Small Molecule Inhibitors of Lck: The Search for Specificity within a Kinase Family

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Abstract: The Src family of non-transmembrane protein kinases is comprised of eleven homologous members in mammals. Together, these kinases regulate a wide variety of cellular processes including cell survival, proliferation, differentiation, and motility. One member of this family, Lck, plays a pivotal role in T-cell signaling. Inhibition of Lck with small molecules has significant potential for therapeutic immunosuppression and treatment of diseases such as rheumatoid arthritis and asthma. Critical for successful clinical use of any Lck inhibitor is high specificity for Lck as inhibition of other members of the Src kinase family may result in unwanted side effects. In this review we provide an overview of the various synthetic compounds currently under investigation as Lck-specific inhibitors. In addition we provide an analysis of the properties of these compounds that account for the specificity required for the inhibition of one of eleven highly similar kinases.

Key Words: Lck, Src, kinase, specificity, inhibitor, A-770041, BMS-243117, pyrrolopyrimidine.

INTRODUCTION

T cell activation is a critical step in cell mediated immunity. Following binding of an antigen to the T cell antigen receptor (TCR), a signaling cascade is activated that results in cytokine release, cell proliferation, and survival. As T cells play such a central role in the immune response, inhibition of T-cell function has the potential to play an important role in a wide variety of diseases. A small molecule inhibitor of T cell activation would have clinical value in a wide array of disease processes, including rheumatoid arthritis, asthma, and multiple sclerosis as well as providing an avenue for immunosuppression [1].

Activation of the non-receptor tyrosine kinase Lck is a required step in T cell activation [2]. Accordingly, development of small molecule inhibitors of Lck as therapeutic agents has been an area of intense research over the past decade. The goal of identifying a Lck inhibitor is significantly complicated by the membership of Lck in the Src family of tyrosine kinases (SFKs). This family of eleven highly homologous kinases plays a critical role in the control of a wide variety of cellular processes [3-5]. The variety of functions performed by the members of this family make it important that any Lck inhibitor be highly specific, with little to no activity towards other members of the SFKs. While this task was initially complicated by a lack of structural information about the SFKs, a decade of research has generated a significant amount of knowledge about the structural similarities and differences and the nature of the small molecule inhibitors that show specificity within this family.

Here, we present a review of the current state of the field in the quest for synthetic Lck specific inhibitors. We review the development of inhibitors currently under investigation as well as what these molecules have taught us about what is required for inhibition of one member of a structurally homologous kinase family. As this review will emphasize inhibitors that demonstrate selectivity within the SFKs, inhibitors published without testing SFK specificity will not be discussed.

THE SRC FAMILY KINASES

Lck is a member of the Src family of non-receptor tyrosine kinases. There are eleven mammalian enzymes in this group (Blk, Brk, Fgr, Frk, Fyn, Hck, Lck, Lyn, Src, Srm, and Yes) with highly conserved primary sequences [4, 5]. SFK members regulate a wide variety of cellular processes, including proliferation, cellular adhesion, growth factor and cytokine signaling, and survival [3, 4, 6]. Recent work further suggests a role for these kinases in the regulation of stem cell pluripotency and differentiation [7].

The SFK members share a characteristic arrangement of structural motifs [8, 9]. At the N-terminus is a short sequence for lipid attachment, involved in the localization of the kinases to the cell membrane. This attachment may be mediated by either myristoylation or, in the case of Lck and Fyn, by palmitylation [10]. Second is an SH3 domain, a domain approximately 70 amino acids in length which binds with some sequence specificity to target sequences rich in proline and other hydrophobic amino acids. Third is an SH2 domain of approximately 100 amino acids which binds specific peptide sequences containing a phosphorylated tyrosine. Fourth is the kinase domain followed by a C-terminal negative regulatory tail. Each of these components has been shown to be critical for the regulation of kinase activity [11, 12].

The tertiary structure of the SFKs is dependent on their activation state [12]. In the inactive form, the SH3 domain binds the short linker sequence connecting the SH2 and kinase domains. In addition, phosphorylation of a highly

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conserved tyrosine residue in the tail region induces intramolecular interaction with the SH2 domain, folding the enzyme into an inactive conformation. In the active form, binding between the various domains is lost and the kinases assume an open conformation.

The SFKs can be activated in at least two ways. Regulation of the phosphorylation of the C-terminal tyrosine provides one mechanism for the regulation of the kinase. *In vivo*, this tail tyrosine residue is phosphorylated by a distinct regulatory kinase known as Csk (for C-terminal Src kinase) and the closely related kinase Chk [13, 14]. Mutation of this tyrosine (Tyr530 in human c-Src) results in a constitutively active form of the kinase and deletions of this region are associated with oncogenic properties [15, 16]. Displacement of the SH3 domain from the linker can also activate the kinase. Multiple examples of SH3-based Src family kinase activation by other proteins have been reported, including the focal adhesion protein p130 Cas [17], the progesterone receptor [18], and the Nef protein of HIV-1 [19]. These studies have led to the idea that recruitment of substrates to Src family kinases through their SH3 domains may induce transient kinase activation, substrate phosphorylation and release, followed by a return of the kinase to its inactive state [20].

The kinase domain structures of Hck, Src, Fyn, and Lck, have been solved, and are representative of the well described structure of other kinase domains [21-24]. In each case, the N-lobe contains a five stranded β-sheet with a single α helix while the C-lobe is comprised of six α-helices and two short β-sheets. A short hinge region joins the lobes, allowing articulation between the two. The catalytic site occurs in the region of the hinge sequence with the ATP binding site being composed of residues from both the N and C lobes. Conserved motifs critical for kinase activity are easily identified in these structures and include the P-loop, the conserved ATP binding lysine, the catalytic domain, and the activation loop (Fig. (**1A**)).

Similar to other kinases, the conformation of the SFK kinase domains differ depending on the activation state of the enzyme [9]. In the inactive form, the two kinase lobes are slightly closed with the activation loop adopting a helical formation that occupies the cleft and prevents the αC helix from accessing the ATP binding site. During activation, repositioning of the activation loop to the catalytic site results in the concurrent movement of the αC helix.

The kinase domains of SFKs have a hydrophobic cavity of approximately 100Å^3 with an opening towards the back of the ATP binding pocket. While this pocket is also found in other kinases, in the SFKs this pocket is accessible due to the small size of the residue at its opening, Thr316 [25]. In closely related kinases (e.g. EGFR) this so called gatekeeper residue is often bulkier and prevents access to the pocket. Interestingly, this pocket is only present in the inactive form of the kinases as the pocket collapses during activation due to the movement of the αC helix [26]. This observation has significant implications in the design of inhibitors that utilize this pocket to provide inhibitor selectivity.

The high degree of homology within the ATP binding pockets of the SFKs suggests that little chemical space exists in this region for the generation of an SFK isoform specific inhibitor. The majority of the residues comprising the ATP binding pocket are highly conserved. Further, the residues that comprise the hydrophobic pocket are completely conserved in all eleven of the SFK isoforms (Fig. (**1B**)). Therefore, while the hydrophobic pocket is useful for improving the activity of inhibitors towards the SFK in general, it likely presents little opportunity to engineer SFK specificity into a Lck inhibitor.

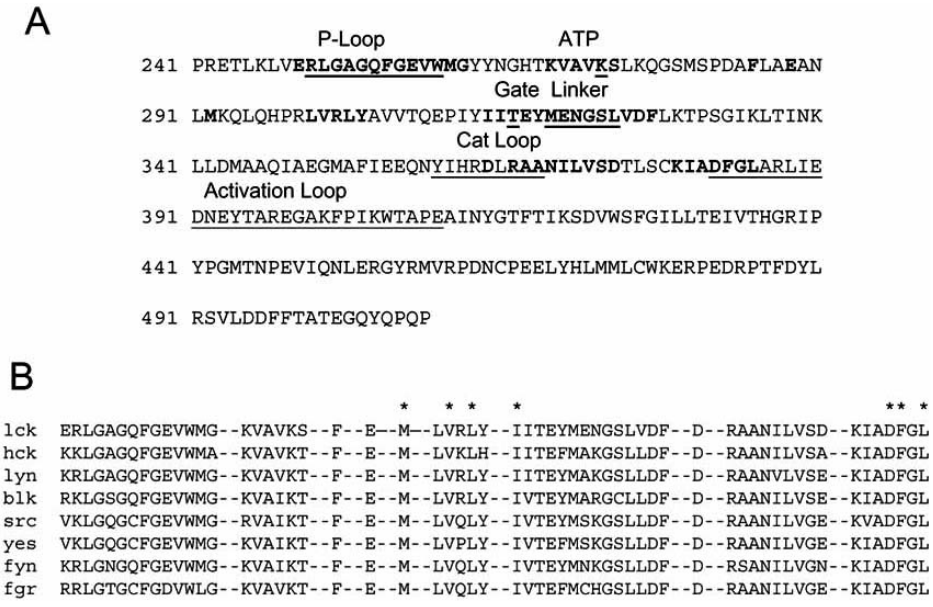
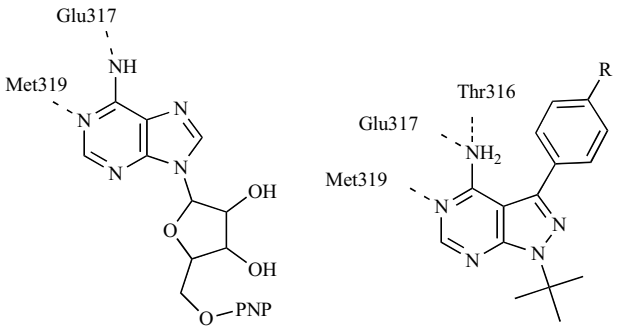


Fig. (1). A. Lck kinase domain primary sequence. Functional motifs are labeled and underlined. Residues comprising the ATP binding pocket (defined as being within 12Å of bound ATP) are in bold. B. Comparison of ATP binding pocket residues in eight well-studied SFK members. Residues marked with an asterisk comprise the hydrophobic pocket.

Structural studies with identical ligands emphasize the challenge of developing SFK isoform specific inhibitors. Crystal structures with the ATP mimetic AMP-PNP show identical hydrogen binding to conserved residues of Lck, Src, and Hck. Fitting into the ATP binding site, the adenine base makes two hydrogen bonds within the hinge sequence (Fig. (**2**)). The carbonyl oxygen of Glu317 and the backbone NH of Met319 bond with N7 and N1 atoms of the adenosine respectively[22]. Each of these residues is conserved in all SFK members.

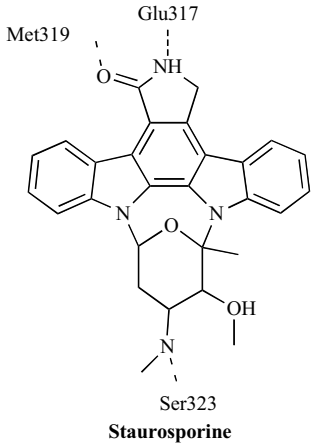


ANP **PP1** R = CH_3 **PP2** R = Cl

Fig. (2). Structure of the ATP mimetic ANP and of PP1 and PP2. Dashed lines represent hydrogen bonds to SFK residues established through analysis of crystal structures. Residue numbering in this figure, and throughout this review, is according to Lck.

Comparison of the binding properties of similar inhibitors in different SFK members further emphasizes the difficulties inherent in the design of a Lck inhibitor that has little activity against other SFK members. The closely related general SFK inhibitor pyrazolopyrimidines PP1 and PP2 have been co-crystalized with Hck and Lck respectively [22, 26]. In each case the inhibitor binds to the ATP binding pocket in manner which closely resembles the binding of purine ring of ATP (Fig. (**2**)). The amino groups of the inhibitors form a hydrogen bond with the side chain hydroxyl of Thr316 of both Hck and Lck as well as with the main chain carbonyl of Glu317. A third hydrogen bond forms between the main chain amide of Met319 and the nitrogen at position 3. The orientation of the molecules within the binding pocket places the phenyl moiety of PP1 and PP2 slightly in the hydrophobic pocket near the gatekeeper residue Thr316 and the compounds' tertbutyl moieties extend towards the solvent exposed ribose binding site. As previously noted, the bulkiness of the phenyl moiety prevents binding to proteins with larger residues blocking entrance to the hydrophobic pocket [26]. In the Zap70 tyrosine kinase, for example, the corresponding residue is a methionine. This is reflected in the poor activity (IC_{50} = 50 μM) of PP1 towards Zap70.

Further evidence of the structural homology that exists between the SFK members is provided by structural studies of Lck and Fyn bound to the general kinase inhibitor staurosporine [22, 24]. In each case, the inhibitor formed three hydrogen bonds with the kinase residues Glu317, Met319, and Ser323 (Fig. (**3**)). As described above, Glu317



Staurosporine

Fig. (3). Structure of Staurosporine. Dashed lines represent hydrogen bonds to SFK residues established through analysis of crystal structures.

and Met319 are conserved across all SFK members. Ser323 is conserved in all kinases except Blk. Further, all hydrophobic interactions in the Fyn–staurosporine complex were conserved in the Lck–staurosporine complex.

Despite the extensive homology that exists in the ATP binding pockets of the SFK, there are some differences that might be utilized in the design of a Lck specific inhibitor. One region that may prove useful is the hinge sequence. Within the SFKs, this region provides one of the least homologous sequences within the ATP binding site with a general sequence of MXXG (Fig. (**1B**)). The conserved hydrogen bonding of Staurosporine to Met319 and Ser323 demonstrates the availability of this region for ligand binding. The unique sequence of Lck in this region may provide enough variation to allow for selectivity.

LCK IN TCR SIGNAL TRANSDUCTION

In T cells Lck plays a critical and essential role in the signaling pathway that connects the T cell receptor to cellular activation [1, 27]. Prior to T cell activation, Lck associates non-covalently with CD4 and CD8 co-receptors *via* a dicysteine motif near the N-terminus [28]. Interaction of the T cell and the antigen-presenting cell leads to Lck activation and phosphorylation of ITAMs (immunoreceptor tyrosine-based activation motifs) with the CD3-TCR complex. These phosphorylation events generate docking sites for the SH2 domains of the tyrosine kinase ZAP-70, which itself must be phosphorylated by Lck in order to be activated. Together, Lck and ZAP-70 stimulate multiple signaling networks including calcium mobilization, Ras/Map kinase, PI3 kinase, and Rho/Rac.

Inhibition of T cell activation by a highly specific inhibitor of Lck activity has potential for significant clinical value in the prevention of transplant rejection. Current therapies generally include the use of calcineurin inhibitors, most notably cyclosporine and tacrolimus, to block the transcriptional associated with T Cell activation [29]. The ubiquitous expression of calcineurin, however, results in serious side effects of these drugs, including nephrotoxicity and hepato-

toxicity. Development of a highly specific inhibitor of T cell activation has the potential to provide therapeutic value without the deleterious side effects of previous therapies [1]. T cell inhibition also has potential therapeutic value in autoimmune diseases. Most notably, there is a direct link between psoriasis and T-cell activation and cyclosporine is currently in use as a treatment for this disease [30]. Links between T-cell activation and other autoimmune diseases such as multiple sclerosis and rheumatoid arthritis have also been suggested [31, 32].

It is important that new inhibitors of Lck also have limited activity towards the other Src kinases. While Lck expression is limited to lymphoid cells, other SFK members play critical roles in other tissues and some (Src, Fyn, and Yes) are ubiquitously expressed. Secondary inhibition of other SFK members has the potential to create serious unintended side effects. The high homology that exists between the members of this family complicates the design of inhibitors specific to a single isoform. Significant effort has been expended over the past decade, however, to identify an inhibitor of Lck which lacks activity towards other SFK members.

LCK INHIBITORS

A-770041

Lck specific inhibitors with modified pyrimidine cores represent the most intensely studied group of compounds that inhibit the SFKs. The earliest members of this family were the pyrazolopyrimidine inhibitors PP1 and PP2, reported by Pfizer in 1996 [33]. PP1 and PP2 block Lck activity *in vitro* with IC_{50} values in the low nanomolar range. While these inhibitors lack specificity within the Src kinase family, the importance of these inhibitors derives from their novel utilization of the hydrophobic pocket near the ATP binding site as a means for providing specificity towards the Src family as a whole.

The development of the Lck specific inhibitor A-770041, a direct descendant of PP1, was published in a series of papers over the past decade by researchers first at BASF and later at the Abbott Laboratories. The final structure of this molecule is a result of both strategic modification and extensive structure activity relationship (SAR) exploration. Throughout development, the goal was to increase the activity towards Lck and limit activity against other members of the Src family while generating compounds with useful pharmacokinetic properties. Analysis of the research that resulted in the development of this inhibitor provides useful insight into the chemical properties needed to engineer Lck specificity into a general SFK inhibitor. An abbreviated summary of the path taken in the development of A-770041 is depicted in Fig. (**4**).

The discovery process that ultimately resulted in A-770041 began with the identification of 4-phenoxyphenyl-quinazoline **1** (Fig. (**4**)) as a potent inhibitor of Lck [34]. This compound demonstrated sub-micromolar activity towards Lck and Src with low activity against the tyrosine kinases Kdr and Tie-2 (Table **1**). Structural analysis of **1** suggested that binding to the kinase active site might resemble that of PP1 in Hck, with two hydrogen bonds connecting the compound to the hinge region. In turn, this raised the interesting possibility that the specificity of the compound towards the SFKs may result in hydrophobic pocket access by the phenoxyphenyl moiety of **1**.

To test the hypothesis that utilization of the hydrophobic pocket could provide increased activity for the SFKs, PP1 was modified to replace the phenyl-methyl substituent with a phenyl-phenoxy group as well as conversion to a pyrrolopyrimidine core structure [34]. The resulting compound (**2,** Fig. (**4**)) was tested for activity against inactive and active forms of Lck. (The inactive form tested, Lck(64-509), contains the SH3, SH2, and kinase domains and is not phosphorylated on the activating tyrosine (Tyr394) while the active form, Lckcd, contains only the kinase domain and is phosphorylated at Tyr394). The compound was shown to be 75 times more potent than PP1 towards inactive Lck with seven-fold less activity towards active Lck. Further, the compound showed increased selectivity for both forms of Lck over Src. Modification of the hydroxyl phenyl moiety to ortho or meta forms led to loss of potency against Lck providing supporting evidence that this group occupies at least a portion of the hydrophobic pocket. Further support for hydrophobic pocket utilization was provided by comparison of the weaker activity of **2** against active Lck, where the hydrophobic pocket is collapsed, versus inactive Lck, where the hydrophobic pocket is available for inhibitor binding.

Further improvements in Lck activity were pursued by exploration of the SAR of the solvent exposed N7 atom of **2** using alkyl and cycloalkyl analogs. Cyclopentyl (**3,** Fig. (**4**)) and 3-hydroxy cyclopentyl analogues provided the greatest improvement in Lck activity. While both analogs showed strong activity in cells, the 3-hydroxy analog lost selectivity against non-SFK kinases. The activity of **3** was not improved by modification of the C6 atom [35]. Compound **3** demonstrates excellent activity *in vitro* and *in vivo* [34, 35]. It is 100-fold more potent than PP1 in Jurkat cell activation assays and inhibits T-cell receptor stimulated IL-2 production in mice at low doses (4 mg/kg) after IP administration. Oral administration, however, diminishes the efficacy of the compound 6-fold.

Modeling of **3** in the active site of Lck suggested that phenoxyphenyl moiety occupied the hydrophobic pocket in the ATP binding site [34]. In an attempt to further increase the interactions with the hydrophobic pocket, modifications to the terminal phenyl ring of **3** were explored [35]. From these experiments it was observed that installation of a functional group at the 2-position (e.g. NH_2, OH, CN) had a negative impact on Lck activity. At the 4-position variable effects were observed with 4-CN and 4-COOH showing a significant decrease in Lck activity. No modifications to the terminal phenyl ring were obtained that provided a compound with an improved profile over **3**.

Development of **3** proceeded with alternating efforts to improve the pharmacokinetic and inhibitory properties of the molecule. This occurred through SAR exploration primarily of N7 and the phenoxy moiety. The N7 moiety projects towards the solvent exposed sugar binding residues of the ATP pocket. Understanding that the carbocyclic framework at N7 was critical for Lck activity, this moiety was modified with

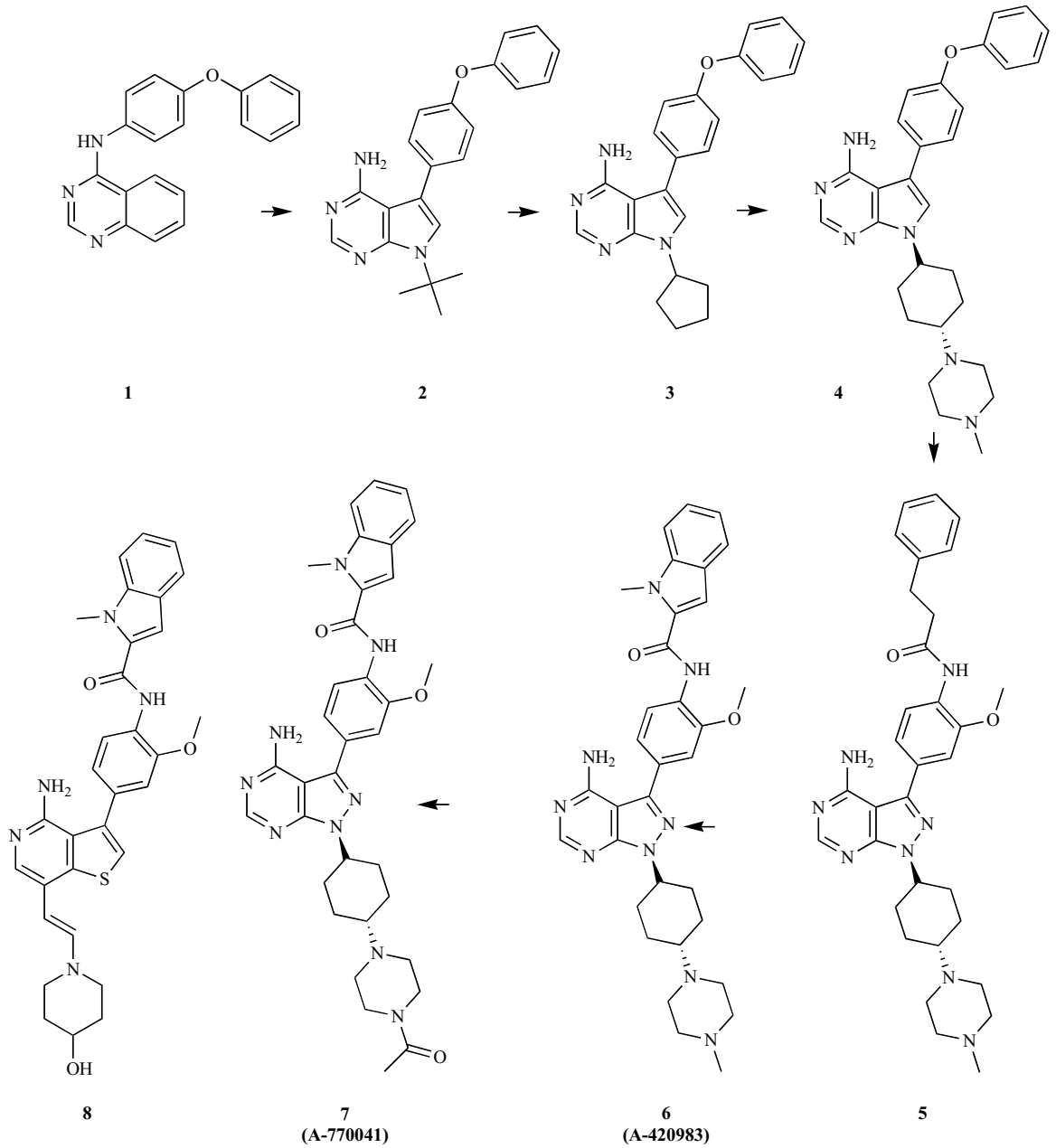


Fig. (4).

analogues designed to increase solubility of the compound. A variety of strategies were adopted to address solubility, including incorporation of alcohols, ethers, basic amines and low molecular weight heterocycles. Compound **4**, for example, demonstrated a significant increase in activity against Lck and Src. In addition to being a potent inhibitor of IL-2 production in stimulated Jurkat cells, orally administered compound **4** showed improved potency *vs*. receptor stimulated IL-2 production with an ED_{50} of 1.5 mg/kg as well as improved pharmacokinetic properties [36].

Modification of the phenoxy extension was pursued with the intent of improving the potential contacts of the inhibitor with the hydrophobic pocket. Alteration of the oxygen bridge with hinges of varying lengths and differing functionalities provided enhancement of both potency for Lck and selectivity against the receptor tyrosine kinases. A key point in this process was the introduction of a hydrogen bond to the gatekeeper residue *via* introduction of a methoxy component of the oxygen bridge in compound **5**. This additional hydrogen bond provided a substantial increase in activity towards Lck *in vitro* while decreasing activity towards Src. *In vivo*, **5** is a potent inhibitor of IL-2 production, possessing an ED_{50} of 5 mg/kg after oral dosing. Problematic with **4** and **5** is a significant drop off of activity in mice by 18 hours [37].

Further exploration of the hydrophobic pocket binding region of **5** resulted in the discovery of compound A-420983

(Compound **6**) [37]. Activity towards Lck remained relatively unchanged, relative to **5**, but A-420983 shows very strong pharmacokinetics and greater than 90% inhibition of IL-2 production at 18 h and an ED_{50} of 1.5 mg/kg. The ability of A-420983 to protect against organ rejection following transplant was tested using a murine cardiac transplantation model. 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 at day 14 while untreated mice showed 0% survival.

X-ray crystallography showed that the 4-amino group of A-420983 makes a key contact to the backbone C=O of Glu317 while the N5 pyrimidine nitrogen contacts the backbone NH of Met319 [37]. The *trans*-cyclohexylpiperazine nitrogen makes a charge-reinforced H bond to the side chain of Asp316. The 3-aryl moiety extends into the hydrophobic pocket. The hydroxyl group in the side chain of the gatekeeper residue Thr316 provides a H-bond interaction with the methoxy group while 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 L303, I314, M292, L385.

Efforts to further increase the selectivity of A-420983, especially with regard to Lck, resulted in the identification of A-770041 (Compound **7**) [38]. Modification of A-420983 focused, in part, on the solvent exposed piperazine moiety. Substitution of distal non-basic groups generated compounds with greater than 450-fold selectivity of Lck versus Fyn. For example, N-methyl piperidine is 45x more potent for Lck than Fyn, while an N-acetyl analog is 450x more selective. Incorporation an N-acetyl group into A-420983 resulted in A-770041.

The pharmacological properties of A-770041 have undergone preliminary investigation [39]. Concanavalin A-stimulated IL-2 production in whole blood is inhibited with an EC_{50} of approximately 80 nM. A-770041 is orally bioavailable and has a $t_{1/2}$ of 4.1 ± 0.1 h. Concanavalin A-induced IL-2 production is also potently inhibited by oral administration of A-770041 (EC_{50} = 78 ± 28 nM). Doses of A-770041 at or above 10 mg/kg/day prevent rejection of hearts transplanted heterotopically in rats from Brown Norway donors to Lewis recipients across a major histocompatibility barrier for least 65 days. Grafts from animals treated with 20 mg/kg/day A-770041 or 10 mg/day Cyclosporin A had minimal microvascular changes or multifocal mononuclear infiltrates.

Thienopyridines

The thienopyridine **8** (Fig. (**4**)) combines the hydrophobic pocket interacting moiety of A-420983 and A-770041 with a novel hinge interacting moiety to improve Lck specificity. The development of A-420983 and A-770041 thoroughly explored the chemical space available in the hydrophobic pocket and solvent exposed binding region [37, 38]. The *trans*-cyclohexylpiperazine moiety of A-420983 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 was seen with A-770041, making this contact with basic amines was detrimental for selectivity. To increase selectivity, an approach was developed to break this interaction and probe alternative extended hinge interactions [40]. Specifically, a goal was set to make productive contacts with the side chains of Tyr318 and the unique Glu320 in the extended hinge region of Lck to drive selectivity within the Src-family [41]. The thienopyridine **8** (Fig. (**4**)) and similar compounds resulted from this work.

To circumvent the difficulty of generating N7 variants of A-420983, the pyrazolopyrimidine core was replaced with a thienopyridine core. SAR exploration of this region confirmed that specificity could be generated through interactions with the hinge region. Initial investigations demonstrated that significant potency increases could be obtained by modification with branching structures at C-7. Critical was the requirement for an Sp2 carbon at the C-7 branch point. Further extending the distance from C-7 to the basic terminus maintained both Lck potency and selectivity against Hck. Analysis of compound **8** against a larger kinase set showed improved selectivity within the Src family with significant decreases in activity against Src and Fyn relative to A-770041 (Table **1**). In mice, compound **8** inhibited TCR stimulated IL-2 production with an ED_{50} of 5 mg/kg. Pharmacokinetic analysis showed poor performance especially with regard to clearance and oral bioavailability.

BMS-243117

The benzothiazole BMS-243117 (**9**, Fig. (**5**)) was identified following SAR exploration of a thiazole compound initially obtained *via* high throughput screening [42]. The inhibitor showed strong activity toward Lck (IC_{50} = 4 nM) with greatly reduced activity against other SFK isoforms (Src IC_{50} = 632 nM, Fyn = 128 nM, Hck = 3.84 μM, Blk = 336 nM, Lyn = 1.32 μM, and Fgr IC_{50} = 240 nM). While the compound showed good *in vitro* activity inhibiting T Cell proliferation with an IC_{50} of 1.1 μM, no *in vivo* data is currently available.

SAR exploration of two regions of BMS-243117 provided interesting information about the regions of Lck that can be exploited for Lck potency and selectivity. First, modification of the aniline substitution resulted in loss of Lck potency, suggesting that alignment of the aniline group with the rest of the molecule is important for the ability to enter the hydrophobic pocket. It is possible that even greater potency could be obtained by extension of this region into the hydrophobic pocket. Second, the presence of the tert-butyl group is less important for Lck potency than for SFK selectivity. For example, replacement of the tert-butyl with a 2,2,6,6-Me_4-4-piperidinyl group has little effect on Lck activity, but significantly increases the potency of the molecule towards Src (1.8x), Fyn (64x), Hck (3x), Blk (5.25x), and Fgr (10x). Interestingly, modification of this portion of the molecule tended to have the greatest effect on selectivity to Fyn. This suggests that Fyn, rather than Src, may be of more clinical significance during eventual trials of Lck specific inhibitors.

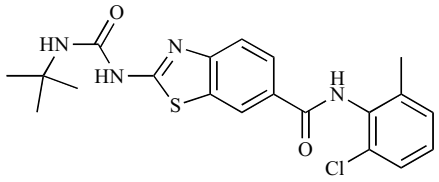
Modeling suggests that BMS-243117 binds in an extended conformation to the ATP binding site of Lck. In this conformation the compound makes four productive hydro-

Table 1.

Compound ATP	Lck (64-509)		Lckcd		Src 5 µM	Src 1 mM	Fyn 1mM	Fgr 1mM	Hck 1mM	Lyn 1mM	Tie2 1mM	Kdr 1mM
	5 µM	1 mM	5 µM	1 mM								
PP1	.151	-	.25	-	.17	-	-	-	-	-	-	-
1	<0.008	-	-	-	-	-	-	-	-	-	17.87	2.72
2	.002	.075	.014	-	.61	-	-	-	-	-	-	-
3	<0.001	.016	.002	1.07	.07	-	-	-	-	-	-	-
4	-	.015	-	-	-	.042	.059	-	-	-	.025	-
5	-	.028	-	-	-	.155	-	-	-	-	-	>50
A-420983	-	.037	-	-	-	.070	-	-	-	-	1.48	>50
A-770041	-	.147	-	-	-	9.05	44.1	14.1	1.22	1.18	1.18	>50
8	-	.21	-	-	-	33.9	35.1	37.9	10	5.3	10.1	>50

Legend Kinase IC_{50} values for compounds depicted in Fig. (**3**). ATP values are the concentration of ATP used in individual assays. See text for discussion and references.

gen bonds with the hinge region of the kinase. The NH of the aniline bonds with the gatekeeper Thr316 side-chain hydroxyl, the benzothiazole nitrogen is in H-bond contact with Tyr318, and the two nitrogens of the urea moiety are in contact with Met319 and Glu320 in the Hck region. Further specificity for the SFK family is likely provided by access of the hydrophobic pocket by the aniline.



9 Lck IC_{50} = 4 nM, Src IC_{50} = 632 nM

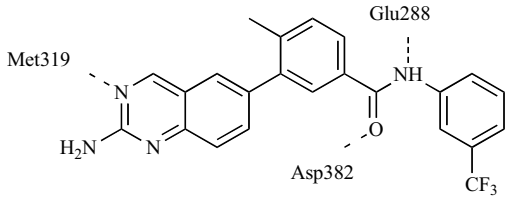
Fig. (5).

Aminoquinazolines

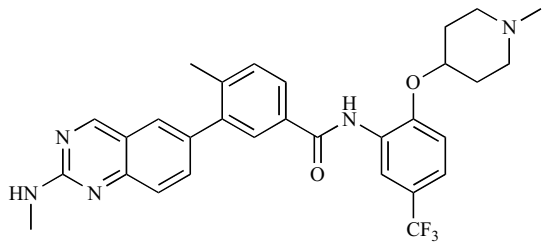
A high throughput screen using Lck as the target identified the aminoquinazoline **10** (Fig. (**6**)) as a potent inhibitor of Lck with an IC_{50} = 0.2 nM, although with poor specificity and pharmacokinetics [43]. Cocrystalization of **10** with Lck revealed four hydrogen bonds. The backbone NH and carbonyl of Met319 form two hydrogen bonds with the quinazoline ring and the amide moiety makes hydrogen bond contacts with the backbone NH of Asp382 from the catalytic loop and with the side chain of Glu288. The aryl ring of the trifluoromethylamide thus sits deep within the extended hydrophobic pocket, making several van der Waals contacts to hydrophobic residues.

SAR and structural analysis of the amide aryl showed the CF_3 group at C5 to be buried in the hydrophobic pocket, forcing C2 towards solvent. Amine groups were added to the C2 position, both increasing solubility as well as providing selectivity against non-Src kinases. Further SAR was performed on the hinge binding region of the quinazoline. Crystal structures with Lck showed this portion of the quinazoline to be primarily exposed to solvent [43]. Accordingly, modifications in this area substantially improved solubility while having little effect on potency. The availability of a hydrogen bond donor was critical for potency. Efficacy against other SFK members, however, was not performed during these initial SAR studies, making it difficult to assess the potential of individual modifications for this purpose.

Of the resulting analogs, **11** (Fig. (**6**)) showed the greatest improvement in Lck activity (IC_{50} = 0.5 nM) while maintaining some selectivity against Src (IC_{50} = 17 nM). Activity against Kdr (IC_{50} = 17 nM) remains quite high, however, suggesting room for improvement might still exist in the access of the compound to the hydrophobic pocket.



10 Lck IC_{50} = 0.2 nM

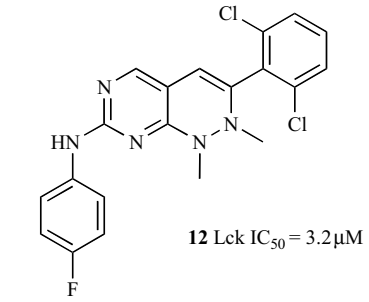


11 Lck IC_{50} = 0.5 nM, Src IC_{50} = 17 nM

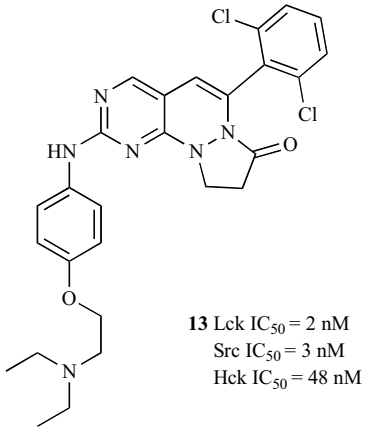
Fig. (6).

Pyrimidopyridazines

Screening of pyrimidopyridazine compounds for Lck activity resulted in the discovery of a novel 1,2 dihyrdropyrimido[4,5-*c*]pyridazine lead with low micromolar activity towards Lck (**12**, Fig. (**7**)) [44]. Modeling of this compound in the active site of Lck suggested two principal hydrogen bonds with Met319, the first bond between N1 of the pyrimidine ring and the protein backbone NH and the second bond between the aniline NH and the carbonyl group. Introduction of p-, m-, o- methoxy aniline moieties at the C-7 position indicated 4-phenyl substitution provided optimal activity against Lck, likely providing positive interactions with the hinge region. The activity of the compound towards Src and Hck was dependent on the nature of this substitution with a morpholine substituent providing greater Lck selectivity than amine moieties with a resulting loss of solubility. The most promising of these compounds, in which the morpholine ring is opened to form a diethylamine tail, showed good solubility and activity towards Lck, although still with strong activity towards Src (**13**, Fig (**7**)). Modeling of this compound suggests that the hydrophobic pocket is not utilized by these compounds. Rather, the dichlorophenyl group may interact outside of the pocket and form a hydrogen bond with Lys273. In addition, the diethyl amine tail of the C-7 moiety may interact with Asp326 in the hinge region. While analysis of these compounds showed modest inhibition of IL-2 release in Jurkat cells, pharmacokinetic parameters need to be improved.



12 Lck IC_{50} = 3.2 μM

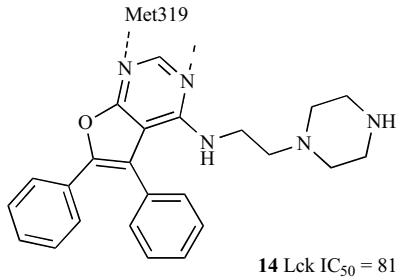


13 Lck IC_{50} = 2 nM
Src IC_{50} = 3 nM
Hck IC_{50} = 48 nM

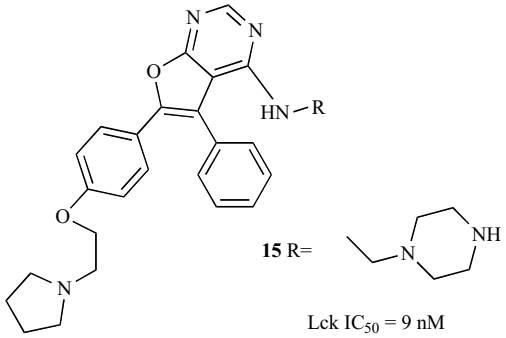
Fig. (7).

Furanopyrimidines

Using HTS, a group of 4-amino-5,6-biaryl-furo[2,3-*d*]pyrimidines were identified as promising leads for a Lck specific inhibitor [45]. Although initial assays were limited in that the only SFK tested was Lck, at least one of these compounds showed high activity towards Lck with an IC_{50} of 81 nM (Compound **14** Fig. (**8**)). A co-crystal structure of this compound showed the inhibitor to occupy the ATP binding sites, with the furanopyrimidine core forming two hydrogen bonds with Met319 in the hinge region.



14 Lck IC_{50} = 81 nM



Lck IC_{50} = 9 nM
Src IC_{50} = 45 nM
Kdr IC_{50} = 3.1 mM

16

Lck IC_{50} = 36 nM
Src IC_{50} = 91 nM
Kdr IC_{50} = 54 nM

Fig. (8).

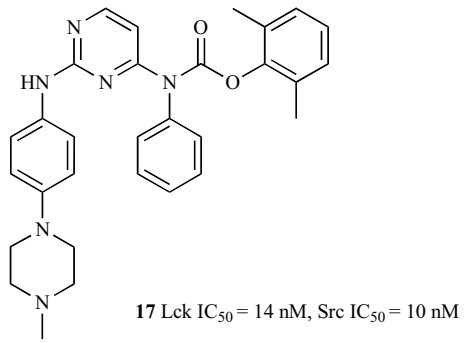
Improvement of the activity of **14** focused on two areas. First, an increase the solubility and activity of the compound were pursued by modification of the phenyl group at C6. Introduction of a polar or hydrophillic group at the para-position resulted in substantial improvement in Lck activity (Compound **15**, Fig. (**8**)). Modeling experiments suggest that novel charge-charge interactions between the amines and Asp326 occur near the end of the hinge region. In addition to providing increased solubility, the properties of this extension also showed significant impact on selectivity towards the non-receptor tyrosine kinase Ack1. Second, substitution of piperazine moiety at C4 with a hydrophobic substituent yielded some improvement in Lck selectivity, but with a significant increase in the potency towards Kdr (Compound **16**, Fig. (**8**)).

Modeling suggests that **15** and **16** approach, but do not access the hydrophobic pocket. This is likely a cause of relatively poor selectivity against non-SFK tyrosine kinases (**15** EGFR IC_{50} = 0.40 μM and Zap70 IC_{50} = 1.0 μM; **16** EGFR

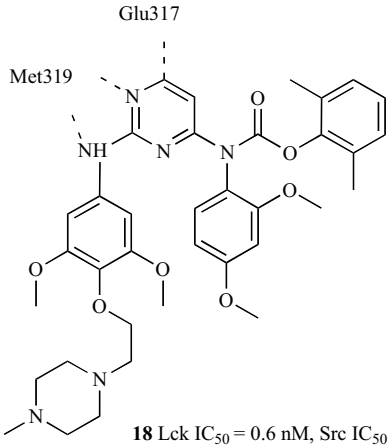
IC_{50} = 1.1 μM; Zap70 IC_{50} = 3.3 μM). Modification of the core to furopyridinamine, followed by further SAR exploration of solvent and hinge exposed regions of the molecule did not result in significant gains in this area [46].

Aminopyrimidine Carbamates

The aminocarbamate **17** (Fig. (**9**)) was isolated using a high throughput screen for Lck inhibitors [47]. The compound is a potent inhibitor of Lck and of Src as well the tyrosine kinase Kdr (IC_{50} = 190 nM). Initial SAR explored substitution of the phenyl group in an attempt to improve the potency and selectivity of the compound. A greater than 10-fold increase in Lck activity was achieved by substitution with phenyl groups containing a hydroxymethyl substitution in the 2 position. Interestingly, substitution of a phenyl group containing a methoxy group in the 2 and 4 position retained excellent Lck activity (IC_{50} = 2 nM) while substantially decreasing activity towards Kdr (IC_{50} = 650 nM).



17 Lck IC_{50} = 14 nM, Src IC_{50} = 10 nM



18 Lck IC_{50} = 0.6 nM, Src IC_{50} = 1 nM

Fig. (9).

Further modification of the aniline in position 2 of the carbamate was performed in an attempt to improve the cellular potency of the inhibitors. Modification of this position had little effect on the *in vitro* activity of the inhibitor towards Lck, but did provide improvements in cellular activity and in selectivity. Substitution with polar groups increased cellular potency by over 10-fold and modification of the piperizine to a morpholine resulted in reduced activity towards Kdr and p38α. The result of this SAR exploration, compound **18** (Fig (**9**)), provides excellent cellular potency, inhibiting CD3 induced T cell IL-2 secretion with an EC_{50}=61 nM, an improvement of 63-fold over **17**. **18** also demonstrates good selectivity against other tyrosine kinases with IC_{50}s of 140 nM and 370 nM against Kdr and Zap70 respectively.

Compound **18** was co-crystallized with Lck [47]. Analysis of the structure demonstrated three hydrogen bonds between the inhibitor and conserved residues in the hinge of Lck (Fig (**9**)). The N3 of the core pyrimidine binds the backbone NH of Met319 and the CH from 4 position binds the carbonyl of Glu317. The third interaction is between the carbonyl of Met319 and the NH at position 2 of the pyrimidine. Both the methylpiperizine and aniline groups are exposed to solvent. Finally, the aryl ether groups are rotated from the plane of the inhibitor to gain entrance to the hydrophobic pocket.

CONCLUSIONS

Fueled by recent successes in the clinic, the development of kinase inhibitor drug molecules has become an area of intense research. With a well defined biochemical function in the immune response, Lck presents an excellent target for the next generation of kinase inhibitors. The high homology of Lck with other members of the Src family of kinases, however, complicates the development of Lck inhibitors. Off-target inhibition of other SFK members has the potential to result in the inhibition of a variety of important cellular functions. Therefore, molecules must be identified that provide high activity towards Lck yet have relatively little activity towards other SFK isoforms.

In this review we have presented the current state in the development of a Lck specific inhibitor. Despite extensive work, the development of a Lck specific inhibitor with good bioavailability and pharmacokinetics has proved to be elusive, although A-770041 and the related compound **8** appear closest to achieving this goal. Analysis of these compounds and closely related analogs provides significant insight into the properties of both the structure of Lck and the molecules designed to inhibit Lck with high specificity. These include the formation of H bonds to the kinase hinge region, and contacts to both the hydrophobic pocket and the unique hinge sequence of Lck. Specificity to Lck can also be generated by contacts with solvent exposed portions of the molecule by mechanisms that are not understood. Further understanding of structural interactions of inhibitor molecules with multiple closely related enzymes has the potential to provide information useful in the rational design of kinase inhibitors and the development of a Lck inhibitor suitable for clinical use.

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