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Discovery of 2-Amino-heteroaryl-benzothiazole-6-anilides as Potent p56^{lck} Inhibitors

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Abstract—A series of structurally novel benzothiazole based small molecule inhibitors of p56^{lck} was prepared to elucidate their structure–activity relationships (SAR), selectivity and cell activity in the T-cell proliferation assay. BMS-350751 (**2**) and BMS-358233 (**3**) are identified as potent Lck inhibitors with excellent cellular activities against T-cell proliferation.

p56^{lck} (Lck), a member of the Src family of non-receptor protein tyrosine kinases is expressed primarily in T-lymphocytes and natural killer cells. In T cells, Lck is absolutely required for T-cell antigen receptor (TCR) signaling, culminating in IL-2 gene expression and effector functions.[1] T-cells lacking Lck are shown to be severely impaired in TCR tyrosine phosphorylation and are unable to undergo activation via the TCR.[2] Selective inhibitors of Lck may have potential therapeutic utility in the treatment of T cell mediated disorders such as autoimmune and inflammatory diseases and in the prevention of solid organ transplant rejection.[3]

In a previous communication[4] we described structure–activity relationship (SAR) studies leading to a novel benzothiazole template, that culminated in the discovery of BMS-243117 (**1**) as a potent, and selective Lck inhibitor. We outline here cognate studies with the goal of discovering suitable replacements for the urea moiety of **1**, leading to 2-aminopyridyl analogue **2** (BMS-350751) and 2-aminopyrimidinyl analogue **3** (BMS-358233) as highly potent Lck inhibitors in vitro with excellent activity in a T-cell proliferation assay (Fig. 1). The synthesis, and SAR studies leading to these analogues are described.

The synthesis of these compounds follows a general synthetic route that is illustrated in Scheme 1. Accordingly, 2-aminobenzothiazole-6-anilide[4] **4** was treated with 2 equiv each of copper(II) bromide and *tert*-butyl nitrite in acetonitrile at 0 °C to room temperature to form the corresponding 2-bromo-benzothiazole-6-anilides **5.** Compound **5** served as an advanced intermediate for the synthesis of the 2-amino-benzothiazole analogues. Reaction of **5** with a primary amine (R_1NH_2) at an elevated temperature (105–110 °C,

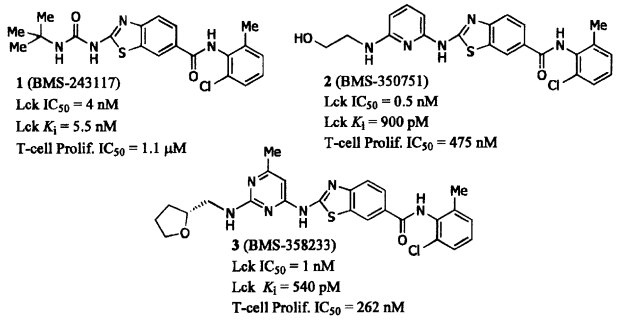
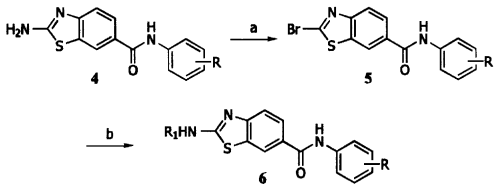


Figure 1. Lck inhibitory activity of benzothiazoles **1**, **2**, and **3**.



Scheme 1. (a) $CuBr_2$, *t*-$BuNO_2$, CH_3CN, 78–95%; (b) R_1NH_2, 105–110 °C or R_1NH_2, THF, NaH, 60 °C, 30–75%.

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neat) afforded the secondary amine **6**. Alternatively, **5** can be treated with the amine (R_1NH_2, 4 equiv) in presence of sodium hydride (8 equiv) at 60 °C for several hours to form **6**. The products were purified either by trituration or automated preparative HPLC on a reversed phase column. A parallel solution-phase approach was used to prepare a wide variety of analogues using these conditions.

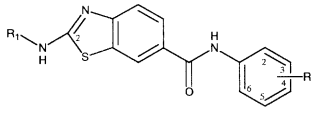
Compound **2** (BMS-350751) and its analogues were tested for their ability to inhibit the phosphorylation of an exogenous substrate[5] by human Lck enzyme (IC_{50}, Tables 1–3).

Table 1 outlines the SAR observed with the C2 amine modification. In our prior communication[4] we demonstrated the importance of the 2,6-substitution in the aniline ring. Therefore, the present SAR studies were carried out using the 2,4,6-trimethylaniline and 2-chloro-6-methylaniline series. In the 2,4,6-trimethylaniline series introduction of an aniline moiety (**6b**) resulted in a 9-fold increase in Lck inhibitory potency compared to the 2-aminoanalogue **6a**. In contrast, cyclopropylmethyl (**6c**) or 5-membered heterocyclic substituents (**6d**–**g** and **6m**) were not tolerated. A more significant increase in Lck inhibitory activity was observed with the 2-pyridyl analogue **6h** while the isomeric 3-pyridyl and 4-pyridyl derivatives (**6i** and **6j**) were less potent. More potent Lck inhibitors were however identified in the 2-chloro-6-methylaniline series (**6r**–**u**).

Because of excellent potency of the 2-pyridyl analogues (**6h** and **6r**) and the 4-pyrimidinyl derivatives (**6l** and **6s**), and ready availability of 2-aminopyridines and 4-aminopyrimidines, further optimization was carried out on both of these series. Table 2 outlines the effect of substitution in the pyridine ring. In the 2,4,6-trimethylaniline series, a methyl substitution at the C4, C5, or C6 position was well tolerated while such substitution at C3 was quite detrimental for potency. The 3,5-dimethyl analogue **6z** was an approximately 8-fold more potent inhibitor than the parent compound **6h**. A somewhat different SAR trend was observed with the 2-chloro-6-methylaniline analogues. Unlike in the 2,4,6-trimethylaniline series, the 6-Me analogue **6ad** was significantly more potent than the 4-Me analogue **6ab** and the 5-Me analogue **6ac**. Compounds **6ad**, **6ae**, and **6af** were identified as some of the more potent Lck inhibitors. A further increase in Lck inhibition was observed by introduction of a polar functionality at either C4 or C6-position of the pyridine ring. Compounds **2**, and **6ah**–**6ao** were identified as highly potent Lck inhibitors. However several of these analogues (**6ah**–**6ak**) were observed to be cytotoxic towards T-cells in an Alamar Blue based assay.[5] Compound **2** was identified as the most potent and non-cytotoxic Lck inhibitor ($IC_{50} = 500$ pM) in this series.

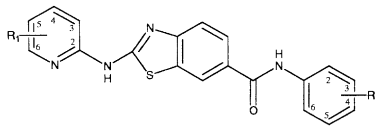
Table 3 summarizes similar studies in the 4-pyrimidine series. Substitution in the pyrimidine ring has led to some very potent Lck inhibitors in this series. A wide variety of substitution at C2, C6 or at both positions was well tolerated. A significant boost in activity was observed with a polar hydroxyethyl amine substituent either at C2 (**6at**), or C6 (**6ba**). Similarly, an ethyl substituent (**6av**), and an amino substituent (**6ay**) or a

Table 1. SAR for C_2-amine modification



Compd	R	R_1	Lck inhibition IC_{50}, μM[5]
6a	2,4,6-Me_3	H	15.07
6b	2,4,6-Me_3	Ph	1.74
6c	2,4,6-Me_3	Cyclopropyl-CH_2	>3.1
6d	2,4,6-Me_3	2-Thiazolyl	>3.1
6e	2,4,6-Me_3	2-Imidazolyl	>3.1
6f	2,4,6-Me_3	4-Imidazolyl	>3.1
6g	2,4,6-Me_3	5-(1,2,4-Triazolyl)	>3.1
6h	2,4,6-Me_3	2-Pyridyl	0.138
6i	2,4,6-Me_3	3-Pyridyl	>3.1
6j	2,4,6-Me_3	4-Pyridyl	>3.1
6k	2,4,6-Me_3	2-Pyrimidinyl	>3.1
6l	2,4,6-Me_3	4-Pyrimidinyl	0.403
6m	2,4,6-Me_3	3-(1,2,4-Triazinyl)	1.89
6n	2,4,6-Me_3	2-Pyrazinyl	0.235
6o	2-Cl,6-Me	Ph	0.327
6p	2-Cl,6-Me	2-Imidazolyl	>3.1
6q	2-Cl,6-Me	3-Pyrazolyl	1.59
6r	2-Cl,6-Me	2-Pyridyl	0.084
6s	2-Cl,6-Me	4-Pyrimidinyl	0.027
6t	2-Cl,6-Me	2-Pyridazinyl	0.041
6u	2-Cl,6-Me	3-Pyrazinyl	0.015

Table 2. SAR for C_2-amino-pyridine modification



Compd	R	R_1	Lck inhibition IC_{50}, μM[5]
6h	2,4,6-Me_3	H	0.138
6v	2,4,6-Me_3	3-Me	>3.1
6w	2,4,6-Me_3	4-Me	0.032
6x	2,4,6-Me_3	5-Me	0.072
6y	2,4,6-Me_3	6-Me	0.18
6z	2,4,6-Me_3	3,5-di-Me	0.017
6aa	2-Cl,6-Me	H	0.084
6ab	2-Cl,6-Me	4-Me	0.02
6ac	2-Cl,6-Me	5-Me	0.035
6ad	2-Cl,6-Me	6-Me	0.009
6ae	2-Cl,6-Me	4,6-di-Me	0.008
6af	2-Cl,6-Me	4-Et	0.005
6ag	2-Cl,6-Me	4-NH_2	0.403
6ah	2-Cl,6-Me	4-Me_2NCH_2	0.009
6ai	2-Cl,6-Me	4-NMe-PiperazinoCH_2	0.001
6aj	2-Cl,6-Me	4-MorpholinoCH_2	0.001
6ak	2-Cl,6-Me	6-NH_2	0.002
6al	2-Cl,6-Me	6-Me_2NCH_2	0.010
6am	2-Cl,6-Me	6-MorpholinoCH_2	0.013
2	2-Cl,6-Me	6-$HOCH_2CH_2NH$	0.0005
6an	2-Cl,6-Me	6-$AcNHCH_2CH_2NH$	0.002
6ao	2-Cl,6-Me	6-$H_2NCMe_2CH_2NH$	0.006

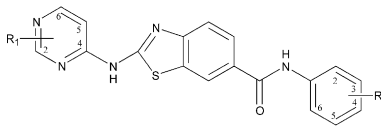
methylamino substituent (**6az**) resulted in Lck inhibitors with improved activity. Some of the most potent inhibitors were the 2,6-disubstituted pyrimidine analogues (**3**, **6bd**–**6bi**). Compounds **3**, **6az**, and **6bg** were selected for further characterization.

Pyridine **2** and the pyrimidine analogues **3**, **6az**, and **6bg** were tested for their activity against other Src family kinases and for cell activity in a T-cell proliferation assay (Table 4). These compounds were potent inhibitors of Src and Fyn kinases, in addition to Lck. Furthermore, these analogues were shown to be potent inhibitors of anti-CD3/anti-CD28 induced PBL proliferation.[5] Compound **3** was found to be the most potent inhibitor ($IC_{50} = 262$ nM) in this assay.

A binding model for these benzothiazole Lck inhibitors was developed based on the published coordinates[6] of the activated Lck kinase domain bound to ANP (phosphoaminophosphonic acid-adenylate ester, a non-hydrolyzable ATP mimic). As illustrated in Figure 2 both BMS-350751 (**2**) and BMS-358233 (**3**) are thought to bind in an extended conformation to the ATP-binding site of Lck. The 2,6-disubstituted aniline of **2** and **3** occupies a clearly defined hydrophobic pocket (common to all Src family kinases) which is not occupied by ATP. The 2,6-disubstitution skews the aniline ring out of plane with respect to the benzothiazole core and this in turn allows the phenyl ring to fit into the narrow and angular hydrophobic pocket. In addition, both of these analogues are likely to make several productive hydrogen bond interactions within the active site: the NH of the aniline is hydrogen bonded to Thr316 side chain hydroxyl, the benzothiazole nitrogen is in H-bond contact with Tyr318, and the two NHs' of the amino-pyridine or the amino-pyrimidine moiety are in contact with the backbone carbonyls of Met319, and Glu320, respectively. The lack of selectivity exhibited by this series of benzothiazoles compared to the urea analogue **1** is not clearly understood but it may be due to differences in the amino acid sidechains found at the edge of the ATP binding pocket in other kinases. For example, Lck residues Glu320 and Asn321 are replaced in Src by Ser 320 and Lys321, respectively, and in Fyn by Asn320 and Lys321, respectively. These differences represent significant alterations in the polarity of the binding site surface features at and near the region surrounding the pendant heterocyclic-substituted benzothiazoles.

In summary, we have described the design and optimization of a novel series of 2-heteroarylamino-benzothiazole Lck inhibitors. SAR studies identified several substituted pyridyl and pyrimidinyl analogues as highly potent inhibitors. BMS-350751 (**2**) and BMS-358233 (**3**) were identified as some of the most highly active Lck inhibitors in this series and displayed excellent potency in a T-cell proliferation assay.

Table 3. SAR for C_2-amino-pyrimidine modification



Compd	R	R_1	Lck inhibition IC_{50}, μM[5]
6l	2,4,6-Me_3	H	0.403
6ap	2,4,6-Me_3	2,6-di-Me	0.053
6aq	2,4,6-Me_3	6-Et	0.077
6s	2-Cl,6-Me	H	0.027
6ar	2-Cl,6-Me	2-Cl	0.031
6as	2-Cl,6-Me	2-MeNH	0.020
6at	2-Cl,6-Me	2-$HOCH_2CH_2NH$	0.003
6au	2-Cl,6-Me	6-Me	0.021
6av	2-Cl,6-Me	6-Et	0.005
6aw	2-Cl,6-Me	6-Cl	0.020
6ax	2-Cl,6-Me	6-OMe	0.013
6ay	2-Cl,6-Me	6-NH_2	0.008
6az	2-Cl,6-Me	6-MeNH	0.002
6ba	2-Cl,6-Me	6-$HOCH_2CH_2NH$	0.008
6bb	2-Cl,6-Me	6-$AcNHCH_2CH_2NH$	0.006
6bc	2-Cl,6-Me	2,6-di-Me	0.020
6bd	2-Cl,6-Me	2-Me,6-$MeNHCH_2$	0.007
6be	2-Cl,6-Me	2-Me,6-MorpholinoCH_2	0.006
6bf	2-Cl,6-Me	2-Me,6-$MeNHCH_2$	0.007
6bg	2-Cl,6-Me	2-$HOCH_2CH_2NH$,6-Me	0.002
6bh	2-Cl,6-Me	2-$HOCMe_2CH_2NH$,6-Me	0.006
3	2-Cl,6-Me	2-(2-tetrahydrofurylCH_2NH),6-Me	0.001
6bi	2-Cl,6-Me	2-MorpholinoCH_2,6-Me	0.010

Table 4. Enzyme selectivity of selected Lck inhibitors

Compd	Lck K_i, nM[5]	Src K_i, nM[5]	Fyn K_i, nM[5]	T-Cell Prolif. IC_{50}, nM[5]
1	5.5	333	100	1100
2	0.9	13	2.4	475
3	0.54	3.2	1.1	262
6az	6.6	17	8.8	690
6bg	1.1	10	4	329

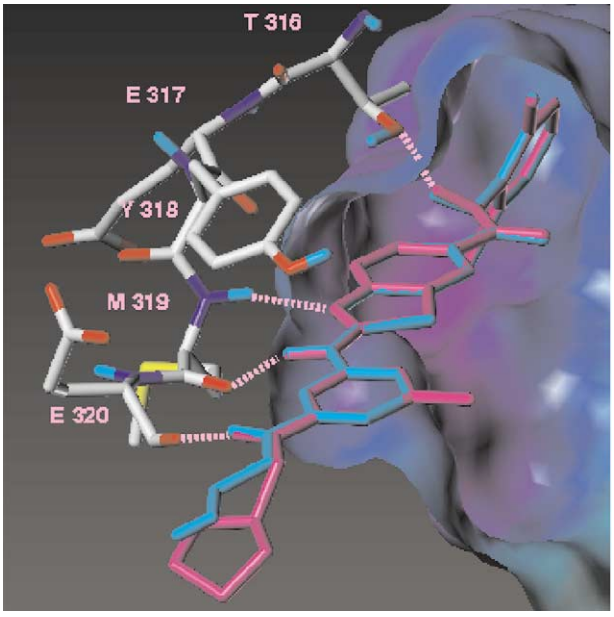


Figure 2. Proposed superimposed conformations of **2** (cyan) and **3** (magenta) bound to the ATP binding site of Lck.

References and Notes

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5. (a) *Lck Enzyme Assay*: For a description of the assay, see ref 4. Lck enzyme assays were run in triplicate and the values are mean of these experiments, standard deviation ±10%. (b) K_i *determination assay:* A coupled assay (see: Cole, P. A.; Grace, M. R.; Phillips, R. S.; Burn, P.; Walsh, C. T. *J. Biol. Chem*. **1995**, *270*, 22105) was used to monitor the tyrosine phosphorylation of the Lck specific peptide AEEEIYGVL-FAKKKK by GST-Lck. The following reaction conditions were used: 50 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 0.15 M NaCl, 200 μM substrate peptide, 1 mM PEP, 200 μM NADH, 1 unit of LDH, 0.6 unit of PK, 9 nM GST-Lck, and 1–2 mM ATP. The assay was carried out in a 96 well plate at 26 °C. For each reaction 78 μL of reaction mixture (all reaction components except ATP) and 2 μL of inhibitor solution in DMSO was added. The reaction was initiated by addition of 20 μL of 5 mM ATP solution. The Cytofluor (Molecular Devices) plate reader was used for fluorescence measurements using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Readings were taken immediately following ATP addition and every 10 to 30 seconds thereafter depending on the reaction rate. Fluorescence measeurements were recorded until the signal reached 90% of the initial signal. At least 50 data points were collected in order to calculate the slope, or rate of the reaction. The rate was then converted to μM product/min and plotted against inhibitor concentration. K_i was determined by nonlinear least squares regression with the Kaleidagraph® software (Synergy Software) according to established procedure (see: Bieth, J. G. *Meth. Enzymol*. **1995**, *248*, 59). Assays for Src, and Fyn were run under the same conditions as for Lck. (c) *PBL Proliferation Assay*: For a description of this assay, see ref 4. (d) *Alamar Blue Assay:* Normal human peripheral blood mononuclear cells (PBMC) were isolated from human heparinized whole blood, and were grown in the presence of 5 μg/mL PHA (phytohemaglutinin) for 2–4 days in RPMI 1640 media with 10% fetal bovine serum to generate PHA blasts. Inhibitor compounds (or DMSO as a control) were diluted to a final concentration of 30 μM in 96-well Costar tissue culture plate. PHA blasts were washed two times, resuspended in RPMI 1640 containing 10% fetal bovine serum, and then were added 3×10^5/well to the plate containing test compounds, in a total volume of 200 μL. The plate was incubated at 37 °C for 24 h. Alamar Blue solution (Biosource catalog # DAL 1100) was then added at 20 μL/well to each well and incubated another 4 h at 37 °C. The plate was read in a CytoFluor Multi-well Plate Reader-4000 (PerSeptive Biosystems) using an excitation wavelength of 530 nm and an emission wavelength of 590 nm.
6. Two crystal structures of the catalytic domain of Lck (Tyr394 phosphorylated) were published. See: (a) Yamaguchi, H.; Hendrickson, W. A. *Nature* **1996**, *384*, 484. (b) Zhu, X.; Kim, J. L.; Newcomb, J. R.; Rose, P. E.; Stover, D. R.; Toledo, L. M.; Zhao, H.; Morgenstern, K. A. *Structure* **1999**, *7*, 651 Protein Data Bank (1QPC pdb, Research Collaboratory for Structural Bioinformatics.