 nM) than the lead molecule **1**. Removal of the 4-fluoro atom from the C-7 anilino-group resulted in a slight decrease in potency (**12b**; Lck IC_{50} = 182 nM). Substitution of methylamine for the aniline group led to a large decrease in potency (**12c**; Lck IC_{50} = 2.8 μM).

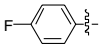
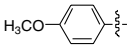
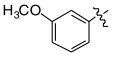
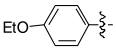
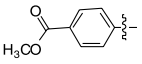
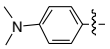
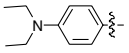
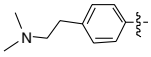
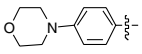
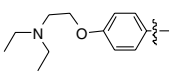
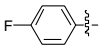
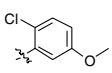
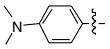
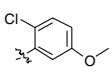
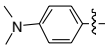
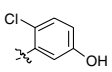
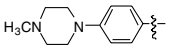
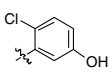
Introduction of *p*-, *m*-, *o*-methoxy aniline moieties at the C-7 position (**12d**, **e**, and **f**) indicated 4-phenyl substitution was optimal. Incorporation of various basic amines at this position of the C-7 anilino-ring resulted in the most potent compounds in the series containing the 2,6-dichlorophenyl group at C-3 (**12i**–**m**; Lck IC_{50} < 50 nM). A 2-chloro-5-methoxy (**12n**–**o**) or 2-chloro-5-hydroxy (**12p**–**q**) group at C-3 was explored as alternative pharmacophores at this position.[11] Only the phenols **12p** and **12q** possessed good potency (Lck IC_{50} = 10 and 47 nM) however with lower aqueous solubility compared to **12m**.

Representative compounds that showed promising Lck inhibition (**12l**–**m**, **p** and **q**) were tested for inhibition of IL-2 production in a Jurkat cellular assay.[12] Compounds **12p** showed the best albeit moderate IL-2 inhibitory activity (0.546 μM). These analogs were also examined for their ability to inhibit the Src family kinases (SFKs), hck (hematopoietic cell kinase) and src kinase (Rous sarcoma oncogene) (Table 2).[13]

The most promising analog **12m** (Lck IC_{50} = 2 nM) was docked in the molecular model proposed earlier for the lead molecule. Inspection of the Lck binding site revealed the principal hydrogen bonds to Met319 were maintained. Additional interactions were suggested

Table 1. IC_{50} values for derivatives **12a–q**



Compound	R^1	R^2	Yield (%)	Lck IC_{50}[a] (nM)	Sol. (μg/ml)
12a			33	124	2
12b			18	182	2
12c	CH_3–		17	2814	—
12d			38	71	5
12e			22	179	—
12f			33	7269	—
12g			15	285	—
12h			18	143	—
12i			59	26	5
12j			66	43	1
12k			10	21	10
12l			21	3	6.0
12m			23	2	33.8
12n[c]			37	4802	7
12o[c]			22	2837	7
12p[c]			2[b]	10	1
12q[c]			12[b]	47	11

[a] IC_{50}s were determined with a commercial Proflour assay (Promega Corp., Cat. #1271).
[b] % yield over two steps, see Ref. 11.
[c] Compounds prepared with a modification of the procedure described in Scheme 2, see Ref. 11.

Table 2. IL-2, Hck kinase, and Src kinase inhibition data for select compounds.

Compound	IL-2 IC_{50}[a] (μM)	Hck IC_{50}[b,13a] (nM)	Src IC_{50}[b,13b] (nM)
12l	2.00	160	104
12m	3.21	48	3
12p	0.546	546	273
12q	2.10	456	764

[a] IL-2 synthesis inhibition measured from Jurkat cells.
[b] IC_{50}s were determined with a commercial Proflour assay (Promega Corp., Cat. #1271). See also Ref. 13.

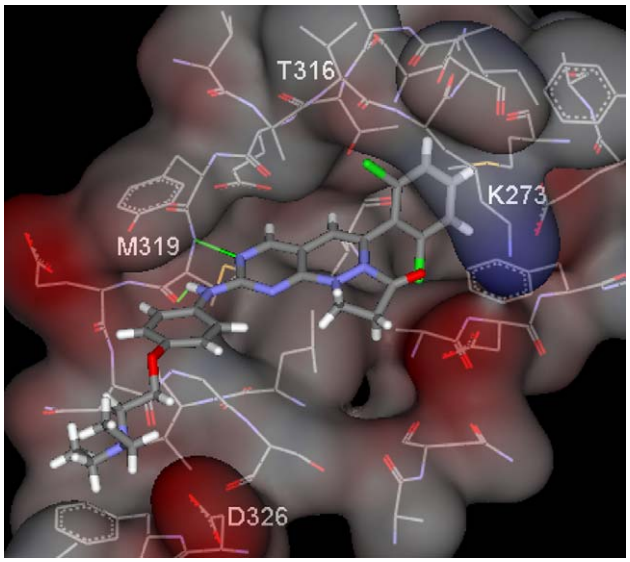


Figure 3. Key hydrogen bonds between **12m** and Lck.

including a hydrogen bond with Lys273, as well as a possible salt bridge with Asp326 (Fig. 3).

Analog **12m** (Lck IC_{50} = 2 nM) also displayed better aqueous solubility (33.8 μg/ml) and was subsequently screened for in vitro metabolism using rat hepatocytes.[14] This compound suffered low metabolic loss with >84% of the parent molecule remaining after 1 h. This molecule was subsequently evaluated in a rat PK study to determine bioavailability and half-life. Pyridazine **12m** displayed modest bioavailability (F = 14%) and a short half-life ($T_{1/2}$ = 1.5 h).

In summary, we have described the design, synthesis, biological activity, and proposed binding mode in Lck kinase of a novel class of tri-cyclic derivatives of 1,2-dihydro-pyrimido[4,5-*c*]pyridazines. SAR studies identified several low nanomolar inhibitors with the most advanced analog **12m** being progressed into pharmacokinetic evaluation.

Acknowledgments

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References and notes

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7. Oxidation with a single equivalent of *m*-CPBA at 0 °C favored formation of sulfoxide. However, small amounts of the sulfone were formed. The ratio favored sulfone when excess oxidant was present or the temperature was raised. Both intermediates underwent nucleophilic displacement.
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9. TsOH acid was found to be the optimal reagent for this cyclization. A more detailed account of the methodology to these ring systems will be disclosed in a separate future publication. See also Ref. 11.
10. The ability of compounds to inhibit human Lck enzyme (IC_{50}) was determined using the commercially available ProFlour Src-family Kinase Assay (Promega Corporation, Madison, WI; cat. #1271). The assay was performed according to manufacturer's instructions, with 2 nM recombinant human active Lck (Upstate Cell Signaling Solutions, Charlottesville, VA; cat. #14-442) at an ATP concentration of 10 mM.
11. A more detailed account of the methodology used to synthesize the various pyridazine analogs including compounds **12n-q** is in preparation and will be disclosed in the literature.
12. IL-2 release was measured by stimulating the Jurkat E6-1 T cell line (human acute T cell leukemia, ATCC, Manassas, VA) with monoclonal anti-human CD3ε antibody and Phorbol Myristate Acetate (PMA) (Sigma, St. Louis, MO). Flat-bottomed 96-well plates were pre-coated with 400 ng/well of anti-human CD3ε mouse antibody UCHT1 (R&D systems, Minneapolis, MN) and incubated for 2 h at 37 °C. Jurkat cells maintained in RPMI-1640 containing 10% fetal bovine serum and 1% antibiotics in the log growth phase (2×10^5–1×10^6 cells/ml) were harvested and incubated in triplicate in 96-well plates for 30 min at 37 ° C in the presence or absence of various concentrations of Lck inhibitors. The cell-inhibitor mixture was then transferred into the wells of the anti-CD3ε-coated 96-well plates, and PMA was added to the wells at a final concentration of 10 ng/ml (1 ng/well). The plates were

incubated overnight at 37 °C. The amount of IL-2 released into the culture media was measured by ELISA (R&D Systems) and the viability of the cells was determined using the MTS assay (Promega, Madison, WI).

13. (a) The ability of compounds to inhibit human Hck enzyme (IC_{50}) was determined using the commercially available ProFlour Src-family Kinase Assay (Promega Corporation, Madison, WI; cat. #1271). The assay was performed according to manufacturer's instructions, with 2.8 nM recombinant human active Hck (Invitrogen, Carlsbad, CA #P2908) at an ATP concentration of 10 mM.; (b) The ability of compounds to inhibit human Src enzyme (IC_{50}) was determined using the commercially available ProFlour Src-family Kinase Assay (Promega Corporation, Madison, WI; cat. #1271). The assay was performed according to manufacturer's instructions, with 14.3 nM recombinant human active Src (Invitrogen, Carlsbad, CA #P3044) at an ATP concentration of 10 mM.
14. (a) Measured as percent loss at 4 h in rat hepatocytes. (b) In vitro metabolism assay procedure: In vitro metabolic stability of analogs in plated rat hepatocytes (Sprague–Dawley) obtained from Cedra Corporation. Metabolic activity was determined in triplicate using a total volume of 0.2 mL containing 0.25 μM NCE incubated in rat hepatocyte and matrigel blank microtiter plates. The plates were maintained at 37 °C throughout the study. Samples were removed from wells at 0, 2, and 4 h, and NCE samples were analyzed by HPLC/MS/MS with reverse phase chromatography. To improve analytical efficiency, compounds were grouped together (post-incubation) into a multi-compound assay. Samples from like time-points containing the different compounds were combined and an internal standard (1.1 ng/mL Stock) was added. Results for each compound were expressed as the ratio of the compound response area over the internal standard response area. Percent loss was calculated by dividing the 2 and 4 h ratios by the 0 h ratio.