Novel 2-Aminopyrimidine Carbamates as Potent and Orally Active Inhibitors of Lck: Synthesis, SAR, and in Vivo Antiinflammatory Activity

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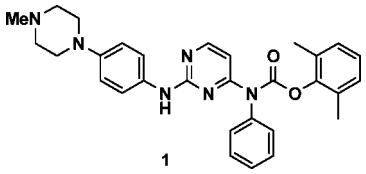
The lymphocyte-specific kinase (Lck) is a cytoplasmic tyrosine kinase of the Src family expressed in T cells and NK cells. Genetic evidence in both mice and humans demonstrates that Lck kinase activity is critical for signaling mediated by the T cell receptor (TCR), which leads to normal T cell development and activation. A small molecule inhibitor of Lck is expected to be useful in the treatment of T cell-mediated autoimmune and inflammatory disorders and/or organ transplant rejection. In this paper, we describe the synthesis, structure−activity relationships, and pharmacological characterization of 2-aminopyrimidine carbamates, a new class of compounds with potent and selective inhibition of Lck. The most promising compound of this series, 2,6-dimethylphenyl 2-((3,5-bis(methyloxy)-4-((3-(4-methyl-1-piperazinyl)propyl)-oxy)phenyl)amino)-4-pyrimidinyl(2,4-bis(methyloxy)phenyl)carbamate (**43**) exhibits good activity when evaluated in in vitro assays and in an in vivo model of T cell activation.

Introduction

T cells serve pivotal roles in the adaptive immune response by acting either as potentiators (CD4+ T helper cells) or effectors (CD8+ cytolytic T effector cells) of immune reactions. The immune specificity of these T cells is imparted by the T cell receptor (TCR)[a] for antigen, or CD3 complex.[1] Signaling pathways used by the TCR have been an intense area of research in the discovery of novel immunosuppressive agents that might serve as improved therapies for graft rejection and T cell-mediated autoimmune diseases. One class of enzymes shown to be important in TCR signal transduction is the protein kinases. The Src-family of cytoplasmic tyrosine kinases is made up of nine members: Src, Lck, Fyn, Lyn, Hck, Fgr, Blk, Yes, and Yrk.[2,3] Of these Src-family kinases, Lck and Fyn have been shown to have important roles in TCR signal transduction.[4−6] TCR signals initiated by Lck ultimately lead to gene regulation events triggering cytokine release, proliferation, and survival of antigen specific T cells thereby amplifying specific immune responses. Lck knock-out mice and patients with Lck mutations effecting expression and/or catalytic activity show defects in T cell maturation and signaling.[7−12] These findings suggest that a small molecule inhibitor of Lck kinase could be a useful immunosuppressive for the treatment of graft rejection and/or T cell-mediated autoimmune diseases.



1

Figure 1. Structure of 2-aminopyrimidine carbamate **1**.

A number of groups have previously reported the synthesis and characterization of Lck kinase inhibitors.[13−25] Potent and bioavailable Lck inhibitors have also been demonstrated to have inhibitory activities in vivo in several models of T cell-dependent immune responses.[22,26,27] Herein we describe the synthesis, structure−activity relationships, and pharmacological characterization of 2-aminopyrimidine carbamates, a new class of compounds with potent and selective inhibition of Lck.[28] Lead optimization led to inhibitors that demonstrated inhibition of T cell activation in in vitro and in vivo biological assays.

Screening of our kinase-preferred compound collection identified 2,6-dimethylphenyl-2-((4-(4-methyl-1-piperazinyl)-phenyl)amino)-4-pyrimidinyl(phenyl)carbamate (compound **1**, Figure 1) as a potent inhibitor of Lck, with an IC_{50} of 14 nM in an Lck kinase assay. Subsequently, we carried out structure−activity relationship studies on **1** designed to identify substituents for increased potency, selectivity, and optimal pharmacokinetic properties. In the course of these studies, we utilized structural biology to obtain enzyme:inhibitor cocrystal structures that assisted in identifying the key components necessary for binding to Lck.

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[a] Abbreviations: Lck, lymphocyte-specific kinase; TCR, T cell receptor; IL-2, interleukin 2; PTK, protein tyrosine kinase; CMGC, kinase group containing CDK, MAPK, GSK3, and CLK families; AGC, kinase group containing PKA, PKG, and PKC families.

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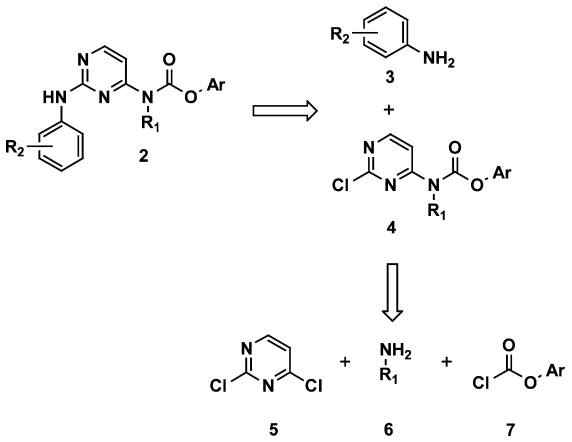
Scheme 1. Retrosynthetic Analysis of 2-Aminopyrimidine Carbamates



Chemistry

Our strategy for the construction of the 2-aminopyrimidine carbamates described in this paper is outlined in the retrosynthetic analysis shown in Scheme 1. In this approach, carbamates **2** were prepared via the nucleophilic addition of anilines **3** to 2-chloropyrimidine carbamates **4** under acidic conditions. Carbamates **4**, in turn, were accessed through the sequential addition of amines **6** to 2,4-dichloropyrimidine (**5**) followed by acylation with chloroformates **7**. This concise route allowed for rapid analoging, as each variation could be introduced by simply employing the desired amine, chloroformate, and aniline.

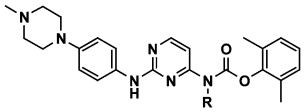
The anilines required for the syntheses of the carbamates shown in Tables 1 and 2 were either obtained from commercial sources or prepared via one of the methods shown in Scheme 2. The 4-alkoxy-substituted anilines **11** were synthesized from the corresponding nitrophenols **8** via an alkylation with either 1-bromo-2-chloroethane or 1-bromo-3-chloropropane. The resulting alkyl chloride **9** then underwent nucleophilic displacement with the desired amine (either dimethylamine, piperidine, morpholine, or *N*-methylpiperazine) to afford alkoxynitro derivative **10**. Catalytic hydrogenation with palladium on carbon afforded the desired alkoxyaniline **11**. For the 4-(*N*-methylpiperazin-1-yl)aniline analogues (**14a**,**b**), 1-halo-4-nitrobenzene derivatives **12** were heated with *N*-methylpiperazine to afford the corresponding 1-methyl-4-(4-nitrophenyl)piperazines **13**. Reduction via catalytic hydrogenation with palladium on carbon afforded the desired anilines **14**.

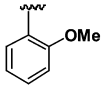
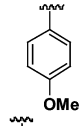
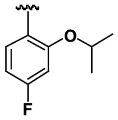
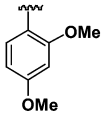
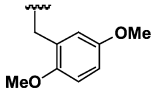
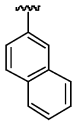
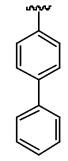
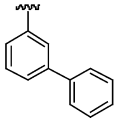
For the synthesis of the 2-chloropyrimidine intermediates **16** and **18**, one of the two methods outlined in Scheme 3 was used. In the cases where the amine was an aniline, heating a solution of 2,4-dichloropyrimidine (**5**), aniline **15**, and diisopropylethylamine in 2-propanol at 100 °C afforded the desired 4-anilino-2-chloropyrimidine **16**. Alternatively, for alkylamines such as benzylamines, more mild conditions could be used. In these cases, addition of a benzylamine, such as **17**, to a 0 °C solution of the 2,4-dichlopyrimidine (**5**) in 2-propanol and warming the solution to room temperature afforded the desired product **18** in good yield.

The desired 2,6-dimethylphenyl chloroformate (**20**) was readily prepared as shown in Scheme 4. A 0 °C solution of 2,6-dimethylphenol (**19**) in dichloromethane was sequentially treated with a solution of triphosgene in dichloromethane and then pyridine. The mixture was slowly warmed to room temperature, affording 2,6-dimethylphenyl chloroformate (**20**) in quantitative yield.

The three previously described building blocks could be combined to form the desired 2-aminopyrimidine carbamates **23** and **24** as shown in Scheme 5. 2-Chloropyrimidine-4-amines (**16** or **18**) were acylated with 2,6-dimethylphenyl chloroformate (**20**) to afford 2-chloropyrimidine carbamates (**21** or **22**) in good yield. Heating compound **21** or **22** with anilines (**11** or **14**) in the presence of trifluoroacetic acid in a solution of 2-propanol promoted nucleophilic displacement of the chloride to afford the desired 2-anilinopyrimidine carbamates (**23** or **24**) in good yield.

Table 1. SAR: Variations of the Amine (IC_{50}, μM)[a]



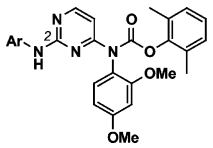
	R	Lck	Src	p38α	KDR
1		0.014	0.010	0.16	0.19
25		0.001	0.004	0.42	0.19
26		0.012	0.003	0.32	0.42
27		0.001	0.013	1.2	4.8
28		0.002	0.006	1.3	0.64
29		0.001	0.001	0.20	0.20
30		0.010	0.014	0.74	0.13
31		0.013	0.098	1.8	0.59
32		0.049	0.50	8.0	1.9

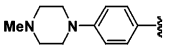
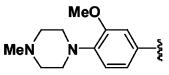
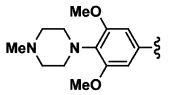
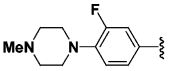
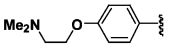
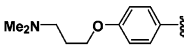
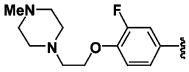
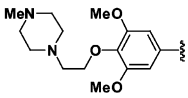
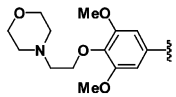
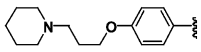
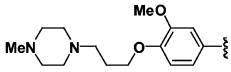
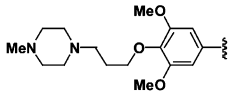
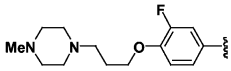
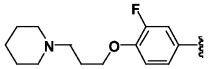
[a] IC_{50} values are the means of two or more separate determinations, in duplicate.

Results and Discussion

Structure−Activity Relationships (SAR). All compounds were screened for inhibitory activity against Lck in a homogeneous time-resolved fluorescent (HTRF) kinase assay. For the purposes of determining selectivity, the compounds were also tested against related kinases Src, p38α, and KDR. Selected compounds were tested for cellular activity by measuring the inhibition of T cell receptor-induced IL-2[a] production in human T cells.

Table 2. SAR: Variations of the Aniline Side Chain (IC_{50}, μM)[a]

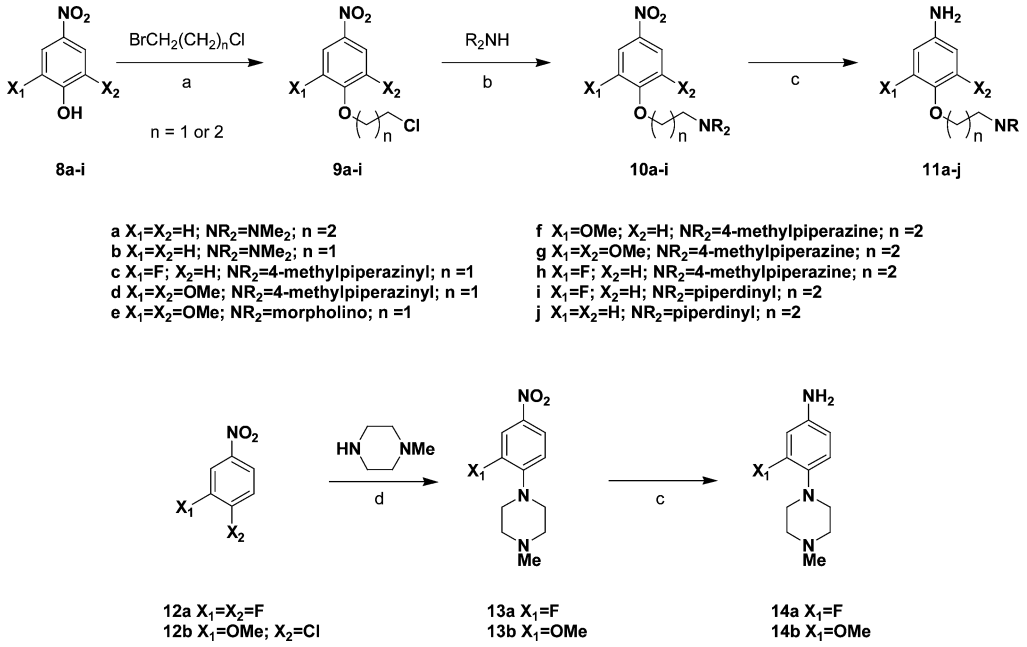


	Ar	Lck	Src	p38α	KDR	IL-2 Sec.[b]
28		0.002	0.006	1.3	0.65	1.9
33		0.001	0.002	0.44	0.14	0.14
34		0.001	0.001	0.43	0.17	0.59[c]
35		0.001	0.002	1.0	0.26	0.20
36		0.001	0.004	2.1	0.22	0.27
37		0.001	0.004	2.1	0.23	0.23
38		0.001	0.006	3.0	0.57	0.54
39		0.001	0.001	0.18	0.23	0.29
40		0.001	0.002	1.2	0.17	0.41
41		0.001	0.007	3.2	0.35	0.36
42		0.001	0.002	2.3	0.21	0.10
43		0.0006	0.001	0.53	0.14	0.061
44		0.001	0.005	1.3	0.59	0.51
45		0.002	0.012	6.8	0.62	0.86

[a] IC_{50} values are the means of two or more separate determinations, in duplicate. [b] IL-2 Sec.:Anti-CD3/CD28-induced T cell IL-2 secretion assay. [c] IC_{50} value is for human mixed lymphocyte reaction; compound **34** was not tested in the T cell receptor-induced IL-2 secretion assay.

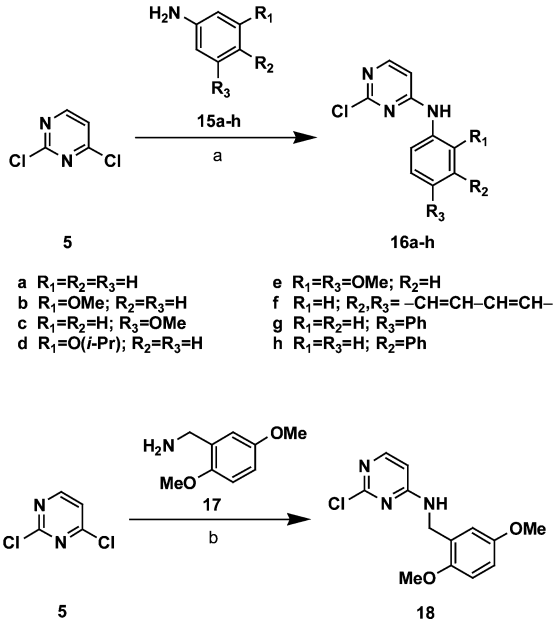
2-Aminopyrimidine carbamate **1** had an IC_{50} of 14 nM in an Lck kinase assay and experienced a 300-fold loss in potency when tested in a T cell receptor-induced IL-2 cellular assay (IC_{50} = 3.9 μM). Our initial work focused on exploring variants of the phenyl group in carbamate **1** and studying the effect this would have on potency and selectivity.[29] As shown in Table 1, phenyl groups with a 2-substituent, such as **25**, provided a 10-fold potency increase over the unsubstituted phenyl. 4-Substituted phenyl groups, such as in **26**, and larger aromatic groups, such as naphthyl (**30**) or biphenyl (**31**), showed no significant improvement (or loss) in potency and selectivity relative to the parent phenyl group. Interestingly, 2,4-disubstituted phenyl groups (**28**) showed a similar potency increase on Lck as 2-substituted variants. However, the disubstitution led to greater than 300-fold selectivity over p38α and KDR. Bulkier 2,4 derivatives, such as **27**, enhanced selectivity to greater than 1000-fold versus these two kinases. We also explored substituting the phenyl group with a benzyl substituent. As exemplified by compound **29**, 2,5-disubstituted benzyl groups were equipotent with the 2,4-disubstituted phenyl derivatives **27** and **28**,

Scheme 2. Synthesis of Aniline Side Chains[a]



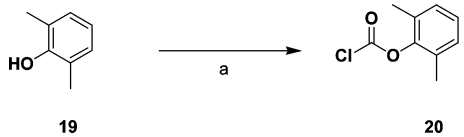
[a] (a) K_2CO_3, MeCN, reflux; (b) K_2CO_3, MeCN, 80 °C; (c) H_2, 10% Pd/C, EtOH, rt, 16 h; (d) 100 °C.

Scheme 3. Synthesis of 2-Chloro-pyrimidine-4-amines[a]



[a] (a) $(i\text{-Pr})_2$NEt, rt → 100 °C, 24 h; (b) *i*-PrOH, 0 °C → rt, 15 h.

Scheme 4. Synthesis of Chloroformates[a]



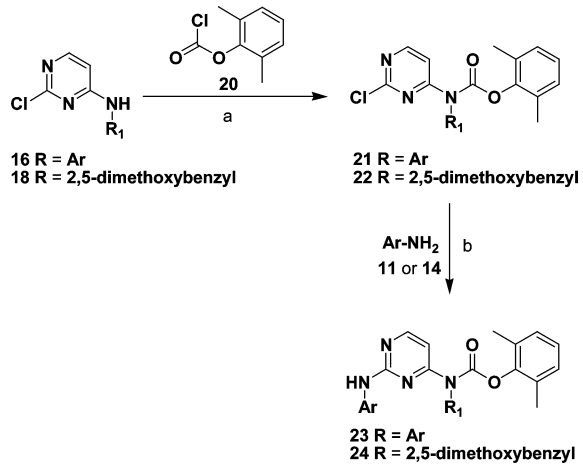
[a] (a) Triphosgene, pyridine, CH_2Cl_2, 0 °C → rt, 100%.

though there was some loss in selectivity. For this reason, we chose to conduct further SAR studies employing anilines with the 2,4-disubstitution.

In an effort to further improve the enzyme activity and optimize the cellular potency of 2,4-dimethoxyaniline-derived carbamate **28**, we varied the aniline group in the 2-position of the pyrimidine ring. As shown in Table 2, all variations led to potent Lck inhibitors, indicating that chemical diversity at this position was well tolerated. The main area of differentiation between these compounds was in their selectivity profile and cellular activity. The introduction of electron-donating or electron-withdrawing groups on the aniline (compounds **33**, **34**, and **35**, respectively) afforded compounds with similar selectivity to **28**, but with a 10-fold increase in cellular activity (presumably due to improved solubility and/or cell permeability). Replacement of the *N*-methylpiperazine with 2-(dimethylamino)-ethoxy or 2-(dimethylamino)propoxy groups (**36** and **37**) also improved cell potency. In these cases p38α selectivity was improved, while selectivity against KDR remained unchanged (200-fold for both **36** and **37**). Replacing the dimethylamino group on the alkoxy side chain with a cyclic amine, such as piperidine (compound **41**), had little impact on potency or selectivity.

We also investigated the impact of combining substituted anilines with variations on the alkoxy side chain. For 3-fluoro-substituted anilines (**38, 44**, and **45**), KDR selectivity was improved slightly, but with a small loss in cell potency, relative to **36**, **37**, and **41**. 3,5-Dimethoxy derivative **39** showed good cellular potency (0.29 μM) and selectivity over KDR and p38α

Scheme 5. Synthesis of 2-Aminopyrimidine Carbamates[a]



[a] (a) $(i\text{-Pr})_2$NEt, THF, rt; (b) CF_3CO_2H, *i*-PrOH, 100 °C, 16 h.

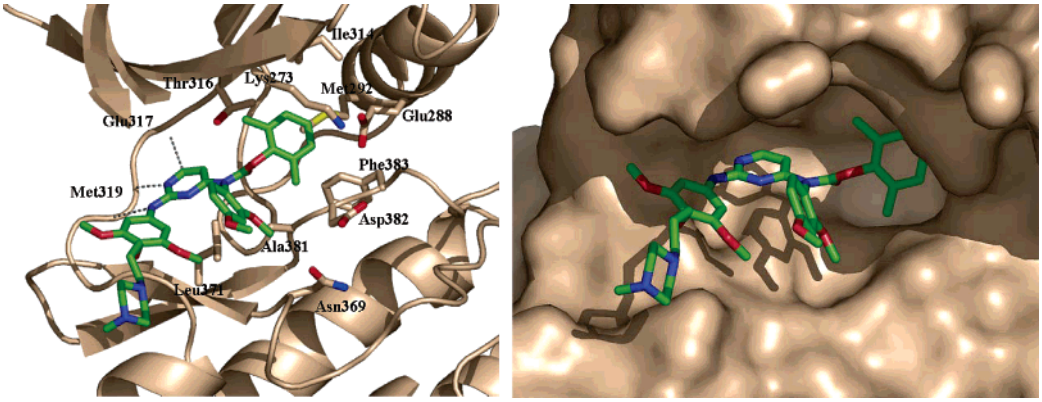


Figure 2. X-ray structure of 2-aminopyrimidine carbamate **43** bound to Lck.

(200-fold for both). Replacing the *N*-methylpiperazine with a morpholine (compound **40**) saw a slight decrease in cell potency, but a 10-fold increase in selectivity over p38α (relative to compound **39**). Increasing the alkoxy side chain to a propoxy linker (compounds **42** and **43**) had the most significant effect. 3-Methoxy derivative **42** maintained selectivity over KDR and p38α (200-fold and 2000-fold, respectively) with a slight improvement in cell potency (0.10 μM). 3,5-Dimethoxy derivative **43** also maintained selectivity over KDR and p38α (200-fold and 800-fold, respectively), but with a significant increase in cell potency (0.061 μM). Because it afforded such a marked increase in cell potency, we chose to further explore the properties of pyrimidine carbamate **43**.

Structural Confirmation. In an effort to confirm the binding elements responsible for potent inhibition of the target protein in this series of compounds, we obtained a cocrystal structure of Lck with carbamate **43**, one of the most promising pyrimidine carbamates in terms of potency and selectivity. Consistent with findings from other researchers, three key H-bond interactions between the inhibitor **43** and the linker region of the protein are observed (Figure 2).[16,19,25] The core pyrimidine in carbamate **43** binds to the linker region through two hydrogen bonds: Methionine 319 donates a backbone NH to the N3 pyrimidine acceptor and the carbonyl from Glu 317 accepts the CH in the 4-position.[30] A third interaction is made between the linker Met 319 carbonyl and the NH substituent at position 2 of the pyrimidine. The aniline side chain extends out of the enzyme, with the *N*-methylpiperazine moiety fully exposed to the solvent. The aryl ether group is rotated 114° from the plane due to the presence of the 2,6-substituents, which appropriately orientates the aromatic ring to enter the deep hydrophobic pocket. This is consistent with previous findings that have shown the 2,6-substitution to be a key element responsible for potency.[15,17] Interestingly, the 2-methoxy group on the aniline fills the small pocket adjacent to the ribose-binding pocket. This novel interaction could explain the improved potency of the 2,4-dimethoxy aniline derivatives relative to compounds lacking a 2-substituent on the aniline ring.

Selectivity Profile. To evaluate carbamate **43** in an in vivo setting, it was critical to understand the selectivity profile of this molecule. To that end, carbamate **43** was assayed against an extended panel of tyrosine and serine/threonine kinases (Table 3).[31] Within the protein tyrosine kinase (PTK)[a] group, **43** was selective (>100-fold) over representatives from the Syk, Tie, JakA, VEGFR, FGFR, Tec, InsR, and Met families. Similar selectivity was seen over kinases outside of the PTK group (CMGC[a] and AGC[a]). Namely, kinases from the MAPK, CDK, and PKC families all showed excellent levels of selectivity (>100-fold). As expected, due to the close homology of Lck to other members of the Src family, no selectivity was shown against Src.

Table 3. Selectivity Profile of Pyrimidine Carbamate **43**

kinase	IC_{50} (μM)[a]
Lck	0.0006
Btk	0.10
CDK2	>40
c-Met	2.4
FGF-1R	0.11
IGF-1R	>25
JAK2	0.24
JAK3	1.2
JNK3	>5
KDR	0.14
p38α	0.53
PKCθ	>5
Src	0.001
Syk	0.20
Tie-2	0.20
Zap-70	0.37

[a] IC_{50} values are the means of two or more separate determinations, in duplicate.

Table 4. Cellular Profile of Pyrimidine Carbamate **43**

cell assay[a]	IC_{50} (μM)[b]
IL-2 Sec.	0.061
T Cell Prolif.	0.49
huWB IL-2	3.0
huMLR	0.21
TCR ζ-chain	0.78
PMA/iono	1.8
JKT	1.7

[a] IL-2 Sec.:Anti-CD3/CD28-induced T cell IL-2 secretion assay; T Cell Prolif.: Anti-CD3/CD28-induced T cell proliferation assay; huWB IL-2: Anti-CD3/CD28-induced T cell IL-2 secretion assay using human whole blood; huMLR: human mixed lymphocyte reaction; TCR ζ-chain: T cell receptor ζ-chain phosphorylation assay; PMA/iono: Anti-CD3/CD28-induced T cell IL-2 secretion assay stimulated with phorbol myristic acid and calcium ionophore; JKT: Jurkat proliferation/survival assay. See Experimental Section for a description of each assay. [b] IC_{50} values are the means of two or more separate determinations, in duplicate.

Cellular Activity. To further understand the cellular properties of pyrimidine carbamate **43**, it was subjected to a series of functional cell assays. As shown in Table 4, carbamate **43** exhibited good potency in the T cell receptor-induced IL-2 secretion assay (IL-2 Sec.) and also inhibited subsequent T cell proliferation (T Cell Prolif.) in the same human T cells. When the IL-2 secretion assay was performed using human whole blood (huWB IL-2), the potency shifted significantly. This is likely due to the relatively high protein binding displayed by this compound (98.7% in humans; 98.4% in rats). Pyrimidine carbamate **43** also inhibited a human mixed lymphocyte reaction

Table 5. Pharmacokinetic Parameters for Compound **43** Following iv Dose in Sprague−Dawley Rats[a]

dose[b] (mg/kg)	$AUC_{0\rightarrow\infty}$ (ng h/mL)	CL (L/h/Kg)	V_{ss} (L/kg)	$t_{1/2}$ (h)
1	568	1.83	11.3	4.6

[a] $n = 3$ animals per study. [b] Dosed as a solution in DMSO.

Table 6. Pharmacokinetic Parameters for Compound **43** Following Oral Dose in Sprague−Dawley Rats[a]

dose[b] (mg/kg)	C_{max} (ng/mL)	t_{max} (h)	$AUC_{0\rightarrow\infty}$ (ng h/mL)	bioavailability, F (%)
5	22	4.0	175	6

[a] $n = 2$ animals per study. [b] Dosed as an aqueous suspension in 1% Tween 80 in 2% HPMC.

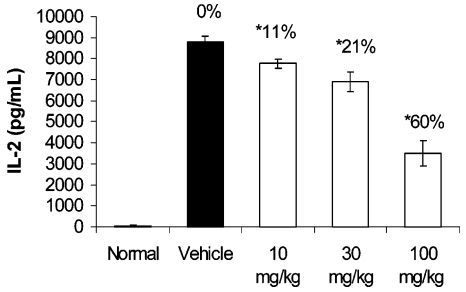


Figure 3. Inhibition of anti-CD3-induced IL-2 production in mice. Mice were dosed p.o. 1 h prior to challenge with compound **43** (eight per group) as described in the Experimental Section. Mice were then challenged i.v. with anti-mouse CD3ϵ monoclonal antibody. Ninety minutes after challenge, IL-2 levels in serum were determined by ELISA. Data points represent the mean IL-2 levels/group ± the standard error. *P* values were determined vs vehicle control by Mann−Whitney U-test. * indicates $p \leq 0.05$.

(huMLR) in the same potency range as the other in vitro cell assays utilizing purified human cells. This compound also displayed inhibition of a mechanism-based biochemical cell assay probing Lck-dependent TCR ζ-chain phosphorylation (TCR ζ-chain). As expected, the compound showed much reduced potency when IL-2 was induced in a receptor-independent fashion by stimulating with phorbol ester and calcium ionophore (PMA/iono). In addition, the compound displayed potency in the μM range when tested in a general proliferation assay using the human T cell line, Jurkat (JKT). With this promising in vitro data, we set out to examine the in vivo properties of pyrimidine carbamate **43**.

Pharmacokinetic Profile. The pharmacokinetic properties of carbamate **43** were assessed in the rat following both intravenous (i.v.) and oral (p.o.) dosing (Tables 5 and 6, respectively). The compound displayed a moderate clearance (1.83 L/h/kg) and high volume of distribution ($V_{ss} = 11.3$ L/kg) after i.v. dosing. After p.o. dosing, the t_{max} of compound **43** was found to be 4.0 h with a relatively low bioavailability (6%).

In Vivo Studies of 2-Aminopyrimidine Carbamate 43. Despite the low bioavailability, the compound was tested in a short-term T-cell receptor dependent mouse model. A single dose of pyrimidine carbamate **43** was administered at 100, 30, and 10 mg/kg orally to mice and evaluated for its ability to inhibit anti-CD3-mediated IL-2 secretion in serum (Figure 3). Terminal plasma levels of compound **43** were determined to derive a pharmacokinetic-pharmacodynamic relationship. The compound showed a dose-dependent inhibition of IL-2 production with an ED_{50} estimated at 80 mg/kg. Based on the measured plasma levels from the three dose groups, the estimated concentration of carbamate **43** at the ED_{50} was calculated to be 1.5 μM. The estimated compound levels at the ED_{50} are consistent with the IC_{50} of compound **43** in the in vitro human whole-blood IL-2 secretion assay (~3 μM; Table 4).

Conclusions

The novel 2-aminopyrimidine carbamates have been shown to be potent and selective inhibitors of Lck. Our investigation of the SAR of this series of compounds combined with structural analysis has identified the key components necessary for kinase inhibition. Optimization efforts led to 2-aminopyrimidine carbamate **43,** which showed inhibition of IL-2 production in in vitro assays and an in vivo model of T cell activation. Future studies will aim to improve the cellular potency and pharmacokinetic properties with the hope of increasing the in vivo efficacy.

Experimental Section

Unless otherwise noted, all materials were obtained from commercial suppliers and used without further purification. 4-(3-Piperidin-1-ylpropoxy)phenylamine (**11j**) was prepared as previously reported.[32] 3,5-Dimethoxy-4-(4-methylpiperazin-1-yl)aniline was obtained from Evotec OAI, Oxon, UK. Anhydrous solvents were obtained from Aldrich and used directly. All reactions involving air- or moisture-sensitive reagents were performed under a nitrogen or argon atmosphere. All final compounds were purified to >95% purity as determined by high-performance liquid chromatography (HPLC). Purity was measured using Agilent 1100 Series high performance liquid chromatography (HPLC) with UV detection at 254 nm (15 min; 1.5 mL/min flow rate; 0 to 100% 0.1% TFA in CH_3CN/100−0% 0.1% TFA in H_2O). Silica gel columns were performed using either glass columns packed with silica gel (200−400 mesh, Aldrich Chemical) or prepacked silica gel cartridges (Biotage). ^{1}H NMR spectra were recorded on a Bruker AV-400 (400 MHz) spectrometer at ambient temperature or on a Varian 400 MHz or on a Varian 300 MHz spectrometer. All observed protons are reported as parts per million (ppm) downfield from tetramethylsilane (TMS) or other internal reference in the appropriate solvent indicated. Data are reported as follows: chemical shift, multiplicity (*s* = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants, and number of protons. Low-resolution mass spectral (MS) data were determined on an Agilent 1100 Series LCMS with UV detection at 254 nm and a low resonance electrospray mode (ESI). High-resolution mass spectra were obtained on a high resonance electrospray time-of-flight mass spectrometer. Combustion analysis was performed by Atlantic Microlab, Inc., Norcross, GA, or Galbraith Laboratories, Knoxville, TN, and were within 0.4% of calculated unless otherwise noted.

***N,N*-Dimethyl-3-(4-nitrophenoxy)propylamine (10a).** Potassium carbonate (24.9 g, 180 mmol) was added to a solution of 4-nitrophenol (10 g, 72 mmol) in acetonitrile (100 mL). 1-Bromo-3-chloropropane (113.2 g, 720 mmol) was added, and the mixture was stirred under reflux overnight. The reaction was cooled to room temperature, the solids were filtered off, and the solvent was evaporated under reduced pressure to afford crude 3-(4-nitrophenoxy)propyl chloride (**9a**) (13 g) which was used without purification.

A sealed tube was charged with 3-(4-nitrophenoxy)propyl chloride (**9a**) (2.00 g, 9.27 mmol), potassium carbonate (7.69 g, 46.4 mmol), and acetonitrile (15 mL). Dimethylamine hydrochloride (3.78 g, 46.4 mmol) was added, and the reaction was mixture stirred overnight at 80 °C. The mixture was cooled to room temperature, and water and dichloromethane were added. The aqueous layer was separated and extracted with dichloromethane. The combined organics were dried over anhydrous sodium sulfate, filtered, and concentrated to afford *N*,*N*-dimethyl-3-(4-nitrophenoxy)propylamine (**10a**) (1.18 g, 57%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.95 (t, $J = 7$ Hz, 2 H), 2.20 (s, 6 H), 2.35−2.45 (m, 2 H), 4.05 (t, $J = 7$ Hz, 2 H), 6.90 (d, $J = 8$ Hz, 2 H), 8.10 (d, $J = 8$ Hz, 2 H).

4-(3-(Dimethylamino)propoxy)aniline (11a). A suspension of palladium on carbon (10%, 0.400 g) in a solution of *N*,*N*-dimethyl-3-(4-nitrophenoxy)propylamine (**10a**) (4.4 g, 19.6 mmol) in ethanol (50 mL) was stirred under a hydrogen (g) atmosphere for 16 h. The catalyst was filtered off and the solvent removed under reduced pressure to afford 4-(3-(dimethylamino)propoxy)aniline (**11a**) (3.8 g, 100%) as a brown oil. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.95 (t, 2H, J = 6.5 Hz, 2 H), 2.25 (s, 6 H), 2.35−2.45 (m, 2 H), 3.95 (t, J = 6.5 Hz, 2 H), 4.70 (s, 2 H), 6.90 (d, J = 8 Hz, 2 H), 8.10 (d, J = 8 Hz, 2 H), 6.65 (d, J = 8 Hz, 2 H), 6.75 (d, J = 8 Hz, 2 H).

4-(2-(Dimethylamino)ethoxy)aniline (11b). 4-(2-(Dimethylamino)ethoxy)aniline (**11b**) was synthesized from 4-nitrophenol, dimethylamine hydrochloride, and 1-bromo-2-chloroethane according to the procedure outlined for the synthesis of 4-(3-(dimethylamino)-propoxy)aniline (**11a**). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 2.25 (s, 6 H), 2.65 (t, J = 7 Hz, 2 H), 3.9 (t, J = 7 Hz, 2 H), 6.5−7 (m, 2 H), 6.65−6.75 (m, 2H).

3-Fluoro-4-(2-(4-methylpiperazin-1-yl)ethoxy)aniline (11c). 3-Fluoro-4-(2-(4-methylpiperazin-1-yl)ethoxy)aniline (**11c**) was synthesized from 2-fluoro-4-nitrophenol, 4-methylpiperazine, and 1-bromo-2-chloroethane according to the procedure outlined for the synthesis of 4-(3-(dimethylamino)propoxy)aniline (**11a**). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 2.20 (s, 3 H), 2.30−2.40 (m, 4 H), 2.40−2.65 (m, 4 H), 2.75 (t, J = 7 Hz, 2 H), 4 (t, J = 7 Hz, 2 H), 6.25−6.3 (m, 1 H), 6.3−6.35 (m, 1 H), 6.75−6.85 (m, 1 H).

3,5-Dimethoxy-4-(2-(4-methylpiperazin-1-yl)ethoxy)aniline (11d). 3,5-Dimethoxy-4-(2-(4-methylpiperazin-1-yl)ethoxy)aniline (**11d**) was synthesized from 2,6-dimethoxy-4-nitrophenol, 4-methylpiperazine, and 1-bromo-2-chloroethane according to the procedure outlined for the synthesis of 4-(3-(dimethylamino)propoxy)-aniline (**11a**). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 2.25 (s, 3 H), 2.40−2.70 (m, 8 H), 2.75 (t, J = 7 Hz, 2 H), 3.70 (s, 6 H), 3.90 (t, J = 7 Hz, 2 H), 5.90 (s, 2 H).

3,5-Dimethoxy-4-(2-morpholinoethoxy)aniline (11e). 3,5-Dimethoxy-4-(2-morpholinoethoxy)aniline (**11e**) was synthesized from 2,6-dimethoxy-4-nitrophenol, morpholine, and 1-bromo-2-chloroethane according to the procedure outlined for the synthesis of 4-(3-(dimethylamino)propoxy)aniline (**11a**). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 2.50−2.55 (m, 4 H), 2.70 (t, J = 7 Hz, 2 H), 3.60−3.70 (m, 4 H), 3.70 (s, 6 H), 4.95 (t, J = 7 Hz, 2 H), 5.8 (s, 2 H).

3-Methoxy-4-(3-(4-methylpiperazin-1-yl)propoxy)aniline (11f). 3-Methoxy-4-(3-(4-methylpiperazin-1-yl)propoxy)aniline (**11f**) was synthesized from 2-methoxy-4-nitrophenol, 4-methylpiperazine, and 1-bromo-3-chloropropane according to the procedure outlined for the synthesis of 4-(3-(dimethylamino)propoxy)aniline (**11a**). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.90−2.10 (m, 2 H), 2.35 (s, 3 H), 2.40−2.60 (m, 10 H), 3.80 (s, 3 H), 4.00 (t, J = 7 Hz, 2 H), 6.20 (dd, J = 2, 8 Hz, 1 H), 6.30 (d, J = 2 Hz, 1 H), 6.80 (d, J = 8 Hz, 1 H).

3,5-Dimethoxy-4-(3-(4-methylpiperazin-1-yl)propoxy)aniline (11g). 3,5-Dimethoxy-4-(3-(4-methylpiperazin-1-yl)propoxy)-aniline (**11g**) was synthesized from 2,6-dimethoxy-4-nitrophenol, 4-methylpiperazine, and 1-bromo-3-chloropropane according to the procedure outlined for the synthesis of 4-(3-(dimethylamino)-propoxy)aniline (**11a**). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.80−1.90 (m, 2 H), 2.20 (s, 3 H), 2.30−2.60 (m, 10 H), 3.70 (s, 6 H), 3.85 (t, J = 7 Hz, 2 H), 5.90 (s, 2 H).

3-Fluoro-4-(3-(4-methylpiperazin-1-yl)propoxy)aniline (11h). 3-Fluoro-4-(3-(4-methylpiperazin-1-yl)propoxy)aniline (**11h**) was synthesized from 2-fluoro-4-nitrophenol, 4-methylpiperazine, and 1-bromo-3-chloropropane according to the procedure outlined for the synthesis of 4-(3-(dimethylamino)propoxy)aniline (**11a**). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.80−1.90 (m, 2 H), 2.2 (s, 3 H), 2.30−2.55 (m, 10 H), 3.90 (t, J = 7 Hz, 2 H), 6.30 (m, 1 H), 6.40 (m, 1 H), 6.70 (m, 1 H).

3-Fluoro-4-(3-(piperidin-1-yl)propoxy)aniline (11i). 3-Fluoro-4-(3-(piperidin-1-yl)propoxy)aniline (**11i**) was synthesized from 2-fluoro-4-nitrophenol, piperidine, and 1-bromo-3-chloropropane according to the procedure outlined for the synthesis of 4-(3-(dimethylamino)propoxy)aniline (**11a**). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.30−1.40 (m, 2 H), 1.40−1.50 (m, 4 H), 1.70−1.80 (m, 2 H), 2.25−2.40 (m, 6 H), 3.90 (t, J = 7 Hz, 2 H), 6.25−6.30 (m, 1 H), 6.35−6.40 (m, 1 H), 6.75−6.85 (m, 1 H).

1-(2-Fluoro-4-nitrophenyl)-4-methylpiperazine (13a). A sealed tube was charged with *N*-methylpiperazine (30 mL, 27.1 g, 270 mmol) and cooled in ice/water. 3,4-Difluoronitrobenzene (2.0 g, 12.8 mmol) was added, and the mixture was heated at 100 °C overnight. The reaction mixture was concentrated to remove excess *N*-methylpiperazine, and the residue dissolved in 1 M hydrochloric acid (30 mL). The solution was washed with dichloromethane (2 × 20 mL). The aqueous phase was separated, basified with 5 M sodium hydroxide (10 mL), and then extracted with dichloromethane (2 × 20 mL). The combined organic phases were dried over anhydrous sodium sulfate, filtered, and concentrated to afford 1-(2-fluoro-4-nitrophenyl)-4-methylpiperazine (**13a**) (1.5 g, 49%) as a low melting solid. ^{1}H NMR ($CDCl_3$) δ ppm 2.39 (s, 3H), 2.63 (m, 4H), 3.33 (m, 4H), 6.92 (m, 1H), 7.91 (m, 1H), 8.00 (m, 1H).

1-(2-Fluoro-4-aminophenyl)-4-methylpiperazine (14a). 1-(2-Fluoro-4-aminophenyl)-4-methylpiperazine (**14a**) was synthesized from 1-(2-fluoro-4-nitrophenyl)-4-methylpiperazine (13a) via hydrogenation over 10% palladium on carbon as detailed for the synthesis of 4-(3-(dimethylamino)propoxy)aniline (**11a**). ^{1}H NMR ($CDCl_3$) δ ppm 2.29 (s, 3 H), 2.53 (m, 4 H), 2.94 (m, 4 H), 3.48 (m, 2 H), 6.33 (m, 2 H), 6.75 (m, 1 H).

3-Methoxy-4-(4-methylpiperazin-1-yl)aniline (14b). 3-Methoxy-4-(4-methylpiperazin-1-yl)aniline (**14b**) was synthesized from 2-chloro-5-nitroanisole according to the two-step procedure outlined for the synthesis of 1-(2-fluoro-4-aminophenyl)-4-methylpiperazine (**14a**). ^{1}H NMR ($CDCl_3$) δ ppm 2.40 (s, 3 H), 2.50−2.70 (m, 4 H), 2.90−3.10 (m, 4 H), 3.80 (s, 3 H), 6.20−6.40 (m, 2 H), 6.80−6.90 (m, 1 H). MS (ESI, pos. ion) *m*/*z*: 222 (M + 1).

2,6-Dimethylphenyl Chloroformate (20). A solution of 2,6-dimethylphenol (1.2 g, 10 mmol) in dichloromethane (20 mL) was cooled to 0 °C, and a solution of triphosgene (1.07 g, 3.6 mmol) in 15 mL of dichloromethane was added with stirring. Pyridine (0.80 mL, 10 mmol) was added dropwise, and the reaction was allowed to warm to room-temperature overnight. The mixture was diluted with ethyl acetate and partitioned between ethyl acetate and 1 N HCl (aq). The organic phase was separated and washed with 1 N HCl (aq) and brine, dried over anhydrous sodium sulfate, filtered, and concentrated to afford 2,6-dimethylphenyl chloroformate (**20**) as a yellow oil (1.84 g, 100%). The product was used without further purification. ^{1}H NMR ($CDCl_3$) δ ppm 2.2 (s, 6 H), 7.15 (m, 3 H).

2,6-Dimethylphenyl 2-Chloropyrimidin-4-yl-(2,4-dimethoxyphenyl)carbamate (22a). A solution of 2,4-dichloropyrimidine (3.0 g, 20 mmol), 2,4-dimethoxyaniline (3.1 g, 20 mmol), and *N*,*N*-diisopropylethylamine (7.8 g, 10 mL, 60 mmol) in 2-propanol (20 mL) was stirred at room temperature for 48 h. The resulting suspension was partitioned between saturated aqueous potassium carbonate solution and ethyl acetate. The organic phase was separated, dried over anhydrous sodium sulfate, filtered, and concentrated to afford crude 2-chloro-*N*-(2,4-dimethoxyphenyl)-pyrimidin-4-amine (**16a**) which was used without purification. MS (ESI, pos. ion) *m*/*z*: 266 (M + 1).

2-Chloro-*N*-(2,4-dimethoxyphenyl)pyrimidin-4-amine (**16a**) (5.3 g, 20 mmol) from above was added to a solution of 2,6-dimethylphenyl chloroformate (3.7 g, 20 mmol) in tetrahydrofuran (75 mL). *N*,*N*-Diisopropylethylamine (7.8 g, 10.0 mL, 60 mmol) was added and the mixture stirred at room temperature for 48 h. The resulting suspension was diluted with ethyl acetate, and the mixture was partitioned between ethyl acetate and saturated aqueous sodium carbonate solution. The organic phase was separated and washed with brine, dried over anhydrous sodium sulfate, filtered, and concentrated to afford an orange oil. This oil was purified via column chromatography on silica gel (eluting with dichloromethane) to afford 2,6-dimethylphenyl 2-chloropyrimidin-4-yl-(2,4-dimethoxyphenyl)carbamate (**22a**) (3.4 g, 41%) as an orange solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.08 (s, 6 H), 3.79 (s, 3 H), 3.83 (s, 3 H), 6.64 (dd, J = 8.65, 2.59 Hz, 1 H), 6.75 (d, J = 2.53 Hz, 1 H), 7.04−7.12 (m, 3 H), 7.37 (d, J = 8.72 Hz, 1 H), 8.08 (d, J =

5.81 Hz, 1 H), 8.66 (d, $J = 5.94$ Hz, 1 H). MS (ESI, pos. ion) *m*/*z*: 414 (M + 1).

2,6-Dimethylphenyl 2,5-Dimethoxybenzyl(2-chloropyrimidin-4-yl)carbamate (22b). A solution of 2,4-dichloropyrimidine (3.16 g, 21.2 mmol) in 2-propanol (40 mL) was cooled to 0 °C, and *N*,*N*-diisopropylethylamine (2.74 g, 3.70 mL, 21.2 mmol) was added dropwise via syringe. 2,5-Dimethoxybenzylamine (3.2 mL, 21.2 mmol) was added dropwise via syringe, and the ice bath was removed. The mixture was stirred at room temperature for 2.5 days and was then concentrated. The residue was purified via column chromatography on silica gel (eluting with 1:1 hexanes−ethyl acetate) to afford *N*-(2,5-dimethoxybenzyl)-2-chloropyrimidin-4-amine (**18a**) (3.99 g, 67%) as an off-white solid. MS (ESI, pos. ion) *m*/*z*: 280 (M + 1).

2-Chloro-*N*-(2,5-dimethoxybenzyl)pyrimidin-4-amine (**18a**) (0.300 g, 1.07 mmol) from above was added to a solution of 2,6-dimethylphenyl chloroformate (0.198 g, 1.07 mmol) in tetrahydrofuran (2.0 mL). *N*,*N*-Diisopropylethylamine (0.138 g, 0.19 mL, 1.07 mmol) was added and the mixture stirred at room temperature for 17 h. The resulting suspension was diluted with ethyl acetate and the mixture was partitioned between ethyl acetate and saturated aqueous sodium carbonate solution. The organic phase was separated and washed with brine, dried over anhydrous sodium sulfate, filtered, and concentrated to afford an orange oil. This oil was purified via column chromatography on silica gel (gradient elution with 0−25% ethyl acetate−hexane) to afford 2,6-dimethylphenyl 2,5-dimethoxybenzyl-(2-chloropyrimidin-4-yl)carbamate (**22b**) (0.351 g, 77%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.98 (s, 6 H), 3.63 (s, 3 H), 3.69 (s, 3 H), 5.36 (s, 2 H), 6.66 (d, $J = 3.03$ Hz, 1 H), 6.83 (dd, $J = 8.78$, 3.09 Hz, 1 H), 6.95 (d, $J = 8.84$ Hz, 1 H), 7.09 (s, 3 H), 8.09 (d, $J = 5.94$ Hz, 1 H), 8.68 (d, $J = 5.94$ Hz, 1 H). MS (ESI, pos. ion) *m*/*z*: 428 (M + 1).

General Procedure for the Synthesis of 2-Aminopyrimidine Carbamates. Synthesis of 2,6-Dimethylphenyl-2,4-bis(methyloxy)phenyl(2-((4-((2-(dimethylamino)ethyl)-oxy)phenyl)amino)-4-pyrimidinyl)carbamate (36). A resealable tube was charged with 2,6-dimethylphenyl 2-chloropyrimidin-4-yl(2,4-dimethoxyphenyl)-carbamate (**22a**) (8.50 g, 20.5 mmol), 4-(2-(dimethylamino)ethoxy)-aniline (**11b**) (4.07 g, 22.6 mmol), trifluoroacetic acid (7.01 g, 4.74 mL, 61.5 mmol), and 2-propanol (160 mL). The system was flushed with argon, the tube was sealed, and the mixture was heated at 100 °C for 24 h. The reaction mixture was cooled to room temperature and concentrated. The residue was diluted with ethyl acetate and partitioned between ethyl acetate and saturated aqueous potassium carbonate solution. The organic layer was separated, washed with brine, dried over anhydrous sodium sulfate, and concentrated. The residue was purified via column chromatography on silica gel (eluting with 90:10:1 dichloromethane/methanol/ammonium hydroxide) to afford 2,6-dimethylphenyl 2,4-bis(methyloxy)phenyl(2-((4-((2-(dimethyl-amino)ethyl)oxy)phenyl)-amino)-4-pyrimidinyl)carbamate (**36**) (4.52 g, 40%) as an off-white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.09 (s, 6 H), 2.22 (s, 6 H,) 2.60 (t, $J = 5.81$ Hz, 2 H), 3.76 (s, 3 H), 3.87 (s, 3 H), 3.94 (t, $J = 5.94$ Hz, 2 H), 6.53 (s, 1 H), 6.56 (s, 1 H), 6.69 (dd, $J = 8.59$, 2.53 Hz, 1 H,) 6.83 (d, $J = 2.53$ Hz, 1 H), 7.02−7.14 (m, 5 H), 7.34 (d, $J = 8.59$ Hz, 1 H), 7.47 (d, $J = 5.81$ Hz, 1 H), 8.34 (d, $J = 5.81$ Hz, 1 H), 9.35 (s, 1 H). MS (ESI, pos. ion) *m*/*z*: 558 (M + 1). HRMS ([M + H]$^+$) calcd: 558.27110 found: 558.27066. Anal. ($C_{31}H_{35}N_5O_5$): C, H, N.

The following compounds were synthesized according to the general procedure detailed above:

2,6-Dimethylphenyl 2-((4-(4-Methyl-1-piperazinyl)phenyl)-amino)-4-pyrimidinyl(phenyl)carbamate (1). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 2.18 (s, 6 H), 2.41 (s, 3 H), 2.72 (2, 4 H), 3.13 (s, 4 H), 6.63 (d, $J = 8.72$, 2 H), 6.89 (br s, 3 H), 7.03 (s, 3 H), 7.34 (d, $J = 8.45$, 2 H), 7.50−7.58 (m, 3 H), 7.63 (d, $J = 5.78$ Hz, 1 H), 8.51 (d, $J = 5.78$ Hz, 1 H). HPLC Purity: 97%. HRMS (M + H]$^+$) calcd: 509.26595 found: 509.26542.

2,6-Dimethylphenyl 2-(Methyloxy)phenyl(2-((4-(4-methyl-1-piperazinyl)phenyl)amino)-4-pyrimidinyl)carbamate (25). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.10 (s, 6 H), 2.23 (s, 3 H), 2.45 (t, 4 H), 2.99 (t, $J = 4.00$ Hz, 4 H), 3.78 (s, 3 H), 6.56 (d, $J = 8.59$ Hz, 2 H), 6.98 (d, $J = 8.84$ Hz, 2 H), 7.04−7.11 (m, 3 H), 7.11−7.16 (m, 1 H), 7.29 (d, $J = 8.084$ Hz, 1 H), 7.42−7.48 (m, 2 H), 7.54 (t, $J = 7.83$ Hz, 1 H), 8.35 (d, $J = 5.56$ Hz, 1 H), 9.26 (s, 1 H). HPLC Purity: 96%. HRMS ([M + H]$^+$) calcd: 539.27652 found: 539.27577.

2,6-Dimethylphenyl 4-(Methyloxy)phenyl(2-((4-(4-methyl-1-piperazinyl)phenyl)amino)-4-pyrimidinyl)carbamate (26). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.11 (s, 6 H), 2.23 (s, 3 H), 2.44−2.48 (m, 4 H), 2.95−3.02 (m, 4 H), 3.86 (s, 3 H), 6.53 (d, $J = 8.97$ Hz, 2 H), 6.99 (d, $J = 7.45$ Hz, 2 H), 7.06−7.15 (m, 5 H,) 7.40−7.47 (m, 3 H), 8.35 (d, $J = 5.68$ Hz, 1 H), 9.30 (s, 1 H). HRMS (M + H]$^+$) calcd: 539.27652 found: 539.27594. Anal. ($C_{31}H_{34}N_6O_3 \cdot 0.9\ H_2O$): C, H, N.

2,6-Dimethylphenyl 4-Fluoro-2-((1-methylethyl)oxy)phenyl-(2-((4-(4-methyl-1-piperazinyl)phenyl)amino)-4-pyrimidinyl)carbamate (27). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.80−1.31 (m, 6 H), 2.18 (s, 6 H), 2.27−2.31 (s, 3 H), 2.52 (t, $J = 5.00$ Hz, 4 H), 3.057 (t, $J = 4.80$ Hz, 4 H), 4.71 (ddd, $J = 12.19$, 6.00, 5.81 Hz, 1 H), 6.64 (d, $J = 8.84$ Hz, 2 H), 7.00 (dd, $J = 8.34$, 2.65 Hz, 1 H), 7.06 (d, $J = 8.84$ Hz, 2 H), 7.11−7.19 (m, 3 H), 7.26 (dd, $J = 11.24$, 2.65 Hz, 1 H), 7.53 (d, $J = 5.81$ Hz, 1 H), 7.58 (dd, $J = 8.34$, 6.57 Hz, 1 H), 8.41 (d, $J = 5.81$ Hz, 1 H), 9.30 (s, 1 H). HRMS (M + H]$^+$) calcd: 585.29839 found: 585.29849. Anal. ($C_{33}H_{37}FN_6O_3$): C, H, N.

2,6-Dimethylphenyl-2,4-bis(methyloxy)phenyl(2-((4-(4-methyl-1-piperazinyl)phenyl)amino)-4-pyrimidinyl)carbamate (28). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.09 (s, 6 H), 2.23 (s, 3 H), 2.43−2.48 (m, 4 H), 2.96−3.02 (m, 4 H), 3.76 (s, 3 H), 3.87 (s, 3 H), 6.55 (d, $J = 8.97$ Hz, 2 H), 6.66−6.71 (m, 1 H), 6.82 (d, $J = 2.53$ Hz, 1 H), 7.00−7.12 (m, 5 H), 7.33 (d, $J = 8.59$ Hz, 1 H), 7.46 (d, $J = 5.81$ Hz, 1 H), 8.33 (d, $J = 5.68$ Hz, 1 H), 9.28 (s, 1 H). HRMS ([M+Na]+) calcd: 591.26902 found: 591.2679. Anal. ($C_{32}H_{36}N_6O_4 \cdot 0.2\ H_2O$): C, H, N.

2,6-Dimethylphenyl (2,5-Bis(methyloxy)phenyl)methyl(2-((4-(4-methyl-1-piperazinyl)phenyl)amino)-4-pyrimidinyl)carbamate (29). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.02 (s, 6 H), 2.23 (s, 3 H), 2.43−2.48 (m, 4 H), 2.98−3.05 (m, 4 H), 3.61 (s, 3 H), 3.77 (s, 3 H), 5.36 (s, 2 H), 6.62 (d, $J = 2.91$ Hz, 1 H), 6.68 (d, $J = 8.21$ Hz, 2 H), 6.83 (dd, $J = 8.84$, 3.03 Hz, 1 H), 6.99 (d, $J = 8.84$ Hz, 1 H), 7.05−7.12 (m, 3 H), 7.30 (d, $J = 7.07$ Hz, 2 H), 7.38 (d, $J = 5.68$ Hz, 1 H), 8.36 (d, $J = 5.68$ Hz, 1 H), 9.34 (s, 1 H). HRMS (M + H]$^+$) calcd: 583.30273 found: 583.30187. Anal. Calcd for $C_{33}H_{38}N_6O_4$: C, 68.02; H, 6.57; N, 14.42. Found: C, 67.59; H, 6.77; N, 14.19.

2,6-Dimethylphenyl 2-((4-(4-Methyl-1-piperazinyl)phenyl)-amino)-4-pyrimidinyl(2-naphthalenyl)carbamate (30). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.15 (s, 6 H), 2.22 (s, 3 H), 2.36−2.45 (m, 4 H), 2.65−2.75 (m, 4 H), 5.71−5.84 (m, 2 H), 6.63−6.72 (m, 2 H), 7.02−7.12 (m, 3 H), 7.53−7.57 (m, 1 H), 7.58−7.68 (m, 3 H), 8.02−8.07 (m, 1 H), 8.08−8.17 (m, 3 H), 8.39 (d, $J = 5.68$ Hz, 1 H), 9.27 (s, 1 H). HRMS (M + H]$^+$) calcd: 559.28160 found: 559.28133. Anal. Calcd for $C_{34}H_{34}N_6O_2$: C, 73.10; H, 6.13; N, 15.04. Found: C, 72.09; H, 6.42; N, 14.21.

2,6-Dimethylphenyl-1,1′-biphenyl-4-yl(2-((4-(4-methyl-1-piperazinyl)phenyl)amino)-4-pyrimidinyl)carbamate (31). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.12−2.19 (m, 9 H), 2.21−2.27 (m, 4 H), 2.59−2.65 (m, 4 H), 6.28−6.34 (m, 2 H), 6.85−6.92 (m, 2 H), 7.05−7.14 (m, 3 H), 7.40−7.46 (m, 1 H), 7.49−7.57 (m, 3 H), 7.59−7.65 (m, 2 H), 7.85−7.91 (m, 2 H), 7.93−7.99 (m, 2 H), 8.38 (d, $J = 5.81$ Hz, 1 H), 9.33 (s, 1 H). HRMS (M + H]$^+$) calcd: 585.29725 found: 585.29747. Anal. ($C_{36}H_{36}N_6O_2$): C, H, N.

2,6-Dimethylphenyl 1,1′-Biphenyl-3-yl(2-((4-(4-methyl-1-piperazinyl)phenyl)amino)-4-pyrimidinyl)carbamate (32). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.15 (s, 6 H), 2.20 (s, 3 H), 2.35−2.41 (m, 4 H), 2.80−2.86 (m, 4 H), 6.37−6.45 (m, 2 H), 6.93−7.01 (m, 2 H), 7.04−7.13 (m, 3 H), 7.35−7.42 (m, 1 H), 7.43−7.52 (m, 4 H), 7.63−7.69 (m, 1 H), 7.75 (s, 1 H), 7.77 (s, 1 H), 7.87−7.95 (m, 2 H), 8.39 (d, $J = 5.68$ Hz, 1 H), 9.31 (s, 1 H).

HRMS (M + H]$^+$) calcd: 585.29725 found: 585.29669. Anal. ($C_{36}H_{36}N_6O_2$·0.9 H_2O): C, H, N.

2,6-Dimethylphenyl 2,4-Bis(methyloxy)phenyl(2-((3-(methyloxy)-4-(4-methyl-1-piperazinyl)phenyl)amino)-4-pyrimidinyl)carbamate (33). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.09 (s, 6 H), 2.21 (s, 3 H), 2.38−2.47 (m, 4 H), 2.80−2.91 (m, 4 H), 3.66 (s, 3 H), 3.75 (s, 3 H), 3.88 (s, 3 H), 6.31−6.39 (m, 1 H), 6.68 (dd, J = 8.72, 2.53 Hz, 1 H), 6.71−6.78 (m, 1 H), 6.79−6.85 (m, 2 H), 7.03−7.12 (m, 3 H), 7.33 (d, J = 8.59 Hz, 1 H), 7.45 (d, J = 5.68 Hz, 1 H), 8.34 (d, J = 5.81 Hz, 1 H), 9.23 (s, 1 H). HPLC purity: 100%. HRMS (M + H]$^+$) calcd: 599.29764 found: 599.29696.

2,6-Dimethylphenyl 2-((3,5-bis(methyloxy)-4-(4-methyl-1-piperazinyl)phenyl)amino)-4-pyrimidinyl(2,4-bis(methyloxy)phenyl)carbamate (34). 1H NMR (400 MHz, DMSO-d_6) δ ppm 2.08 (s, 6 H), 2.20 (s, 3 H), 2.36 (s, 4 H), 2.95 (s, 4 H), 3.62 (s, 6 H), 3.78 (s, 3 H), 3.81 (s, 3 H), 6.61 (dd, J = 8.72, 2.40 Hz, 1 H), 6.72 (d, J = 2.40 Hz, 1 H), 6.89 (s, 2 H), 7.03−7.10 (m, 3 H), 7.15 (d, J = 5.81 Hz, 1 H), 7.33 (d, J = 8.59 Hz, 1 H), 8.36 (d, J = 5.68 Hz, 1 H), 9.14 (s, 1 H). HPLC purity: 100%. HRMS (M + H]$^+$) calcd: 629.3009 found: 629.30863.

2,6-Dimethylphenyl 2,4-Bis(methyloxy)phenyl(2-((3-fluoro-4-(4-methyl-1-piperazinyl)phenyl)amino)-4-pyrimidinyl)carbamate (35). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.09 (s, 6 H), 2.22−2.29 (m, 3 H), 2.86−2.93 (m, 4 H), 3.76 (s, 3 H), 3.85 (s, 3 H), 6.59−6.69 (m, 2 H), 6.80 (d, J = 2.53 Hz, 1 H), 6.90 (d, J = 8.34 Hz, 1 H), 6.99−7.12 (m, 4 H), 7.34 (d, J = 8.59 Hz, 1 H), 7.51 (d, J = 5.81 Hz, 1 H), 8.38 (d, J = 5.68 Hz, 1 H), 9.51 (s, 1 H). HRMS (M + H]$^+$) calcd: 587.27766 found: 587.27708. Anal. ($C_{32}H_{35}FN_6O_4$): C, H, N.

2,6-Dimethylphenyl 2,4-Bis(methyloxy)phenyl(2-((4-((3-(dimethylamino)propyl)oxy)phenyl)amino)-4-pyrimidinyl)carbamate (37). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.75−1.89 (m, 2 H), 2.05−2.13 (m, 6 H), 2.17 (s, 6 H), 2.37 (t, J = 7.07 Hz, 2 H), 3.77 (s, 3 H), 3.84−3.94 (m, 5 H), 6.54 (d, J = 8.84 Hz, 2 H), 6.70 (dd, J = 8.59, 2.53 Hz, 1 H), 6.83 (d, J = 2.53 Hz, 1 H), 7.02−7.17 (m, 5 H), 7.34 (d, J = 8.59 Hz, 1 H), 7.48 (d, J = 5.81 Hz, 1 H), 8.35 (d, J = 5.81 Hz, 1 H), 9.33 (s, 1 H). HPLC Purity: 96%. HRMS (M + H]$^+$) calcd: 572.28675 found: 572.28597.

2,6-Dimethylphenyl-2,4-bis(methyloxy)phenyl(2-((3-fluoro-4-((2-(4-methyl-1-piperazinyl)ethyl)oxy)phenyl)amino)-4-pyrimidinyl)carbamate (38). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.09 (s, 6 H), 2.16 (s, 3 H), 2.24−2.39 (m, 4 H), 2.66 (t, J = 5.81 Hz, 3 H), 3.77 (s, 3 H), 3.84 (s, 3 H), 4.02 (t, J = 5.81 Hz, 2 H), 6.62−6.69 (m, 1 H), 6.72−6.81 (m, 2 H), 6.91 (d, J = 10.11 Hz, 1 H), 7.02−7.16 (m, 4 H), 7.34 (d, J = 8.59 Hz, 1 H), 7.50 (d, J = 5.68 Hz, 1 H), 8.38 (d, J = 5.68 Hz, 1 H), 9.52 (s, 1 H). HRMS (M + H]$^+$) calcd: 631.30387 found: 631.30582. Anal. ($C_{34}H_{39}FN_6O_5$·0.5 H_2O): C, H, N.

2,6-Dimethylphenyl 2-((3,5-Bis(methyloxy)-4-((2-(4-methyl-1-piperazinyl)ethyl)oxy)phenyl)amino)-4-pyrimidinyl(2,4-bis(methyloxy)phenyl)carbamate (39). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.07−2.12 (m, 6 H), 2.15 (s, 3 H), 2.19−2.42 (m, 8 H), 2.57 (t, J = 5.94 Hz, 2 H), 3.64 (s, 6 H), 3.79 (s, 3 H), 3.82 (s, 3 H), 3.85 (t, J = 6.06 Hz, 2 H), 6.62 (dd, J = 8.59, 2.53 Hz, 1 H), 6.73 (d, J = 2.53 Hz, 1 H), 6.94 (s, 2 H), 7.03−7.12 (m, 3 H), 7.14 (d, J = 5.81 Hz, 1 H), 7.34 (d, J = 8.59 Hz, 1 H), 8.37 (d, J = 5.56 Hz, 1 H), 9.15 (s, 1 H). HRMS (M + H]$^+$) calcd: 673.33442 found: 673.33418. Anal. ($C_{36}H_{44}N_6O_7$·0.7 H_2O): C, H, N.

2,6-Dimethylphenyl 2-((3,5-Bis(methyloxy)-4-((2-(4-morpholinyl)ethyl)oxy)phenyl)amino)-4-pyrimidinyl(2,4-bis(methyloxy)-phenyl)carbamate (40). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.08 (s, 6 H), 2.40−2.48 (m, 4 H), 2.54−2.63 (m, 2 H), 3.51−3.60 (m, 4 H), 3.63 (s, 6 H), 3.78 (s, 3 H), 3.81 (s, 3 H), 3.83−3.90 (m, 2 H), 6.57−6.65 (m, 1 H), 6.72 (d, J = 2.53 Hz, 1 H), 6.94 (s, 2 H), 7.02−7.16 (m, 4 H), 7.34 (d, J = 8.59 Hz, 1 H), 8.36 (d, J = 5.68 Hz, 1 H), 9.16 (s, 1 H). HRMS (M + H]$^+$) calcd: 660.30279 found: 660.30315. Anal. ($C_{35}H_{41}N_5O_8$·0.7 H_2O): C, H, N.

2,6-Dimethylphenyl-2,4-bis(methyloxy)phenyl(2-((4-((3-(1-piperidinyl)propyl)oxy)phenyl)amino)-4-pyrimidinyl)carbamate (41). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.34−1.42 (m, 2 H), 1.46−1.54 (m, 4 H), 1.78−1.87 (m, 2 H), 2.09 (s, 6 H), 2.28−2.41 (m, 6 H), 3.75 (s, 3 H), 3.85−3.91 (m, 5 H), 6.51 (s, 1 H), 6.54 (s, 1 H), 6.69 (dd, J = 8.59, 2.53 Hz, 1 H), 6.82 (d, J = 2.53 Hz, 1 H), 7.04−7.12 (m, 5 H), 7.34 (d, J = 8.59 Hz, 1 H), 7.47 (d, J = 5.81 Hz, 1 H), 8.34 (d, J = 5.81 Hz, 1 H), 9.34 (s, 1 H). HRMS (M + H]$^+$) calcd: 612.31805 found: 612.31809. Anal. ($C_{35}H_{41}N_5O_5$): C, H, N.

2,6-Dimethylphenyl 2,4-Bis(methyloxy)phenyl(2-((3-(methyloxy)-4-((3-(4-methyl-1-piperazinyl)propyl)oxy)phenyl)amino)-4-pyrimidinyl)carbamate (42). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.78−1.88 (m, 2 H), 2.093 (s, 6 H), 2.24 (s, 3 H), 2.36−2.47 (m, 6 H), 2.50 (m, 4 H), 3.65 (s, 3 H), 3.76 (s, 3 H), 3.83−3.91 (m, 5 H), 6.40 (s, 1 H), 6.68 (dd, J = 8.59, 2.53 Hz, 1 H), 6.76 (m, 1 H), 6.80 (d, J = 2.53 Hz, 1 H), 6.84−6.92 (m, 1 H), 7.01−7.14 (m, 3 H), 7.34 (d, J = 8.59 Hz, 1 H), 7.43 (d, J = 5.81 Hz, 1 H), 8.35 (d, J = 5.81 Hz, 1 H), 9.22 (s, 1 H). HPLC Purity: 100%. HRMS (M + H]$^+$) calcd: 657.33951 found: 657.33974.

2,6-Dimethylphenyl 2,4-Bis(methyloxy)phenyl(2-((3-fluoro-4-((3-(4-methyl-1-piperazinyl)propyl)oxy)phenyl)amino)-4-pyrimidinyl)carbamate (44). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.79−1.88 (m, 2 H), 2.09 (s, 6 H), 2.15 (s, 3 H), 2.23−2.44 (m, 10 H), 3.76 (s, 3 H), 3.85 (s, 3 H), 3.92−3.98 (m, 2 H), 6.63−6.69 (m, 1 H), 6.70−6.77 (m, 1 H), 6.79 (d, J = 2.53 Hz, 1 H), 6.87−6.93 (m, 1 H), 7.04−7.14 (m, 4 H), 7.34 (d, J = 8.46 Hz, 1 H), 7.51 (d, J = 5.81 Hz, 1 H), 8.38 (d, J = 5.81 Hz, 1 H), 9.51 (s, 1 H). HRMS (M + H]$^+$) calcd: 645.31952 found: 645.31855. Anal. ($C_{35}H_{41}FN_6O_5$·0.5 H_2O): C, H, N.

2,6-Dimethylphenyl 2,4-Bis(methyloxy)phenyl(2-((3-fluoro-4-((3-(1-piperidinyl)propyl)oxy)phenyl)amino)-4-pyrimidinyl)carbamate (45). 1H NMR (400 MHz, DMSO-d_6) δ ppm 1.34−1.42 (m, 2 H), 1.46−1.53 (m, 4 H), 1.79−1.87 (m, 2 H), 2.09 (s, 6 H), 2.28−2.40 (m, 6 H), 3.76 (s, 3 H), 3.85 (s, 3 H), 3.91−3.99 (m, 2 H), 6.66 (dd, J = 8.72, 2.53 Hz, 1 H), 6.73 (t, J = 9.35 Hz, 1 H), 6.79 (d, J = 2.53 Hz, 1 H), 6.87−6.94 (m, 1 H), 7.02−7.14 (m, 4 H), 7.34 (d, J = 8.59 Hz, 1 H), 7.51 (d, J = 5.81 Hz, 1 H), 8.38 (d, J = 5.68 Hz, 1 H), 9.51 (s, 1 H). HRMS (M + H]$^+$) calcd: 630.30862 found: 630.30831. Anal. ($C_{35}H_{40}FN_5O_5$·0.25 H_2O): C, H, N.

2,6-Dimethylphenyl 2-((3,5-Bis(methyloxy)-4-((3-(4-methyl-1-piperazinyl)propyl)-oxy)phenyl)amino)-4-pyrimidinyl(2,4-bis(methyloxy)phenyl)carbamate (43). A resealable tube was charged with 2,6-dimethylphenyl 2-chloropyrimidin-4-yl(2,4-dimethoxyphenyl)carbamate (**22a**) (0.27 g, 0.55 mmol), 3,5-dimethoxy-4-(3-(4-methylpiperazin-1-yl)propoxy)aniline (**11g**) (0.19 g, 0.65 mmol), trifluoroacetic acid (0.29 g, 0.20 mL, 1.2 mmol), and 2-propanol (3.0 mL). The system was flushed with argon, and the tube was sealed. The mixture was heated at 100 °C for 18 h. The reaction mixture was cooled to room temperature and concentrated to afford a brown oil. The oil was diluted with ethyl acetate and partitioned between ethyl acetate and saturated aqueous potassium carbonate. The organic layer was separated, washed with brine, dried over anhydrous sodium sulfate, and concentrated to afford 2,6-dimethylphenyl 2-((3,5-bis(methyloxy)-4-((3-(4-methyl-1-piperazinyl)-propyl)oxy)phenyl)amino)-4-pyrimidinyl(2,4-bis(methyloxy)phenyl)-carbamate (**43**) (0.189 g, 42%) as an off-white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.67−1.76 (m, 2 H), 2.10 (s, 6 H), 2.15 (s, 3 H), 2.24−2.38 (m, 6 H), 2.38−2.45 (m, 3 H), 3.64 (s, 6 H), 3.74−3.88 (m, 8 H), 6.62 (dd, J = 8.65, 2.59 Hz, 1 H), 6.73 (d, J = 2.53 Hz, 1 H), 6.94 (s, 2 H), 7.02−7.17 (m, 5 H), 7.34 (d, J = 8.59 Hz, 1 H), 8.37 (d, J = 5.68 Hz, 1 H), 9.14 (s, 1 H). MS (ESI, pos. ion) m/z: 687 (M + 1). HRMS (M + H]$^+$) calcd: 687.35007 found: 687.34959. Anal. ($C_{37}H_{46}N_6O_7$·0.3 H_2O): C, H, N.

Lck Kinase Assay. The Lck HTRF kinase assay involved ATP-dependent phosphorylation of a biotinylated substrate peptide of gastrin in the presence or absence of inhibitor compound. The final concentration of gastrin was 1.2 μM. The final concentration of ATP was 0.5 μM (K_m app = 0.6 ± 0.1 μM), and the final concentration of Lck (a GST-kinase domain fusion (AA 225−509)) was 250 pM. Buffer conditions were as follows: 50 mM HEPES pH = 7.5, 50 mM NaCl, 20 mM MgCl, 5 mM MnCl, 2 mM DTT, 0.05% BSA. The assay was quenched and stopped with 160 μL of

detection reagent. Detection reagents were as follows: Buffer made of 50 mM Tris, pH = 7.5, 100 mM NaCl, 3 mM EDTA, 0.05% BSA, 0.1% Tween20. Prior to reading, Streptavidin allophycocyanin (SA-APC) was added at a final concentration in the assay of 0.0004 mg/mL, along with europilated anti-phosphotyrosine Ab (Eu-anti-PY) at a final conc of 0.025 nM. The assay plate was read in a Discovery fluorescence plate reader with excitation at 320 nm and emission at 615 and 655 nm.

Assays for other kinases were done in a similar way as described above, varying the concentrations of enzyme, peptide substrate, and ATP added to the reaction, depending on the specific activity of the kinase and measured K_ms for the substrates.

Human Mixed Lymphocyte Reaction (huMLR). The purpose of this assay was to test the potency of T cell activation inhibitors in an in vitro model of allogeneic T cell stimulation. Human peripheral blood lymphocytes (hPBL; 2×10^5/well) were incubated with mitomycin C-treated B lymphoblastoid cells (JY cell line (ATCC, Rockville, MD); 1×10^5/well) as allogeneic stimulators in the presence or absence of dilutions of potential inhibitor compound in 96-well round-bottom tissue culture plates. These cultures were incubated at 37 °C in 5% CO_2 for 6 days total. The proliferative response of the hPBL was measured by ^{3}H-thymidine incorporation overnight between days 5 and 6 after initiation of culture. Cells were harvested onto glass fiber filters and ^{3}H-thymidine incorporation into DNA was analyzed by liquid scintillation counter.

Jurkat Proliferation/Survival Assay. The purpose of this assay was to test the general antiproliferative/cytotoxic effect of compounds on the Jurkat human T cell line (ATCC, Rockville, MD). Jurkat cells (1×10^5/well) were plated in 96-well flat-bottom tissue culture plates with or without compound dilutions and cultured for 72 h at 37 °C in 5% CO_2. Viable cell number was determined during the last 4 h of culture by adding 10 μL/well WST-8 dye (Alexis; San Diego, CA). WST-8 dye conversion relied on active mitochondrial electron transport for reduction of the tetrazolium dye. The dye conversion was read by OD at 450−600 nM.

Anti-CD3/CD28-Induced T Cell IL-2 Secretion and Proliferation Assay. The purpose of this assay was to test the potency of T cell receptor (TCR; CD3) and CD28 signaling pathway inhibitors in human T cells. T cells were purified from human peripheral blood lymphocytes (hPBL) and preincubated with or without compound prior to stimulation with a combination of an anti-CD3 and an anti-CD28 antibody in 96-well tissue culture plates (1×10^5 T cells/well). Cells were cultured for ~20 h at 37 °C in 5% CO_2 and then secreted IL-2 in the supernatants was quantified by cytokine ELISA (Pierce/Endogen, St. Louis, MO). The cells remaining in the wells were then pulsed with ^{3}H-thymidine overnight to assess the T cell proliferative response. Cells were harvested onto glass fiber filters and ^{3}H-thymidine incorporation into DNA was analyzed by liquid scintillation counter. For comparison purposes, phorbol myristic acid (PMA) and calcium ionophore were used in combination to induce IL-2 secretion from purified T cells. Potential inhibitor compounds were tested for inhibition of this response as described above for anti-CD3 and -CD28 antibodies. Human whole-blood anti-CD3/CD28-induced IL-2 secretion assays were run in a similar fashion as described above using whole blood from normal volunteers diluted 50% in tissue culture medium prior to stimulation.

TCR ζ-Chain Phosphorylation Assay. Jurkat cells (1.25×10^6/well) were seeded in a 96-well plate (round-bottom) in 150 μL of RPMI + 10% FCS and then preincubated with 50 μL compound or without compound (50 μL medium) at 37 °C for 30 min. For CD3 stimulation, cells were cooled on ice for 15 min and then incubated with 10 μL of anti-CD3 mAb (UCHT-1) at a final concentration of 1.2 μg/mL for 30 min. 20 μL of GAM-IgG$_1$ was added to cells at 30 μg/mL final and incubated for 20 min on ice. Cells were transferred to a 37 °C water bath and stimulated for 1 min. Cells were spun at speed 1000 rpm in the centrifuge for 5 min. Supernatant was aspirated, and cells were lysed in 30 μL of cold lysis buffer (1% Triton X-100 in NaCl/HEPES) with phosphatase/protease inhibitors for 30 min on ice. Lysates were spun at 3000 rpm in the centrifuge for 5 min to remove nuclei before 25 μL of supernatant was transferred to new tubes for the Igen ζ-chain phosphorylation detection assay.

A master mix was made containing (vol. per sample tube): 21 μL of Igen dilution buffer (PBS, 1% BSA, 0.1% tween-20; Igen/BioVeris; Gaithersburg, MD), 4 μL of the capture antibody, anti-human CD247 mAb, clone G3 (BD Coulter, San Diego, CA) coupled with Dynabeads M-280, 200 μL of the detection antibody, ruthenylated p-CD3-ζ (415.9A; Santa Cruz Biotech., Santa Cruz, CA) at 1 to 16000 dilution in Igen dilution buffer. 25 μL per sample lysate was transferred from the lysate plate to a tube including the negative and positive controls. Sample tubes were shaken at room temperature overnight. Tubes then were placed in the carousel of Igen tube analyzer and run on the analyzer. Signal-to-noise ratio was generally around 15−20.

The procedure of coupling the capture antibody, anti-human CD247 mAb, clone G3 to Dynabeads M-280 was based on the protocol provided by Dynal Biotech when the beads were purchased. The procedure of p-CD3-ζ ruthenylation is based on the protocol provided by Bio Veris when BV-TAG NHS-Ester was purchased.

Pharmacokinetic Studies. Sprague−Dawley rats were administered a solution of compound in DMSO at the indicated doses i.v. For oral dosing, a suspension in 2% hydroxypropyl methylcellulose with 1% Tween-80 was administered. Samples were taken at the indicated times and analyzed for parent compound by LC-MS method.

Anti-CD3-Induced IL-2 Production in Mice. 12 week old (20 g) BALB/c mice were dosed 1 h prior to challenge with compound p.o. (8 per group) at the indicated doses in 2% hydroxypropyl methylcellulose with 1% Tween-80. Mice were then challenged i.v. with anti-mouse CD3ϵ monoclonal antibody (145.2C11, BD PharMingen, San Diego, CA; 3 μg/mouse) diluted in PBS. Ninety minutes after anti-CD3 challenge, blood was collected via cardiac puncture. IL-2 levels were measured in serum by ELISA (BioSource, Camarillo, CA). Data points represent the mean IL-2 levels/group ± the standard error. *P* values were determined vs vehicle control by Mann−Whitney U-test.

Supporting Information Available: Analysis data for the compounds synthesized and data collection and refinement statistics for the X-ray structure of compound **43** bound to Lck. This material is available free of charge via the Internet at http://pubs.acs.org.

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