Optimization of 2-Phenylaminoimidazo[4,5-*h*]isoquinolin-9-ones: Orally Active Inhibitors of lck Kinase

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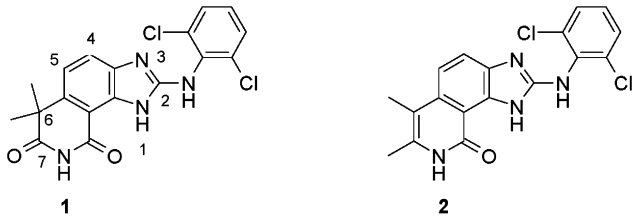
The tyrosine kinase p56lck (lck) is essential for T cell activation; thus, inhibitors of lck have potential utility as autoimmune agents. Our initial disclosure of a new class of lck inhibitors based on the phenylaminoimidazoisoquinolin-9-one showed reasonable cellular activity but did not work in vivo upon oral administration. Our current work highlights the further use of rational drug design and molecular modeling to produce a series of lck inhibitors that demonstrate cellular activity below 100 nM and are as efficacious as cyclosporin A in an in vivo mouse model of anti-CD3-induced IL-2 production.

Tyrosine kinases play an essential role in the regulation of cell signaling and cell proliferation by phosphorylating tyrosine residues of peptides and proteins.[1−3] We have been exploring a novel series of molecules that inhibit the catalytic activity of p56lck (lck), a member of the src family of protein tyrosine kinases (vide infra).[4]

The lck (−) Jurkat cell lines are unable to proliferate, produce cytokines, and generate increases in intracellular calcium, inositol phosphate, and tyrosine phosphorylation in response to T cell receptor (TCR) stimulation.[5,6] Therefore, an agent inhibiting lck would effectively block T cell function, act as an immunosuppressive agent, and have potential utility in autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, and lupus, as well as in the area of transplant rejection and allergic diseases.[7,8]

The TCR is the antigen-specific component of the T cell, which is activated upon presentation of antigenic peptides.[9] TCR activation initiates a series of enzyme-mediated signal transduction cascades that result in the production of proinflammatory cytokines such as interleukin-2 (IL-2).[10−12]

Our initial disclosure of phenylaminoimidazoisoquinolinones as a new class of lck inhibitors highlighted the use of a model of inhibitors bound to lck to advance our screening hit **1** with micromolar potency against lck, to a nanomolar inhibitor of lck, **2** (Figure 1).[4] Although quite potent in vitro, **2** only demonstrated in vivo efficacy when dosed at 100 mg/kg, ip. Our current work highlights modifications to **2** that improve in vitro potency to subnanomolar levels and also provide oral efficacy in a mouse model of IL-2 production.



1 **2**

Figure 1. Initial lead **1** and optimized compound **2**.

Chemistry

Our previously reported synthetic route to the phenylaminoimidazoisoquinolinone inhibitor **2** involved a 12-step linear sequence.[4] We therefore looked for opportunities to shorten our synthesis and allow for flexibility to introduce a variety of functionality. We could accomplish our first goal through the use of a selective S_NAr reaction starting with 2,6-dichloro-3-nitrobenzonitrile **3** as the key building block (Scheme 1).[13] Treatment of **3** with ammonia or an alkylamine provided benzonitrile **4** or **5**, respectively. This ultimately allowed access to products alkylated at the N-1 position, e.g., methylamine **12**, which were not accessible via our previous synthetic route. A subsequent S_NAr reaction with ethyl 2-methylacetoacetate to form **6** allowed ready introduction of the atoms necessary for formation of the isoquinoline core.[14] Formation of the benzimidazole **11** was best accomplished by reacting diamine **7**, 2,6-dichlorophenyl isocyanate, and HgO in refluxing THF. The isoquinolone was then formed by treatment of **11** with a mixture of concentrated sulfuric acid, water, and acetic acid to provide **2** or **12**. The last step in the reaction included the hydrolysis of the nitrile, condensation with the ketone, and hydrolysis and decarboxylation of the ester functionality in one step.

Reacting **5** with methyl acetoacetate to provide intermediate **13** accomplished introduction of a methyl ester directly attached to C-6 (Scheme 2). Isoquinolone formation as for **12** and **2** provided a mixture of C-6

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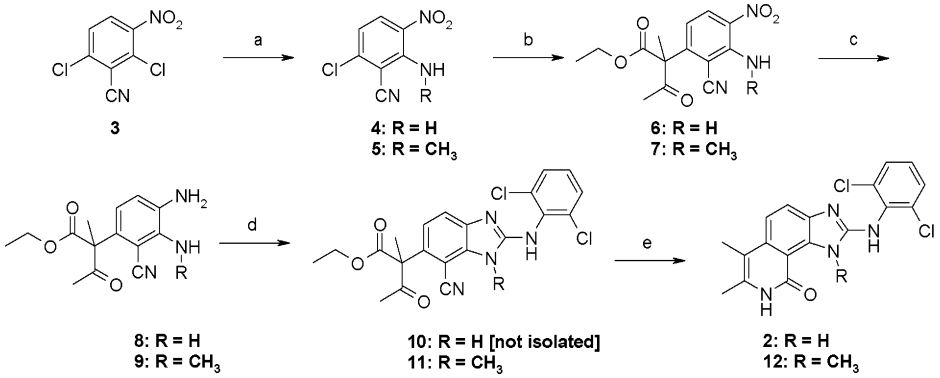
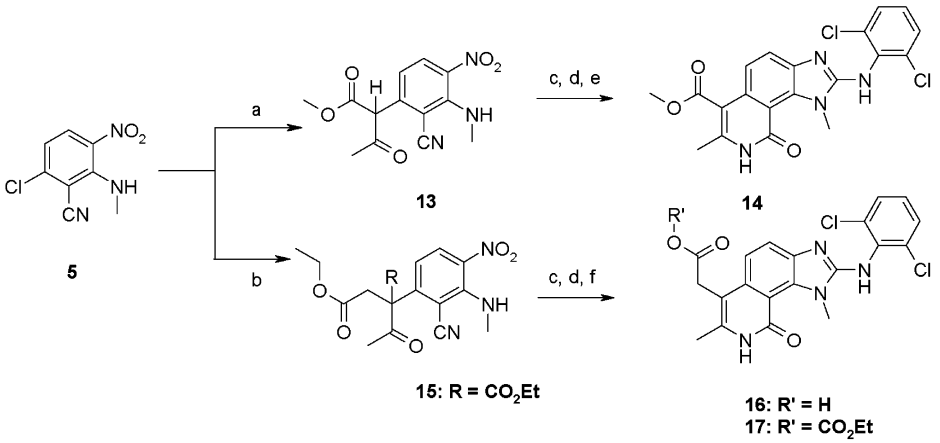
Scheme 1. Improved Synthesis of **2**[a]



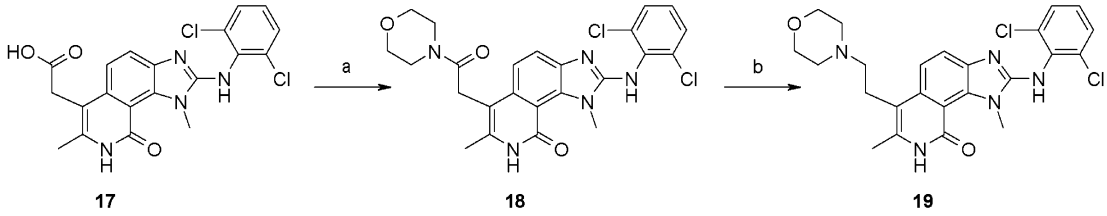
[a] Reagents: (a) H_2NR, MeOH, 80 °C; (b) ethyl 2-methylacetoacetate, K_2CO_3, DMF; (c) H_2, 10% Pd/C, MeOH; (d) 2,6-$Cl_2C_6H_3NCS$, EtOAc, or 2,6-$Cl_2C_6H_3NCS$, HgO, THF, 80 °C; (e) concentrated H_2SO_4, H_2O, HOAc, 100 °C.

Scheme 2. β-Keto Ester Additions[a]



[a] Reagents: (a) 3-oxobutyric acid methyl ester, K_2CO_3, DMF; (b) 2-acetylmalonic acid diethyl ester, $CsCO_3$, DMF; (c) H_2, 10% Pd/C, MeOH; (d) 2,6-$Cl_2C_6H_3NCS$, EtOAc; (e) concentrated H_2SO_4; (f) (1) concentrated H_2SO_4 H_2O, HOAc, 100 °C, (2) EtOH, H_2SO_4.

Scheme 3. Amide and Amine Synthesis at C-6[a]



[a] Reagents: (a) morpholine, TBTU, DMF; (b) BH_3, SMe_2, THF.

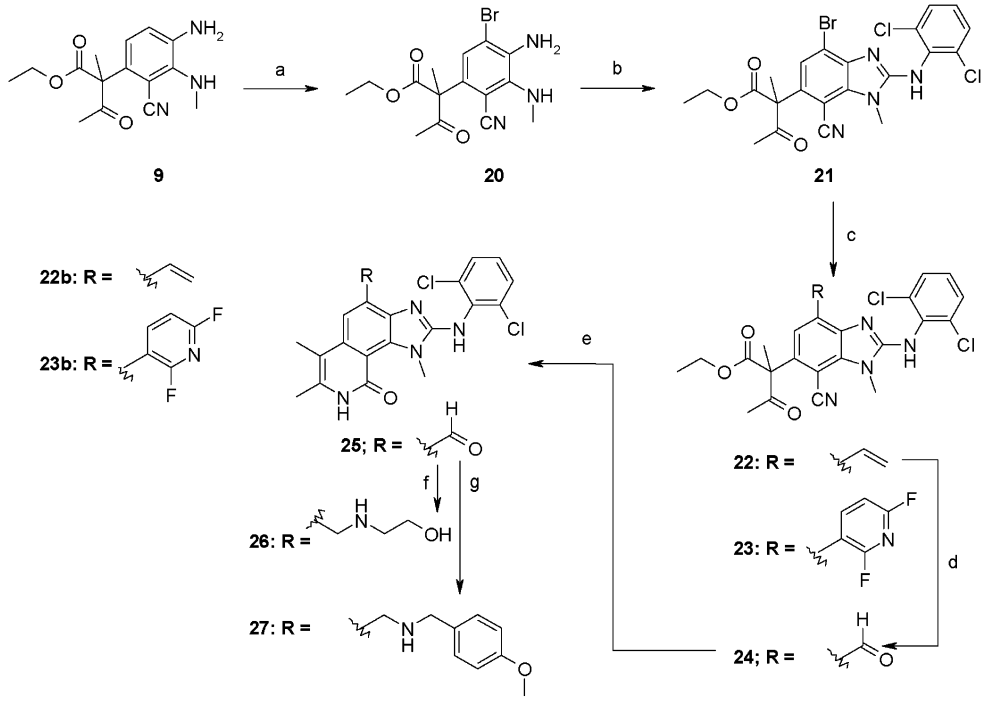
ester and decarboxylated material that could not be separated easily. However, performing the reaction at room temperature with concentrated sulfuric acid solved this issue and provided **14** in good yield. The homologous ester, **15**, was prepared in a similar manner with diethylacetyl succinate. Isoquinolone formation under the standard acidic conditions resulted in ester hydrolysis of the final product to provide **16**. Consequently, reesterification with ethanol and sulfuric acid was required to obtain **17**.

Ester **14** proved to be resistant to further manipulation perhaps because of steric congestion around the ester carbonyl caused by the C-7 methyl group and the isoquinoline phenyl. The homologated analogue **16** did not suffer from this liability and could be transformed, via hydrolysis to the acid, into amide derivatives, e.g., **18** (Scheme 3). The resulting amides could be reduced to the corresponding amines, e.g., **19**, with BH_3–dimethyl sulfide in THF, without affecting the isoquinolone amide.

Functionalization of the C-4 position of the isoquinolone was accomplished via a bromine atom as a functional handle. Of all the potential precursors for introduction of the bromine atom at C-4, diamine **9** was the only intermediate that was readily brominated in the desired manner (Scheme 4). The sequence progressed as described above to provide benzimidazole **21**. The bromine in compound **21** was further manipulated before isoquinolone formation via palladium cross-coupling with vinyl- or aryltin reagents to provide **22** and **23**. Conversion to the corresponding isoquinolone derivative occurred as described above. Vinyl compound **22** could be converted to aldehyde **25** that could undergo reductive aminations, e.g., **26** and **27** (Scheme 4).

Attempts to introduce a functional handle at the C-7 position through the use of substituted β-keto esters had

Scheme 4. Introduction of C-4 Bromine and Cross-Coupling Reactions[a]



[a] Reagents: (a) Br_2, $CHCl_3$; (b) ArNCS, HgO, THF, 80 °C; (c) $RSnBu_3$, $(PPh_3)_2PdCl_2$; (d) $NaIO_4$, OsO_4, THF; (e) H_2SO_4,100 °C; (f) $NaBH_4$, MeOH; (g) *p*-methoxybenzylamine, $NaCNBH_3$, MeOH.

limited success either because of poor yields of the S_NAr reaction or difficulties in obtaining appropriately substituted keto esters. Literature precedent demonstrated that 3-methyl-1*H*-quinolin-2-one undergoes oxidation with selenium dioxide to the corresponding aldehyde.[15] Reaction of **12** with SeO_2 in dioxane resulted in selective oxidation at the C-7 methyl to provide the corresponding aldehyde **28** exclusively with no sign of oxidation at the C6 methyl group (Scheme 5). The 2D NMR experiments with NOESY (nuclear Overhauser effect spectroscopy) confirmed the site of oxidation. The predicted greater acidity of the C-7 methyl hydrogens due to conjugation with the isoquinolone carbonyl could explain the observed selectivity.

Aldehyde **28** constitutes an intermediate for a wide range of functionality at C-7 (Scheme 5). **28** can be reduced directly to alcohol **29**, converted to an amine through reductive amination, e.g. **30**, or converted to secondary alcohol **31** by addition of vinylmagnesium chloride. Although Wittig olefination procedures with unstabilized ylides were not successful, Horner−Wadsworth−Emmons olefination with trimethyl phosphonoacetate and lithium hydroxide provided α,β-unsaturated ester **36**.[16] Alternatively, **28** could be transformed to the simple vinyl analogue **32** via a Peterson olefination.[17] α,β-unsaturated ester **36** underwent 1,4-reduction to the corresponding alkyl ester **37** upon hydrogenation over PtO_2 in methanol. Reduction of **36** to the corresponding allylic alcohol **38** was more troublesome than initially expected. Hydride reducing agents such as $LiAlH_4$ had no effect on the ester, and returned only un-reacted starting material. We found that if the amide NH of the isoquinolone was deprotonated initially with sodium bis(trimethylsilyl)amide, followed by addition of $LiAlH_4$, the desired alcohol could be obtained successfully.

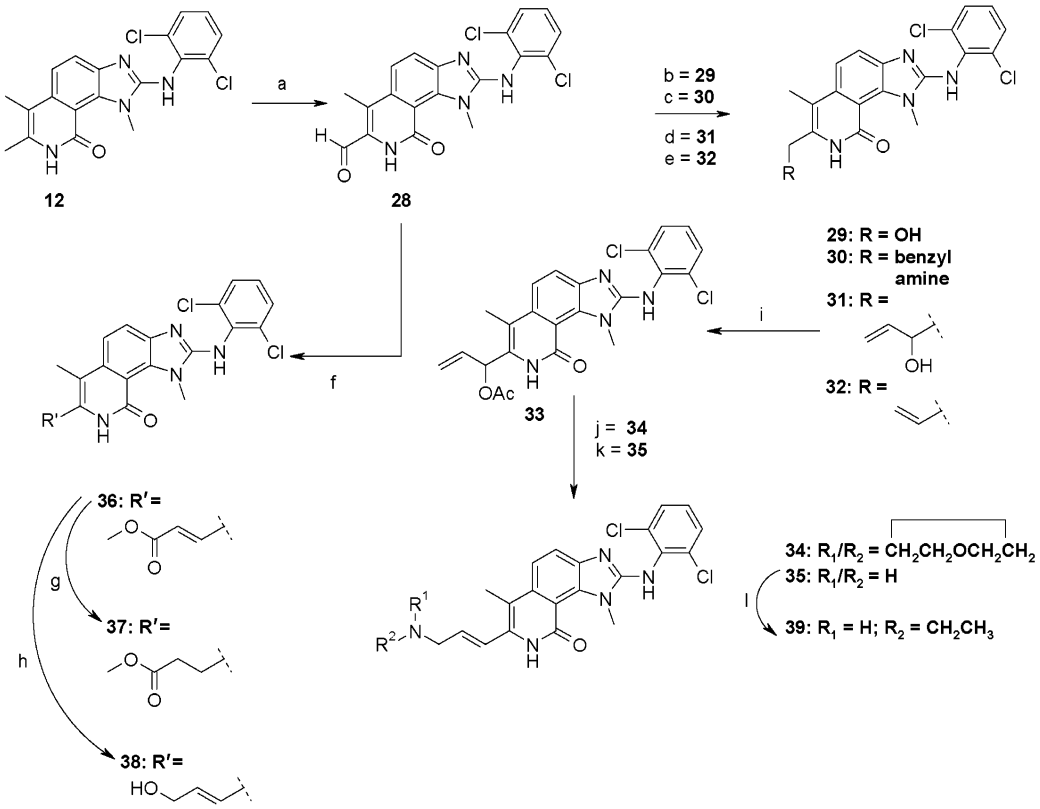
Secondary alcohol **31** provides another functional handle when transformed into allylic acetate **33**. Palladium-catalyzed allylic transposition of **33** worked well with secondary amines to provide compounds such as **34**.[18] However, primary amines failed to react in the allylic transposition reaction. To obtain secondary amines, **33** was treated with NaN_3 as a nucleophile followed by a Staudinger reaction to provide **35**. Reductive amination with acetaldehyde provided amine **39**.

Results and Discussion

The two issues we felt were most important in obtaining inhibitors with oral efficacy in our mouse model of IL-2 production were to improve in vitro potency and to improve physicochemical properties, most notably aqueous solubility. One of the benefits of our improved synthetic route was the ready access it allowed for providing N-1 alkylated products. We found that the corresponding N-1 methyl analogue **12** shows a 5-fold increase in potency over **2** (Table 1). The methyl appears to be optimal because the corresponding ethyl analogue **40** is over 100-fold less potent.

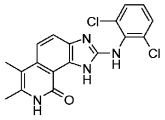
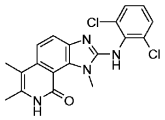
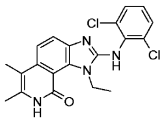
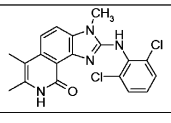
This important result also helped to further validate our previously developed binding model, which predicted the 2,6-dichlorophenyl group to be pointing toward the N-3 nitrogen of the benzimidazole (conformer **A**, Figure 2).[19] Because the introduction of a methyl group on either nitrogen would be expected to strongly favor a conformation in which the dichlorophenyl group is oriented away from the methyl, the favorable potency of **12** strongly supports conformer **A** (Figure 2) as representing the bioactive conformation of the 2,6-dichlorophenyl group. Previous SAR studies showed that the N-3 methylated compound **41** is over 20-fold less potent than compound **2**.[4] However, the improved in vitro potency is likely due to a

Scheme 5. C-7 Oxidation and Functionalization[a]



[a] Reagents: (a) SeO_2, dioxane, 100 °C; (b) $LiAlH_4$, THF; (c) (1) benzylamine, THF, (2) $NaBH_4$, MeOH; (d) vinylmagnesium bromide, THF, 0 °C to room temp; (e) (1) $TMSCH_2MgCl$, THF, −78 °C to room temp, (2) $BF_3(OEt)_2$, CH_2Cl_2, 0 °C to room temp; (f) $(MeO_2)P(O)CH_2CO_2Me$, LiOH, THF, H_2O; (g) H_2, PtO_2, HOAc, EtOAH; (h) (1) $NaN[Si(CH_3)_3]_2$, (2) $LiALH_4$, THF, 0 °C to room temp; (i) Ac_2O, Et_3N; (j) morpholine, $Pd_2(dba)_3$, PPh_3, THF; (k) NaN_3, $Pd_2(dba)_3$, PPh_3, THF, (2) PPh_3, THF; (l) acetaldehyde, $Na(OAc)_3BH$, MeOH.

Table 1. Methylation Studies on Benzimidazole Core[c]

Compound Code	Structure	Enzyme IC_{50} (μM)[a]	Calcium assay EC_{50} (μM)[b]
2		0.11	0.18±0.02
12		0.023	0.13±0.02
40		> 2.5	NT
41		> 2.0	NT

[a] IC_{50} values for inhibition of lck. For active compounds, they are the mean values of two or more separate determinations, in duplicate. [b] EC_{50} values for inhibition of Ca release in Jurkat cells. Values are the mean of two or more separate experiments in duplicate ± SD. [c] NT = not tested.

combination of locking the dichlorophenyl group into the bioactive conformation, increasing hydrophobic interactions with lck, and reducing desolvation factors.

On the basis of our model highlighted in Figure 3, we had little expectation of improving potency through

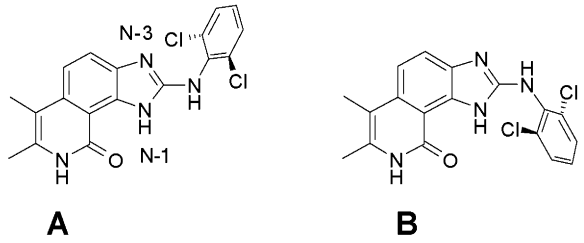


Figure 2. Bioactive conformer of **2**.

further modification of the *N*-phenyl ring. SAR at this position tracked with our previous reported work with no noticeable improvement in potency. Thus, we concentrated on functionalizing the isoquinoline core. We had previously shown the requirement for 2,6-disubstitution on the phenyl ring with either chlorine atoms or methyl groups.

The spine of the isoquinolone core (Figure 3, C-4 through C-7) points out toward the cleft between the N and C lobes of lck. We therefore reasoned that substitution along this side of the molecule would be tolerated and could provide an opportunity to improve the potency and physicochemical properties of this inhibitor class.

We were able to introduce a wide range of functionality at both C-4 and C-6 that improved solubility; however, most modifications provided less potent compounds compared to **12** (Tables 2 and 3).

Although we were successful in introducing amine and alcohol functionality at the C-7 position, the initial SAR was also discouraging (Table 4). However, on

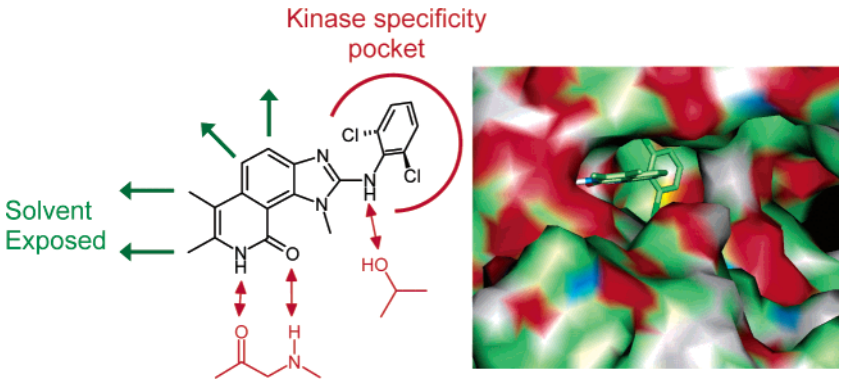
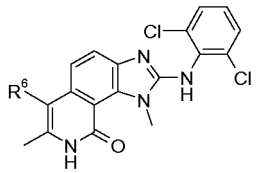
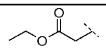
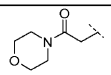
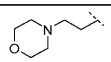
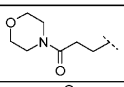
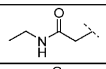
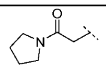
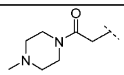
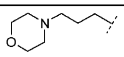


Figure 3. Opportunities suggested by the binding model of **12**.

Table 2. Effects of Substitution at C-6

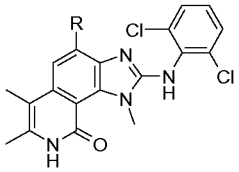


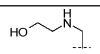
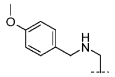
Compound Code	R^6	Enzyme IC_{50} (μM)[a]	Calcium assay EC_{50} (μM)[b]
12		0. 023	0.13±0.02
14		0.087	0.09±0.01
16		0.70	0.14±0.04
18		0.12	0.66±0.01
19		0.03	0.27±0.05
49		0.12	0.27±0.03
50		0.46	0.60±0.10
51		0.74	0.27±0.10
52		0.44	1.2±0.21
53		0.11	0.30±0.04
54		0.027	0.09±0.01

[a] IC_{50} values for inhibition of lck. For active compounds, they are the mean values of two or more separate determinations, in duplicate. [b] EC_{50} values for inhibition of Ca release in Jurkat cells. Values are the mean of two or more separate experiments in duplicate ± SD.

conversion of **28** to the α,*β*-unsaturated ester **36**, we observed our first signs, albeit modest, of improved cellular potency. This prompted a deeper investigation of the newly introduced ester and olefin functionality. While the saturated ester **37** showed a 16-fold loss in molecular potency and nearly a 2-fold loss in cellular potency compared to **12**, remarkably, the simple vinyl group **32** maintained molecular potency and improved cell potency nearly 10-fold when compared to **12**. The relatively poor potency of the ethyl analogue **42** further demonstrated the importance of an olefin substituent at C-7.

Table 3. Effects of Substitution at the C-4 Position



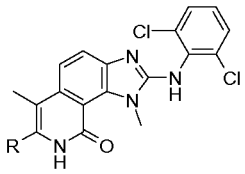
Compound Code	R	Enzyme IC_{50} (μM)[a]	Calcium assay EC_{50} (μM)[b]
12		0. 023	0.13±0.02
23		0.05	0.37±0.01
26		0.026	0.12±0.01
27		0.18	0.50±0.01

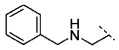
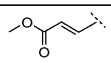
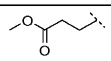
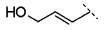
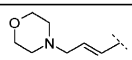
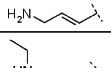
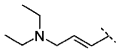
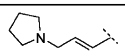
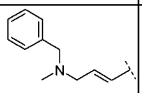
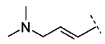
[a] IC_{50} values for inhibition of lck. For active compounds, they are the mean values of two or more separate determinations, in duplicate. [b] EC_{50} values for inhibition of Ca release in Jurkat cells. Values are the mean of two or more separate experiments in duplicate ± SD.

The loss in activity on going from an sp^2 to an sp^3 group at C-7 can be rationalized on the basis of our lck inhibitor binding model. As shown in Figure 4, the protein is very close above and below the plane of the molecule at the C-7 position. Therefore, planar substituents at the C-7 position fit well in this narrow cleft. Compounds that contain functionality attached to an sp^3 atom adopt a conformation that would position the attached group out of the plane of the molecule toward the protein, thereby lowering binding affinity. These results parallel our initial discovery of **2** from **1** in which removing the *gem*-dimethyl groups at C-6 and creating a planar system resulted in a significant boost in potency.[4]

By maintainance of the olefin substituent and introduction of polar functionality, further enhancements in cellular and molecular potency were achieved. Furthermore, compounds of this nature also resulted in an increase in aqueous solubility. Allylic alcohol **38** showed a small improvement in molecular potency but a significant boost in cellular potency. Furthermore, introduction of an allylic amine substituent provided an enhancement in both molecular and cellular potency. Although the polar allylic group does not appear to

Table 4. Effect of C-7 sp^2 Substitution[d]



Compound Code	R	Enzyme IC_{50} (μM)[a]	Calcium assay EC_{50} (μM)[b]	EC_{50} SEB IL-2 Whole Blood (μM)[c]	EC_{50} Human Whole Blood IL-2(μM)[c]
12		0. 023	0.13±0.01	NT	1.1±0.8
28		0.17	0.45±0.05	NT	NT
29		0.77	0.24±0.02	NT	NT
30		2.0	4.2±0.01	NT	NT
31		0.570	1.3±0.10	NT	NT
32		0.045	0.02±0.04	0.90±0.8	12.0±0.04
36		0.086	0.07±0.01	NT	NT
37		0.36	0.23±0.04	NT	NT
42		0.78	0.08±0.01	NT	NT
38		0.009	0.008±0.01	0.22±0.1	3.0±0.05
34		0.004	0.030±0.01	0.170±0.1	NT
35		0.002	0.021±0.01	0.92±0.1	3.3±0.05
39		0.002	0.009±0.05	NT	NT
43		0.0007	0.007±0.01	0.13±0.02	1.8±0.5
44		0.002	0.005±0.01	0.15±0.1	0.30±0.1
45		0.01	0.20±0.01	NT	NT
46		0.003	0.01±0.01	0.56±0.01	0.68±0.2

[a] IC_{50} values for inhibition of lck. For active compounds, they are the mean values of two or more separate determinations, in duplicate. [b] EC_{50} values for inhibition of Ca release in Jurkat cells. Values are the mean of two or more separate experiments in duplicate ± SD. [c] EC_{50} values for SEB IL2 and human whole blood IL2 values are the mean of two or more separate experiments in duplicate ± SD. [d] NT = not tested.

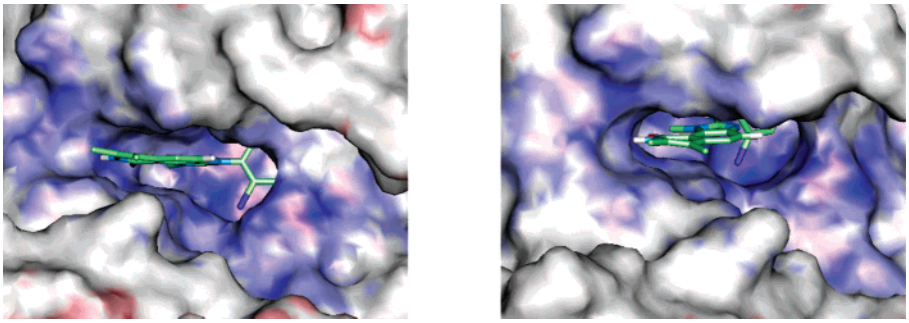


Figure 4. Views of **12** in relationship to C-7 and the enzyme cleft.

interact with the protein directly (vide infra), overall improvements in the physicochemical properties likely contribute to enhanced cellular potency. Further profiling this series of compounds showed them to be effica-

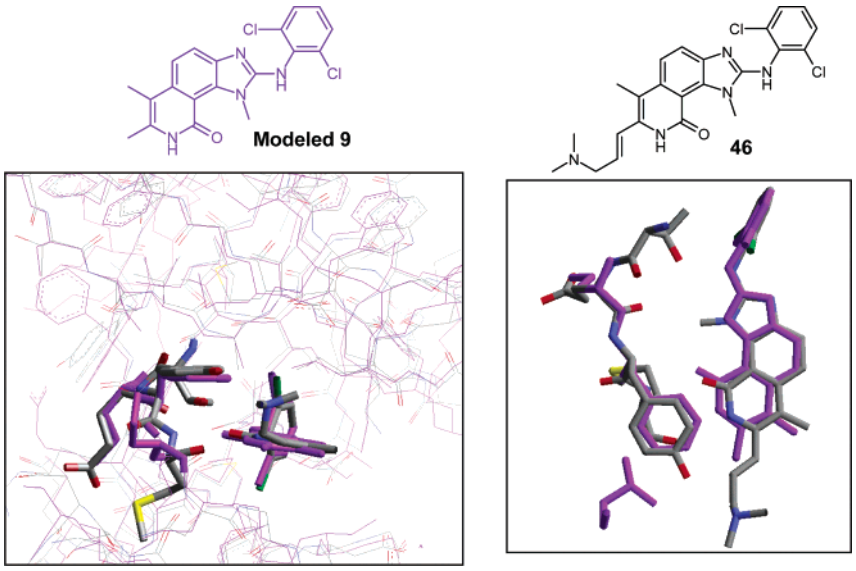


Figure 5. Overlay of X-ray of **46** with homology model of **12**.

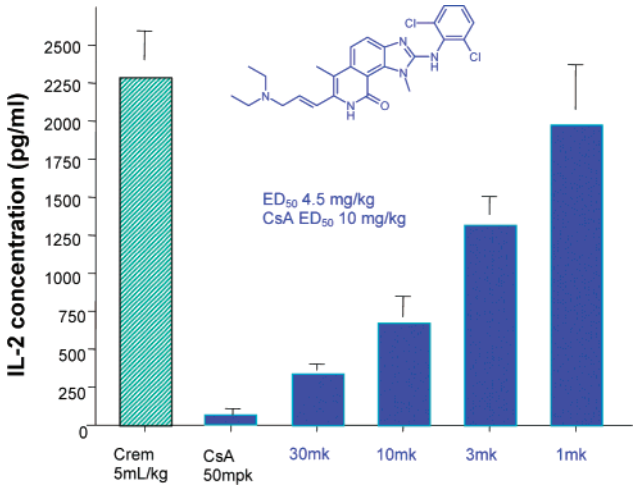


Figure 6. IL-2 inhibition by **43** when dosed orally.

cious in both the Jurkat IL-2 and human whole blood IL-2 assay.

X-ray. Through the course of this work, our binding model proved to be very useful in guiding the SAR studies. After compound **43** was profiled, we obtained an inhibitor−lck cocrystal structure, which confirmed the accuracy of this model with one of our more potent allylic amines, **46**. As shown (Figure 5), our model structure and the X-ray structure essentially overlap identically. Although the vinyl group is clearly resolved, the amine functionality is disordered and was therefore modeled into the crystal structure.

In Vivo Activity. Compounds were tested for in vivo efficacy in mice by measuring inhibition of IL-2 production following stimulation with anti-CD3. In this model, **2** was only moderately active when dosed ip as reported previously.[4] However, **43** demonstrated an ED_{50} of 4.5 mg/kg, which was comparable with that of cyclosporin A (ED_{50} = 10 mg/kg, Figure 6). Tertiary amines **34**, **44**, and **46** gave similar results in this model (data not shown). Interestingly, allylic alcohol **38**, which has in vitro potency comparable to that of **43**, proved to be ineffective when tested in this model. The lack of adequate oral exposure of **38** (∼0 μM at 100 and 30 mg/kg) might simply be due to its poor solubility compared to **43** (0.25 vs 195 μg/mL at pH 7.4, respectively).

Table 5. Selectivity Profile of **43**

kinase	enzyme IC_{50} (μM)[a]	kinase	enzyme IC_{50} (μM)[a]
lck	0.0005	EGFR	>10
lyn	0.002	PDGFR	0.008
src	0.0002	HER2	>10
zap70	0.40	insulinRK	>100
syk	>40	IKKα & β	50
PKC	>300	IGFR	>100
PKA	>100	VEGFR	>100
p38	0.15	HGFR	>100
Btk	0.0004	Erk	>30
Itk	>10		

[a] IC_{50} values for inhibition of listed kinases. For active compounds, they are the mean of two or more separate determinations, in duplicate. ATP concentrations are at or below k_m of the listed kinases.

Table 6. Selectivity Profile of **43**

kinase	enzyme IC_{50} (μM)[a]	kinase	enzyme IC_{50} (μM)[a]
CDK4	>100	P70S6K	>1.0
CaMK	>100	GSK3β	>1.0
JNK	>1.0	ROCK−II	>1.0
MAPKAP-1b&2	>1.0	AMPK	>1.0
MSK-1	>1.0	CHK1	>1.0
PRAK	>1.0	CK2	>1.0
PDK1	>1.0	PHK	>1.0
PKBα	>1.0	CDK2	>1.0
SGK	>1.0	CSK	87% inh @ 1.0

[a] IC_{50} values for inhibition of listed kinases. ATP concentrations are run at 100 μM through the Dundee consortium.

Selectivity Profile. Selectivity over other kinases will be an important consideration for developing a chronically administered therapeutic agent. **43** demonstrates high selectivity when compared to a variety of both tyrosine and serine/threonine kinases (Tables 5 and 6). As expected, **43** shows no significant selectivity over two other members of the src family. Two kinases that demonstrated significant activity were PDGFR and Btk, both of which have a high homology in the ATP binding site, although less so in the whole kinase domain.

Conclusions

We have improved our class of phenylaminoimidazoisoquinolinones as potent inhibitors of lck kinase by 1000-fold over our screening hit. Our ability to accomplish this was aided through the use of a binding model, whose high level of accuracy has been confirmed by an X-ray structure of one of our inhibitors. Two key discoveries that led to significant improvements of this novel series were the N-1 alkylation of the benzimidazole and the conversion of the C-7 methyl group to a planar, sp^2 vinyl group that provided an increase in both molecular and cellular potency. Furthermore, introduction of an allylic polar substituent at the C-7 position, in particular an allylic amine, not only provided picomolar inhibitors of lck and improved aqueous solubility but also provided in vivo oral efficacy with activity comparable to that of cyclosporin A.

Experimental Section

Melting points were determined with an electrothermal capillary melting point apparatus or a Fisher-Johns apparatus and are uncorrected. Proton NMR (^{1}H NMR) spectra were recorded on Bruker Avance 400, Varian Gemini 2300, and Bruker AF270 spectrometers operating at 400, 300, and 270 MHz, respectively. Chemical shifts are reported as δ values in ppm downfield from TMS, using the solvent peak as an internal reference. Electron impact mass spectra (EIMS) were run on a Finnigan SSQ7000 instrument at 70 eV. Chemical ionization mass spectra (CIMS) were run on the same machine, with NH_3 as reagent gas. Electrospray mass spectra (ESMS) were run on a MicroMass Platform LCZ instrument, at a cone voltage of 30 V. Flash column chromatography was carried out on silica gel (230−400 mesh). Organic solutions that had been in contact with water were dried over $MgSO_4$ prior to concentration in a rotary evaporator.

2-Amino-6-chloro-3-nitrobenzonitrile (4). To 2,6-dichloro-3-nitrobenzonitrile (30 g, 139 mmol) was added a solution (200 mL, 5.15 M) of freshly prepared ammonia in ethanol. The flask was sealed and then placed in a preheated oil bath at 80 °C for 1.5 h. After the mixture was cooled, the resulting precipitate was taken up in ethyl acetate, washed with water and brine, dried over $MgSO_4$, and concentrated to afford 18.4 g (68%) of the title compound. ^{1}H NMR ($CDCl_3$, 270 MHz): δ 6.80 (d, 1H, J = 8.0 Hz), 8.33 (d, 1H, J = 8.0 Hz), 8.69 (bs, 2H).

6-Chloro-2-methylamino-3-nitrobenzonitrile (5). A solution of 2,6-dichloro-3-nitrobenzonitrile (98.7 g, 455 mmol) in EtOAc (910 mL) was cooled to 5 °C. A 40% aqueous methylamine (79.5 mL, 1.14 mol) solution was added with vigorous mechanical stirring, keeping the temperature at 10−15 °C. After addition was complete, stirring was continued for 3 h at the same temperature. More methylamine (16 mL, 230 mmol) was added, and the mixture was stirred for a further 1.5 h at room temperature. Water (300 mL) was added, followed by hexane (450 mL). The mixture was stirred for 15 min and filtered and the solid was washed with water and MeOH to provide 80.3 g (83%) of the title compound. Mp 168−171 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.45−3.50 (m, 3H), 6.80 (d, 1H, J = 9.0 Hz), 8.31 (d, 1H, J = 9.0 Hz), 8.69 (bs, 1H).

2-(3-Amino-2-cyano-4-nitrophenyl)-3-oxobutyric Acid Ethyl Ester (6). To a solution of 2-amino-6-chloro-3-nitrobenzonitrile (1.97 g, 10 mmol) in DMF was added ethyl 2-methylacetoacetate (3.60 g, 25 mmol) and potassium carbonate (1.50 g, 11 mmol). The reaction was stirred for 24 h at room temperature. The reaction mixture was diluted with EtOAc, then washed with 1 M HCl, water, and brine, and dried over $MgSO_4$. Column chromatography using hexanes/EtOAc (2:1) afforded 1.78 g (58%) of the title compound. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.13 (t, 3H, J = 4.0 Hz), 1.72 (s, 3H), 2.26 (s, 3H), 4.14−4.17 (m, 2H), 6.55 (d, 1H, J = 8.0 Hz), 7.45 (bs, 2H), 8.23 (d, 1H, J = 8.0 Hz).

2-(2-Cyano-3-methylamino-4-nitrophenyl)-2-methyl-3-oxobutyric Acid Ethyl Ester (7). To a stirred solution of potassium *tert*-butoxide (24.3 g, 206 mmol) in DMSO (500 mL) was added ethyl 2-methylacetoacetate (34.3 g, 233 mmol) dropwise over 5 min. The temperature rose to 30 °C. 6-Chloro-2-methylamino-3-nitrobenzonitrile (43.6 g, 190 mmol) was added in portions over 15 min. The temperature rose to 40 °C. The solution was stirred for 1 h with no external heating or cooling. The mixture was poured into 10% aqueous NH_4Cl (500 mL) and was extracted with EtOAc. The combined extracts were washed with water and brine and were evaporated. MeOH (200 mL) was added to the residue, and the mixture was stirred for 1.5 h. The yellow solid was filtered, washed with cold MeOH (25 mL), and dried to provide 36.2 g (60%) of the title compound. Mp 84−87 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 1.22 (t, 3H, J = 7 Hz), 1.81 (s, 3H), 2.36 (s, 3H), 3.55 (bs, 3H), 4.18−4.30 (bs, 2H), 6.64 (d, 1H, J = 9 Hz), 8.06 (bs, 1H), 8.24 (d, 1H, J = 9 Hz).

2-(3,4-Diamino-2-cyanophenyl)-3-oxobutyric Acid Ethyl Ester (8). To a solution of 2-(3-amino-2-cyano-4-nitrophenyl)-3-oxobutyric acid ethyl ester (850 mg, 2.79 mmol) in methanol (50 mL) was added 10% palladium on carbon (85 mg). The flask was pressurized with hydrogen to 50 psi and hydrogenated until no loss of H_2 could be detected. The reaction mixture was filtered over Celite and concentrated to afford the title compound. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.3 (t, 3H, J = 4.0 Hz), 1.80 (s, 3H), 2.30 (s, 3H), 4.25−4.35 (m, 2H), 6.42 (d, 1H, J = 8.0 Hz), 6.77 (d, 1H, J = 8.0 Hz), 8.10 (bs, 2H), 8.69 (bs, 2H).

2-(4-Amino-2-cyano-3-methylaminophenyl)-2-methyl-3-oxobutyric Acid Ethyl Ester (9). A solution of **7** (10.5 g, 32.5 mmol) in EtOAc (130 mL) was hydrogenated over 10% palladium on carbon (0.5 g) at 50 psi for 24 h. The catalyst was removed by filtration through diatomaceous earth, and the filtrate was evaporated. A mixture of EtOAc/hexane (1:1, 10 mL) was added to the residue, and the resulting mixture was stirred for 0.5 h. The crystals were filtered and washed with hexane to provide 7.74 g (81%) of the title compound. Mp 130−131.5 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 1.21 (t, 3H, J = 7.0 Hz), 1.67 (s, 3H), 2.23 (s, 3H), 2.96−3.0 (m, 3H), 4.18−4.21 (m, 2H), 4.89−4.91 (m, 1H), 5.17 (s, 2H), 6.35 (d, 1H, J = 8 Hz), 6.72 (d, 1H, J = 8 Hz). CIMS m/z: 290 (MH^+). Anal. ($C_{15}H_{19}N_3O_3$) C, H, N.

2-(2,6-Dichlorophenylamino)-6,7-dimethyl-1,8-dihydroimidazo[4,5-*h*]isoquinolin-9-one (2). To a solution of **8** (767 mg, 2.79 mmol) in ethyl acetate (30 mL) was added 2,6-dichlorophenyl isothiocyanate (626 mg, 3.07 mmol). The reaction mixture was stirred at room temperature overnight. The reaction mixture was diluted with ethyl acetate (20 mL), washed with water (3 × 20 mL), dried ($MgSO_4$), and concentrated. The crude residue was chromatographed using hexanes/EtOAc (1:1) to afford the thiourea. To the intermediate thiourea (1.30 g, 2.72 mmol) was added DCC (600 mg, 2.91 mmol) and THF. The reaction mixture was heated at 80 °C for 3 h. After the mixture was cooled, solvent was concentrated and crude residue was taken back up in ethyl acetate. Insoluble material was filtered off. The filtrate was concentrated to afford 600 mg (50%) of the title compound. The ^{1}H NMR results were identical to the results reported in ref 4.

2-[4-Cyano-2-(2,6-dichlorophenylamino)-3-methyl-3*H*-benzimiazol-5-yl]-2-methyl-3-oxobutyric Acid Ethyl Ester (11). A solution of **9** (7.7 g, 26.6 mmol) and 2,6-dichlorophenyl isothiocyanate (5.43 g, 26.6 mmol) in THF (150 mL) was stirred at room temperature for 5 h. Mercuric oxide (6.34 g, 29.3 mmol) was then added in one portion, and stirring continued overnight. The mixture was filtered through diatomaceous earth, washing well with THF. The filtrate was evaporated and the residue was triturated with ether to provide 7.7 g (63%) of the title compound. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.17 (t, 3H, J = 8.0 Hz), 1.82 (s, 3H), 2.30 (s, 3H), 4.08 (s, 3H), 4.16−4.25 (m, 2H), 7.16 (d, 1 H, J = 8 Hz), 7.48 (t, 1 H, J = 8 Hz), 7.50 (d, 1 H, J = 8 Hz), 7.67 (d, 2 H, J = 8 Hz).

2-(2,6-Dichlorophenylamino)-6,7-dimethyl-1,8-dihydroimidazo[4,5-*h*]isoquinoline-9-one (12). To a stirred mixture of concentrated H_2SO_4 (40 mL), HOAc (40 mL), and water (40 mL) at 60 °C was added **11** (7.4 g, 16 mmol) in one portion. The solution was heated at 100 °C for 2.5 h and then stirred overnight at room temperature. The reaction mixture was poured onto ice and neutralized with concentrated NH_4-OH, with ice-cooling. The precipitate was filtered and washed well with water. The solid was slurried in MeOH, stirred well, filtered, washed with MeOH until the washings were colorless, and dried to provide 5.5 g (88%) of the title compound. Mp >300 °C. ^{1}H NMR (DMSO-d_6, TFA, 400 MHz): δ 2.22 (s, 3H), 2.32 (s, 3H), 4.28 (s, 3H), 7.60 (t, 1H, J = 8 Hz), 7.70 (d, 1H, J = 9 Hz), 7.73 (d, 1H, J = 9 Hz), 7.77 (d, 2H, J = 8 Hz). CIMS m/z: 387 (MH^+). Anal. ($C_{19}H_{16}Cl_2N_4O$) C, H, N.

2-(2,6-Dichlorophenylamino)-1-ethyl-6,7-dimethyl-1,8-dihydroimidazo[4,5-*h*]isoquinolin-9-one (40). 40 was prepared as described for compound **12** with ethylamine. ^{1}H NMR (DMSO-d_6, TFA, 400 MHz): δ 2.25 (t, 3H, J = 7.0 Hz), 2.16 (s, 3H), 2.25 (s, 3H), 5.23 (q, 2H, J = 7, 8 Hz), 7.54 (dd, 1H, J = 1, 8 Hz), 7.64 (dd, 2H, J = 1, 8 Hz), 7.70 (d, 2H, J = 8 Hz), 11.44 (s, 1H). ESMS m/z: 431 (MH^+).

2-(2-Cyano-3-methylamino-4-nitrophenyl)-3-oxobutyric Acid Methyl Ester (13). 13 was prepared as described for **6** and **7**. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.95 (s, 3H), 3.48 (s, 3H), 3.91 (s, 3H), 6.52 (d, 1H, J = 8 Hz), 8.37 (d, 1H, J = 8 Hz), 8.61 (bs, 1H).

1,7-Dimethyl-2-dichlorphenylamino-9-oxo-8,9-dihydro-1*H*-imidazo[4,5-*h*]isoquinoline-6-carboxylic Acid Methyl Ester (14). 14 was prepared as described above for **12**. ^{1}H NMR (TFA-*d*, 400 MHz): δ 2.62 (s, 3H), 4.19 (s, 3H), 4.42 (s, 3H), 7.46−7.50 (m, 1H), 7.51 (d, 2H, J = 8 Hz), 7.87 (d, 1H, J = 9 Hz), 7.93 (d, 1H, J = 9 Hz). ESMS m/z: 431 (MH^+).

2-Acetyl-2-(2-cyano-3-methylamino-4-nitrophenyl)succinic Acid Dimethyl Ester (15). 15 was prepared as described for **6** and **7**. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.25−1.29 (m, 5H), 1.37 (t, 3H, J = 8 Hz), 2.08 (s, 3H), 2.43 (s, 3H), 3.41−3.45 (m, 2H), 4.15 (apt t, 2H, J = 8 Hz), 4.39 (apt t, 2H, J = 8 Hz), 6.72 (d, 1H, J = 8 Hz), 8.33 (d, 1H, J = 8 Hz), 8.39 (bs, 1H).

2-(2,6-Dichlorophenylamino)-1,7-dimethyl-9-oxo-1,8-dihydroimidazo[4,5-*h*]isoquinolin-6-ylacetic Acid (16). 16 was prepared as described for compounds **2** and **12.** ^{1}H NMR (TFA-*d*, 400 MHz): δ 2.92 (s, 3H), 4.37 (s, 2H), 4.61 (s, 3H), 7.59 (t, 1H, J = 7 Hz), 7.72 (d, 2H, J = 7 Hz), 8.06 (s, 2H), 7.98 (d, 1H, J = 8 Hz). ESMS m/z: 431.

2-(2,6-Dichlorophenylamino)-1,7-dimethyl-9-oxo-1,8-dihydroimidazo[4,5-*h*]isoquinolin-6-ylacetic Acid Ethyl Ester (17). 17 was prepared from **16** by refluxing in ethanol and H_2SO_4. Mp 280−285 °C. ^{1}H NMR (TFA-*d*, 400 MHz): δ 1.45 (bs, 3H), 2.75 (s, 3H), 4.20 (s, 2H), 4.50 (s, 3H), 7.35−7.40 (m, 1H), 7.75 (s, 1H), 7.95−8.0 (m, 2H), 8.37−8.39 (m, 1H), δ 8.97 (s, 1H), 11.08 (s, 1H). ESMS m/z: 500 (MH^+). MSCI m/z: 459, 461 (MH^+). Anal. ($C_{22}H_{20}Cl_2N_4O_3 \cdot 1H_2O$) C, H, N.

2-(2,6-Dichlorophenylamino)-1,7-dimethyl-6-(2-morpholin-4-yl-2-oxoethyl)-1,8-dihydroimidazo[4,5-*h*]isoquinoline-9-one (18). To a solution of **17** (1.0 g, 2.3 mmol) in DMF (7 mL) was added *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) (0.82 g, 2.6 mmol) and morpholine (0.24 mL, 2.8 mmol), and the mixture was stirred for 18 h at room temperature. Ice/water was added and the precipitate was collected, washed with water, and dried to give 0.97 g (84%) of the title compound. Mp >300 °C. ^{1}H NMR (TFA-*d*, 400 MHz): δ 2.58 (s, 3H), 3.89−3.99 (m, 2H), 4.03−4.14 (m, 4H), 4.15−4.23 (m, 2H), 4.28 (s, 2H), 4.44 (s, 3H), 7.51 (t, 1H, J = 8 Hz), 7.63 (d, 2H, J = 8 Hz), 7.70 (d, 1H, J = 9 Hz), 7.90 (d, 1H, J = 9 Hz). ESMS m/z: 500, 502 (MH^+). Anal. ($C_{24}H_{23}Cl_2N_5O_3 \cdot 0.3H_2O$) C, H, N.

2-(2,6-Dichlorophenylamino)-1,7-dimethyl-6-(2-(ethylamino)-2-oxoethyl)-1,8-dihydroimidazo[4,5-*h*]isoquinoline-9-one (50). 50 was prepared as described above. Mp >300 °C. ^{1}H NMR (TFA-*d*, 400 MHz): δ 1.26 (t, 3H, J = 8 Hz), 2.67 (s, 3H), 3.45−3.61 (m, 2H), 4.32 (s, 2H), 4.49 (s, 3H), 7.55 (t, 1H, J = 8 Hz), 7.67 (d, 2H, J = 8 Hz), 7.88 (d, 1H, J = 8 Hz), 7.98 (d, 1H, J = 8 Hz). ESMS m/z: 458, 460 (MH^+).

2-(2,6-Dichlorophenylamino)-1,7-dimethyl-6-(2-pyrrolidin-1-yl-2-oxoethyl)-1,8-dihydroimidazo[4,5-*h*]isoquinoline-9-one (51). 51 was prepared as described above. Mp >300 °C. ^{1}H NMR (TFA-*d*, 400 MHz): δ 2.12−2.20 (m, 2H), 2.25−2.30 (m, 2H), 2.60 (s, 3H), 3.70−3.88 (m, 2H), 3.92−4.16 (m, 2H), 4.32 (s, 2H), 4.44 (s, 3H), 7.51 (t, 1H, J = 8 Hz), 7.62 (d, 2H, J = 8 Hz), 7.67 (d, 1H, J = 9 Hz), 7.89 (d, 1H, J = 9 Hz). ESMS m/z: 484, 486 (MH^+).

2-(2,6-Dichlorophenylamino)-1,7-dimethyl-6-[2-(4-methylpiperazin-1-yl)-2-oxoethyl)-1,8-dihydroimidazo[4,5-*h*]isoquinoline-9-one (52). 52 was prepared as described as above. Mp >300 °C. ^{1}H NMR (TFA-*d*, 400 MHz): δ 2.66 (s, 3H), 3.30 (s, 3H), 3.41 (t, 1H, J = 7 Hz), 3.50−3.66 (m, 2H), 3.94 (d, 1H, J = 12 Hz), 4.07 (d, 1H, J = 12 Hz), 4.14−4.26 (m, 1H), 4.36 (d, 1H, J = 17 Hz), 4.46 (d, 1H, J = 17 Hz), 4.55 (s, 3H), 4.77 (d, 1H, J = 14 Hz), 5.08 (1H, d, J = 14 Hz), 7.61 (1H, t, J = 8 Hz), 7.63 (2H, d, J = 8 Hz), 7.75 (d, 1H, J = 8 Hz), 7.98 (d, 1H, J = 9 Hz). ESMS m/z: 513, 515 (MH^+).

2-(2,6-Dichlorophenylamino)-1,7-dimethyl-6-(2-morpholin-4-ylethyl)-1,8-dihydroimidazo[4,5-*h*]isoquinoline-9-one (19). A stirred suspension of **18** (85 mg, 0.17 mmol) in THF (9 mL) was heated to reflux, and borane methyl sulfide (0.09 mL, 0.9 mmol) added. Stirring was continued for 3.5 h at reflux and overnight at room temperature. Then 6 M HCl was added and the solution was stirred for 2 h. The solution was applied to a Varian SCX column and was washed with MeOH/CH_2Cl_2, 50:50. Then the product was eluted with MeOH/CH_2Cl_2/NH_4OH, 50:50:1. The product was further purified on a silica column, eluting with CH_2Cl_2/MeOH, 98:2, to provide 32 mg (39%) of the title compound. Mp 285−290 °C. ^{1}H NMR (TFA-*d*, 400 MHz): δ 2.65 (s, 3H), 3.46−3.65 (m, 6H), 4.01 (d, 2H, J = 12 Hz), 4.20 (t, 2H J = 12 Hz), 4.38−4.50 (m, 2H), 4.43 (s, 3H), 7.51 (t, 1H, J = 8 Hz), 7.63 (d, 1H, J = 8 Hz), 7.98 (d, 1H, J = 8 Hz), 7.97−8.0 (m, 2H). ESMS m/z: 486, 488 (MH^+).

2-(2,6-Dichlorophenylamino)-1,7-dimethyl-6-(3-morpholin-4-yl-3-oxopropyl)-1,8-dihydroimidazo[4,5-*h*]isoquinoline-9-one (49). 49 was prepared as described as above. Mp 277−282 °C. ^{1}H NMR (TFA-*d*, 400 MHz): δ 2.20−2.36 (m, 2H), 2.67 (s, 3H), 3.18 (t, 2H, J = 9 Hz), 3.43 (d, H, J = 9 Hz), 3.54−3.62 (m, 2H), 3.80 (d, 2H, J = 12 Hz), 4.05−4.15 (m, 2H), 4.38 (d, 2H, J = 12 Hz), 4.45 (s, 3H), 7.54 (t, 1H, J = 8 Hz), 7.65 (d, 2H, J = 8 Hz), 8.00 (s, 2H). ESMS m/z: 500, 502 (MH^+). Anal. ($C_{25}H_{27}Cl_2N_5O_2 \cdot 0.2H_2O$) C, H, N.

2-(2,6-Dichlorophenylamino)-1,7-dimethyl-6-(3-morpholin-4-ylpropyl)-1,8-dihydroimidazo[4,5-*h*]isoquinolin-9-one (53). 53 was prepared as described above. Mp 277−282 °C. ^{1}H NMR (TFA-*d*, 400 MHz): δ 2.18 (br, 2H), 2.67 (s, 3H), 3.17 (t, 2H, J = 8 Hz), 3.42 (t, 2H, J = 8 Hz), 3.3−3.6 (m, 2H), 3.81 (d, 2H, J = 12 Hz), 4.11 (t, 2H, J = 6 Hz), 4.39 (d, 2H, J = 12 Hz), 4.45 (s, 3H), 7.53 (t, 1H, J = 8 Hz), 7.65 (d, 2H, J = 8 Hz), 8.00 (s, 2 H). CIMS m/z: 500 (MH^+).

2-(4-Amino-5-bromo-2-cyano-3-methylaminophenyl)-2-methyl-3-oxobutyric Acid Ethyl Ester (20). To a solution of **9** (9.02 g, 31.2 mmol) in $CHCl_3$ (90 mL) was added bromine (4.98 g, 31.2 mmol) dropwise at ambient temperature. After the addition of bromine, the reaction mixture was diluted with EtOAc (800 mL). This solution was washed successively with saturated $NaHCO_3$ solution and brine and dried. The residue after evaporation was purified by flash chromatography in hexanes/EtOAc, 2:1, to provide 6.1 g (53%) of the title compound. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.3 (t, 3H, J = 16 Hz), 1.89 (s, 3H), 2.45 (s, 3H), 2.95 (s, 3H), 4.30−4.40 (m, 2H), 7.01 (s, 1 H), 8.40 (bs, 2H), 8.6 (bs, 1H).

2-[8-Bromo-4-cyano-2-(2,6-dichlorophenylamino)-3-methyl-3*H*-benzimidazol-5-yl]-2-methyl-3-oxobutyric Acid Ethyl Ester (21). To **20** (3.32 g, 9.0 mmol) in 1,4-dioxane (45 mL) was added 2,6-dichlorophenyl isothiocyanate (2.02 g, 9.9 mmol) and mercuric oxide (2.54 g, 11.7 mmol) under nitrogen atmosphere. The resulting mixture was stirred and heated at 95 °C overnight. The reaction mixture was cooled to room temperature and filtered though a short pad of diatomaceous

earth and SiO_2. The filtrate was concentrated and the residue was purified by flash chromatography in hexanes/EtOAc, 2:1, to provide 3.44 g (71%) of the title compound. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.36 (t, 3H, J = 4.0 Hz), 1.96 (s, 3H), 2.39 (s, 3H), 4.14 (s, 3H), 4.36−4.40 (m, 2H), 7.23 (s, 1 H), 7.55 (t, 1H, J = 8.0 Hz), 7.77 (d, 2H, J = 8.0 Hz), 9.62 (s, 1H).

2-[4-Cyano-2-(2,6-dichlorophenylamino)-3-methyl-8-vinyl-3*H*-benzimiazol-5-yl]-2-methyl-3-oxobutyric Acid Ethyl Ester (22). A mixture of 2-[8-bromo-4-cyano-2-(2,6-dichlorophenylamino)-3-methyl-3*H*-benzimiazol-5-yl]-2-methyl-3-oxobutyric acid ethyl ester (600 mg, 1.11 mmol), $(PPh_3)_2$-$PdCl_2$ (78 mg, 0.11 mmol), and tributyl(vinyl)tin (0.49 mL, 1.67 mmol) in NMP (4 mL) was degassed and heated at 100 °C for 3 days under argon. The mixture was concentrated, and the residue was purified by flash chromatography in hexanes/EtOAc, 3:1, to provide 530 mg (98%) of the title compound. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.38 (t, 3H, J = 8 Hz), 1.97 (s, 3H), 2.45 (s, 3H), 4.05 (s, 3H), 4.37−4.40 (m, 2H), 5.50 (d, 1H, J = 8 Hz), 6.10−6.30 (m, 1H), 6.97 (s, 2H), 7.10 (d, H, J = 8 Hz), 7.39 (d, H, J = 8 Hz).

2-(2,6-Dichlorophenylamino)-1,6,7-trimethyl-4-vinyl-1,8-dihydroimidazo-[4,5-*h*]isoquinoline-9-one (22b). 22 (66 mg, 0.14 mmol) in a mixture of H_2SO_4 (0.6 mL), acetic acid (0.6 mL), and water (0.6 mL) was heated at 100 °C for 2 h. The resulting mixture was cooled to room temperature and diluted with water (10 mL). The solution was adjusted to pH 8 with 10% NaOH solution. The precipitated brown solid was filtered and purified by flash chromatography in CH_2Cl_2/MeOH, 30:1, to provide 15 mg (27%) of the title compound. Mp >250 °C (dec). ^{1}H NMR (TFA-*d*, 400 MHz): δ 2.11 (s, 3H), 2.22 (s, 3H), 4.13 (s, 3H), 5.49 (d, 1H, J = 11 Hz), 6.35 (d, 1H, J = 16 Hz), 7.05 (dd, 1H, J = 11, 16 Hz), 7.31 (t, 1H, J = 8 Hz), 7.45 (d, 1H, J = 8 Hz), 7.68−7.71 (m, 1H), 8.86 (s, 1H), 10.9 (s, 1H). CIMS m/z: 413(MH^+).

2-[4-Cyano-2-(2,6-dichlorophenylamino)-7-formyl-3-methyl-3*H*-benzoimidazol-5-yl]-2-methyl-3-oxobutyric Acid Ethyl Ester (24). To a solution of **22** (0.58 g, 1.20 mmol) in THF (30 mL) was added osmium tetroxide (3 mL), followed by sodium periodate (0.77 g, 3.59 mmol) and water (3.0 mL). The mixture was stirred at room temperature for 30 min before dilution with water and extraction of the product with ethyl acetate. The organic layer was then diluted with an aqueous $NaHCO_3$ solution. The organic layer was then dried with $MgSO_4$ filtered, and concentrated to an oil. This was then loaded onto a silica gel column (2:1 hexanes/ethyl acetate) to provide 0.46 g (100% g) of the title compound. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.40 (t, 3H, J = 16 Hz), 1.60 (s, 3H), 1.97 (s, 3H), 2.45 (s, 3H), 4.01 (s, 3H), 4.05 (s, 3H), 4.31−4.45 (m, 2H), 7.01 (d, 1H, J = 8 Hz), 7.19 (bs,1H), 7.45 (d, 1H, J = 8 Hz), 8.95 (bs, 1H), 10.01 (bs, 1H).

2-(2,6-Dichlorophenylamino)-1,6,7-trimethyl-9-oxo-1,8-dihydroimidazo[4,5-*h*]isoquinoline-4-carbaldehyde (25). 24 (35 mg, 0.07 mmol) in a mixture of H_2SO_4 (0.6 mL), acetic acid (0.6 mL), and water (0.6 mL) was heated at 100 °C for 1.5 h and cooled to room temperature. The resulting mixture was diluted with water (10 mL), and the pH was adjusted to 7 with ammonium hydroxide solution. The precipitated orange powder was filtered to provide 18 mg (60%) of the title compound. ^{1}H NMR (TFA-*d*, 400 MHz): δ 2.34 (s, 3H), 2.38 (s, 3H), 4.14 (s, 3H), 7.25 (t, 1H, J = 8 Hz), 7.60 (d, 2H, J = 8 Hz), 8.10 (s, 1H), 10.1 (s, 1H).

2-(2,6-Dichlorophenylamino)-4-(2-hydroxyethylaminomethyl)-1,6,7-trimethyl-1,8-dihydroimidazo[4,5-*h*]isoquinolin-9-one (26). A suspension of **25** (30 mg, 0.07 mmol) in methanol (5 mL) was treated with ethanolamine (44 μL, 0.72 mmol) and sodium cyanoborohydride (14 mg, 0.22 mmol) and stirred at room temperature for 16 h. The resulting mixture was concentrated, and the residue was diluted with water. The precipitated solid was filtered and dried to give 12 mg (36%) of the title compound. Mp >300 °C. ^{1}H NMR (MeOH-d_4, 400 MHz): δ 2.36 (s, 3H), 2.38 (s, 3H), 2.71 (t, 2H, J = 6.0 Hz), 3.62 (t, 2H, J = 6.0 Hz), 4.14 (s, 2H), 4.20 (s, 3H), 7.25 (s, 1H), 7.49−7.52 (m, 3H). ESMS m/z: 460, 462 (MH^+).

2-(2,6-Dichlorophenylamino)-4-[(4-methoxybenzylamino)methyl]-1,6,7-trimethyl-1,8-dihydroimidazo[4,5-*h*]isoquinolin-9-one (27). A suspension of **25** (30 mg, 0.07 mmol) in methanol (5 mL) was treated with 4-methoxybenzylamine (94 μL, 0.72 mmol) and sodium cyanoborohydride (14 mg, 0.22 mmol) and stirred at room temperature for 16 h. The resulting mixture was concentrated and preparative TLC (silica gel/5% MeOH in CH_2Cl_2) purified the residue to give 7 mg (18%) of the title compound. Mp 247−250 °C. ^{1}H NMR (MeOH-d_4, 400 MHz): δ 2.34 (s, 3H), 2.38 (s, 3H), 3.76 (s, 2H), 3.79 (s, 3H), 3.82 (s, 2H), 4.24 (s, 3H), 6.85 (d, 2H, J = 8 Hz), 7.13 (d, 2H, J = 8 Hz), 7.24 (t, 1H, J = 8 Hz), 7.45−7.47 (m, 3H). ESMS m/z: 536, 538 (MH^+).

2-(2,6-Dichlorophenylamino)-1,6,7-trimethyl-4-(2,6-difluoropyridin-3-yl)-1,8-dihydroimidazo[4,5-*h*]isoquinoline-9-one (23b). To 2-[8-bromo-4-cyano-2-(2,6-dichlorophenylamino)-3-methyl-3*H*-benzimidazol-5-yl]-2-methyl-3-oxobutyric acid ethyl ester (100 mg, 0.186 mmol) in dry NMP (3 mL) was added 2,6-difluoro-3-tributylstannanylpyridine (113 mg, 0.28 mmol) and dichlorobis(triphenylphosphine)palladium(II) (10 mg). The reaction mixture was heated in a sealed tube at 100 °C overnight. The reaction mixture was cooled to room temperature and extracted with EtOAc (25 mL). The organic fraction was washed with water, dried over anhydrous sodium sulfate, filtered, and evaporated. Column chromatography (1:1 EtOAc/hexanes) followed by preparative TLC (silica gel, 40% ethyl acetate/hexanes) provided 28 mg (26%) of the title compound. ESMS m/z: 572 (MH^+).

To the coupled product (25 mg, 0.044 mmol) was added 1:1 concentrated sulfuric acid/water (3 mL). The reaction mixture was heated at 100 °C for 2 h. The reaction mixture was cooled to room temperature, ice/water (2 mL) was added, and the reaction was made basic by addition of aqueous NH_4OH. The solid was filtered off and purified by preparative TLC (silica gel/5% CH_3OH/CH_2Cl_2) to provide 7 mg (32%) of the title compound. Mp >300 °C. ^{1}H NMR (DMSO-d_6, TFA, 400 MHz): δ 2.21 (s, 3H), 2.29 (s, 3H), 4.13 (s, 3H), 7.23−7.30 (m, 2H), 7.48 (s, 1H), 7.53−7.55 (m, 2H), δ 8.37−8.39 (m, 1H), 8.97 (s, 1H), 11.08 (s, 1H). ESMS m/z: 500 (MH^+).

2-(2,6-Dichlorophenylamino)-1,6-dimethyl-9-oxo-8,9-dihydro-1*H*-imidazo[4,5-*h*]isoquinoline-7-carbaldehyde (28). To a suspension of **12** (521 mg, 1.3 mmol) in dioxane (30 mL) was added selenium dioxide (430 mg, 3.9 mmol), and the mixture was heated at 100 °C for 5 h. The reaction mixture was then cooled to room temperature, filtered through diatomaceous earth with 10% MeOH/CH_2Cl_2, and then concentrated in vacuo. The crude material was triturated with CH_2Cl_2 to provide 476 mg (92%) of the title compound. Mp >300 °C. ^{1}H NMR (DMSO-d_6, TFA, 400 MHz): δ 2.27 (s, 3H), 4.30 (s, 3H), 7.38 (t, 1H, J = 8 Hz), 7.60 (d, 2H, J = 8 Hz), 7.69 (s, 2H), 8.68 (bs, 1H), 10.21 (s, 1H), 10.51 (bs, 1H), 11.72 (bs, 1H). NOSEY (DMSO-d_6, TFA): correlation between C5−H and C6−CH_3 and between C6−CH_3 and C7−CHO. CIMS m/z: 401, 403 (MH^+).

2-(2,6-Dichlorophenylamino)-1,6-dimethyl-7-(1-hydroxyprop-2-en-1-yl)-1,8-dihydroimidazo[4,5-*h*]isoquinolin-9-one (31). A suspension of **28** (100 mg, 0.25 mmol) in THF (3 mL) was cooled to −78 °C. Vinylmagnesium bromide (1 M in THF, 2.0 mmol) was added dropwise, and the brown suspension was warmed gradually to −10 °C over 2 h. The solution was quenched with saturated NH_4Cl, extracted with EtOAc, and concentrated in vacuo to provide the title compound, which was used in the next step without purification. Mp 235−236 °C. ^{1}H NMR (MeOH-d_4, 400 MHz): δ 2.35 (s, 3H), 4.24 (s, 3H), 5.26 (d, 1H, J = 10 Hz), 5.40 (d, 1H, J = 17 Hz), 5.56 (d, 1H, J = 5 Hz), 6.03 (ddd, 1H, J = 5, 10, 17 Hz), 7.32 (t, 1H, J = 8 Hz), 7.53 (d, 2H, J = 8 Hz), 7.61 (d, 1H, J = 9 Hz), 7.72 (d, 1H, J = 9 Hz). ESMS m/z: 429 (MH^+). Anal. ($C_{21}H_{18}Cl_2N_4O_3$) C, H, N.

3-[2-(2,6-Dichlorophenylamino)-1,6-dimethyl-9-oxo-8,9-dihydro-1*H*-imidazo[4,5-*h*]isoquinolin-7-yl]acrylic Acid Methyl Ester (36). To a suspension of **28** (329 mg, 0.82 mmol) in THF (5 mL) was added sequentially trimethyl phosphonoacetate (164 mg, 0.90 mmol), lithium hydroxide monohy-

drate (76 mg, 1.8 mmol), and water (0.9 mL). The blood-red solution was stirred for 2 h and quenched with water, and the resulting solid was collected and dried in vacuo. Column chromatography (5% MeOH/CH_2Cl_2) provided 300 mg (80%) of the title compound. Mp >300 °C. ^{1}H NMR (DMSO-d_6, TFA, 400 MHz): δ 2.45 (s, 3H), 3.76 (s, 3H), 4.20 (s, 3H), 6.89 (d, 1H, J = 16 Hz), 7.56 (t, 1H, J = 8 Hz), 7.73−7.77 (m, 3H), 7.81 (d, 1H, J = 16 Hz), 7.87 (d, 1H, J = 9 Hz), 11.29 (s, 1H). ESMS m/z 457, 459 (MH^+). Anal. ($C_{22}H_{18}Cl_2N_4O_3 \cdot 1.5H_2O$) C, H, N.

3-[2-(2,6-Dichlorophenylamino)-1,6-dimethyl-9-oxo-8,9-dihydro-1*H*-imidazo[4,5-*h*]isoquinolin-7-yl]propionic Acid Methyl Ester (37). To a solution of **36** (30 mg, 0.06 mmol) in EtOH (3 mL) and AcOH (4 mL) in a Parr reactor was added PtO_2 (2 mg, 0.007 mmol). The Parr reactor was charged with 50 psi of H_2 and shaken for 12 h. The crude reaction mixture was filtered through diatomaceous earth with EtOH and concentrated in vacuo. Column chromatography (2% MeOH/CH_2Cl_2) provided 9 mg (30%) of the title compound. Mp 268 °C (dec). ^{1}H NMR (DMSO-d_6, TFA, 400 MHz): δ 2.24 (s, 3H), 2.66 (t, 2H, J = 8 Hz), 2.90 (t, 2H, J = 8 Hz), 3.75 (s, 3H), 4.24 (s, 3H), 7.54 (t, 1H, J = 8 Hz), 7.70−7.76 (m, 4H), 11.37 (s, 1H). ESMS m/z: 458 (MH^+).

2-(2,6-Dichlorophenylamino)-1,6-dimethyl-7-vinyl-1,8-dihydroimidazo[4,5-*h*]isoquinoline-9-one (32). To a suspension of **28** (100 mg, 0.25 mmol) in THF (5 mL) was added trimethylsilylmethylmagnesium chloride (2 mL, 2 mmol) at −78 °C. The reaction mixture was warmed to room temperature for 1 h, cooled to 0 °C, quenched with water, and extracted with EtOAc to provide the silyl alcohol (85 mg, 70%). The crude silyl alcohol was suspended in CH_2Cl_2 and cooled to 0 °C. Boron trifluoride etherate (42 μL, 0.32 mmol) was added, and the slurry was warmed to room temperature for 1 h. The reaction mixture was quenched with water, and the CH_2Cl_2 was removed in vacuo. Collection of the resulting solid followed by CH_2Cl_2 trituration provided 17 mg (61%) of the title compound. Mp >300 °C. ^{1}H NMR (DMSO-d_6, TFA, 400 MHz): δ 2.34 (s, 3H), 4.14 (s, 3H), 5.52 (1H, J = 8 Hz), 6.13 (d, 1H, J = 8 Hz), 7.69 (d, 1H, J = 8 Hz), 7.9 (bs, 1H), 7.67 (bs, 4H), 11.1 (s, 1H). ESMS m/z 399, 401 (MH^+).

2-(2,6-Dichlorophenylamino)-1,6-dimethyl-7-ethyl-1,8-dihydroimidazo[4,5-*h*]isoquinoline-9-one (42). 32 (50 mg, 0.13 mmol) was suspended in 3 mL of acetic acid and 3 mL of EtOH. PtO_2 (6 mg, 0.03 mmol) was added, and the reaction mixture was stirred under 1 atm of H_2(g) for 1 h. The reaction mixture was filtered, concentrated, and extracted with EtOAc. Column chromatography (0−10% MeOH/CH_2Cl_2) provided 25 mg (52%) of the title compound. Mp >300 °C. ^{1}H NMR (DMSO-d_6, TFA, 400 MHz): δ 1.14 (t, 3H, J = 6 Hz), 2.27 (s, 3H), 2.61 (q, 2H, J = 6 Hz), 4.24 (s, 3H), 7.75 (t, 1H, J = 8 Hz), 7.70 (s, 2H), 7.76 (d, 2H, J = 8 Hz), 11.42 (s, 1H). ESMS m/z: 401 (MH^+). Anal. ($C_{20}H_{18}Cl_2N_4O \cdot 1.5H_2O$) C, H, N.

2-(2,6-Dichlorophenylamino)-1,6-dimethyl-7-(3-hydroxypropen-1-yl)-1,8-dihydroimidazo[4,5-*h*]isoquinolin-9-one (38). A suspension of **36** (100 mg, 0.22 mmol) in THF (7 mL) was cooled to −78 °C. Sodium bis(trimethylsilyl)amide (1 M in THF, 0.44 mmol) was added dropwise. The bright-red solution was warmed to 0 °C for 15 min. Then lithium aluminum hydride (1 M in THF, 2.6 mmol) was added, and the orange solution was then warmed to room temperature for 0.5 h. The mixture was cooled to 0 °C, quenched with saturated ammonium chloride, and extracted with EtOAc. Column chromatography (3−6% MeOH/CH_2Cl_2) provided 32 mg (34%) of the title compound. Mp 298−300 °C. ^{1}H NMR (DMSO-d_6, TFA, 400 MHz): δ 2.32 (s, 3H), 4.16 (s, 2H), 4.19 (s, 3H), 6.63 (d, 1H, J = 8 Hz), 6.82 (d, 1H, J = 8 Hz), 7.42 (bs, 1H), 7.64−7.68 (m, 4H), 10.92 (s, 1H). ESMS m/z: 429, 431 (MH^+).

2-(2,6-Dichlorophenylamino)-7-(1-acetoxyprop-3-en-1-yl)-1,6-dimethyl-1,8-dihydroimidazo[4,5-*h*]isoquinolin-9-one (33). To a solution of **31** (106 mg, 0.25 mmol) in THF (1 mL) was added acetic anhydride (1 mL). Triethylamine (35 μL, 0.25 mmol) was added, and the reaction mixture was stirred for 14 h and then concentrated in vacuo. Column chromatography (2% MeOH/CH_2Cl_2) provided 85 mg (79%) of the title compound. Mp 169−171 °C. ^{1}H NMR (MeOH-d_4, 400 MHz): δ 2.16 (s, 3H), 2.40 (s, 3H), 4.18 (s, 3H), 5.34−5.48 (m, 2H), 6.08−6.16 (m, 1H), 6.55 (s, 1H), 7.27 (bs, 1H), 7.58 (d, 2H, J = 8 Hz), 7.59 (d, 1H, J = 8 Hz), 7.68 (bs, 1H). ESMS m/z: 471 (MH^+).

2-(2,6-Dichlorophenylamino)-1,6-dimethyl-7-(3-morpholin-4-yl-propen-1-yl)-1,8-dihydroimidazo[4,5-*h*]isoquinolin-9-one (34). Tris(dibenzylideneacetone)dipalladium-(0) (1.8 mg, 0.002 mmol) and triphenylphosphine (1.6 mg, 0.006 mmol) were stirred in THF (0.5 mL) for 20 min under inert atmosphere until the red solution turned yellow. To this solution was added sequentially **33** (20 mg, 0.04 mmol) in THF (0.5 mL), triethylamine (17 μL, 0.12 mmol), and morpholine (11 μL, 0.12 mmol). The solution was stirred for 14 h and then concentrated to an oil. Column chromatography (10% MeOH/CH_2Cl_2) provided 10 mg (50%) of the title compound. Mp 175−177 °C. ^{1}H NMR (DMSO-d_6, TFA, 400 MHz): δ 2.35 (s, 3H), 3.16 (t, 2H, J = 12 Hz), 3.48 (d, 2H, J = 12 Hz), 3.66 (t, 2H, J = 12 Hz), 4.02 (d, 4H, J = 7 Hz), 4.24 (s, 3H), 6.47 (dt, 1H, J = 8, 16 Hz), 7.15 (d, 1H, J = 16 Hz), 7.58 (dd, 1H, J = 8, 9 Hz), 7.74−7.77 (m, 3H), 7.82 (d, 1H, J = 9 Hz). ESMS m/z: 498 (MH^+).

7-(3-Aminopropen-1-yl)-2-(2,6-dichlorophenylamino)-1,6-dimethyl-1,8-dihydroimidazo[4,5-*h*]isoquinolin-9-one (35). A suspension of tris(dibenzylideneacetone)dipalladium(0) (185 mg, 0.25 mmol) and triphenylphosphine (320 mg, 1.2 mmol) in THF (40 mL) was stirred for 20 min under N_2. A solution of **33** (1.88 g, 4.0 mmol) in THF (5 mL) was added, and the mixture was stirred for 20 min. Sodium azide (280 mg, 4.4 mmol) and water (4.0 mL) were added, and the reaction mixture was heated at 60 °C for 3 h. The solution was cooled to room temperature, and triphenylphosphine (1.0 g, 3.8 mmol) was added. After the mixture was stirred for 45 min, ammonium hydroxide (4 mL) was added and stirring continued overnight. The resulting solution was dried over $MgSO_4$ and then concentrated to an oil. Column chromatography on silica eluting with CH_2Cl_2/MeOH (90:10 increasing to 50:50) provided 1.2 g (70%) of the title compound. Mp >300 °C. ^{1}H NMR (DMSO-d_6, TFA, 400 MHz): δ 2.35 (s, 3H), 3.68 (bs, 2H), 4.23 (s, 3H), 6.45−6.60 (m, 1H), 6.90−7.00 (m, 1H), 7.55−7.65 (m, 1H), 7.70−7.85 (m, 4H), 8.05−8.20 (m, 1H). ESMS m/z 428 (MH^+).

2-(2,6-Dichlorophenylamino)-1,6-dimethyl-7-(3-(ethylamino)propenyl)-1,8-dihydroimidazo[4,5-*h*]isoquinolin-9-one (39). A solution of **35** (100 mg, 0.234 mmol) in acetic acid (1 mL) was cooled to −10 °C. Sodium triacetoxyborohydride (87 mg, 0.410 mmol) was added, and the mixture was stirred for 20 min at −10 °C. Acetaldehyde (10 mg, 0.234 mmol) was taken up in THF (3 mL) and added dropwise to the reaction mixture (at −10 °C). Once all the aldehyde had been added, the reaction mixture was warmed to room temperature and was stirred overnight. The crude reaction mixture was diluted with EtOAc (10 mL), washed with water (5 mL), dried ($MgSO_4$), and concentrated under reduced pressure. Chromatography [CH_2Cl_2/MeOH/NH_4OH (9:1:0.1)] afforded 28 mg (26%) of the title compound. Mp 218−221 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.15−1.25 (m, 3H), 2.33 (s, 3H), 3.03−3.13 (m, 2H), 3.80 (bs, 2H), 4.21 (s, 3H), 6.45−6.60 (m, 1H), 7.09 (d, 1H, J = 15 Hz), 7.55 (t, 1H, J = 8 Hz), 7.7−7.9 (m, 4H).

2-(2,6-Dichlorophenylamino)-7-(3-diethylaminopropenyl)-1,6-dimethyl-1,8-dihydroimidazo[4,5-*h*]isoquinolin-9-one (43). 43 was prepared as described above. ^{1}H NMR (DMSO-d_6, TFA, 400 MHz): δ 1.27 (t, 6H, J = 7.0 Hz), 2.38 (s, 3H), 3.04−3.40 (m, 4H), 4.01 (d, 2H, J = 7 Hz), 4.26 (s, 3H), 6.54 (dt, 1H, J = 7, 16 Hz), 7.26 (d, 1H, J = 16 Hz), 7.60 (t, 1H, J = 8 Hz), 7.78 (d, 3H, J = 8 Hz), 7.84 (d, 1H, J = 9 Hz).

2-(2,6-Dichlorophenylamino)-1,6-dimethyl-7-(3-pyrrolidin-1-ylpropenyl)-1,8-dihydroimidazo[4,5-*h*]isoquinolin-9-one (44). 44 was prepared as described above. ^{1}H NMR (DMSO-d_6, TFA, 400 MHz): δ 1.91 (br, 2H), 2.06 (br, 2H), 2.38 (s, 3H), 3.11 (br, 2H), 3.59 (br, 2H), 4.04 (d, 2H, J = 7 Hz), 4.26 (s, 3H), 6.55 (dt, 1H, J = 7, 15 Hz), 7.16 (d, 1H, J = 15

Hz), 7.60 (dd, 1H, $J = 8$, 8 Hz), 7.78 (d, 3H, $J = 8$ Hz), 7.84 (d, 1H, $J = 8$ Hz). Anal. ($C_{25}H_{25}Cl_2N_5O_1 \cdot 0.2H_2O$) C, H, N.

2-(2,6-Dichlorophenylamino)-1,6-dimethyl-7-[3-(benzylmethylamino)propenyl]-1,8-dihydroimidazo[4,5-*h*]isoquinolin-9-one (45). 45 was prepared as described above. ^{1}H NMR (DMSO-d_6, TFA, 400 MHz): δ 2.38 (s, 3H), 2.76 (s, 3H), 3.81−4.10 (m, 2H), 4.27 (s, 3H), 4.31 (d, 1H, $J = 13$ Hz), 4.51 (d, 1H, $J = 15$ Hz), 6.62 (dt, 1H, $J = 7$, 15 Hz), 7.20 (d, 1H, $J = 16$ Hz), 7.40−7.67 (m, 6H), 7.71−7.92 (m, 3H), 7.85 (d, 1H, $J = 8$ Hz). Anal. ($C_{29}H_{27}Cl_2N_5O_1 \cdot 0.1H_2O$) C, H, N.

2-(2,6-Dichlorophenylamino)-1,6-dimethyl-7-(3-dimethylaminopropenyl]-1,8-dihydroimidazo[4,5-*h*]isoquinolin-9-one (46). 46 was prepared as described above. ^{1}H NMR (DMSO-d_6, TFA, 400 MHz): δ 2.37 (s, 3H), 2.85 (s, 6H), 3.96 (d, 2H, $J = 7$ Hz), 4.26 (s, 3H), 6.52 (dt, 1H, $J = 7$, 15 Hz), 7.16 (d, 1H, $J = 15$ Hz), 7.60 (t, 1H, $J = 8$ Hz), 7.77 (d, 3H, $J = 8$ Hz), 7.84 (d, 1H, $J = 9.0$ Hz). Anal. ($C_{23}H_{23}Cl_2N_5O_1 \cdot 0.2H_2O$) C, H, N.

2-(2,6-Dichlorophenylamino)-1,7-dimethyl-6-(2-hydroxyethyl)-1,8-dihydroimidazo[4,5-*h*]isoquinoline-9-one (54). To a stirred solution of 2-(2,6-dichlorophenylamino)-1,7-dimethyl-9-oxo-1,8-dihydroimidazo[4,5-*h*]isoquinolin-6-ylacetic acid ethyl ester (25 mg, 0.05 mmol) in THF (2 mL) was added a solution of lithium aluminum hydride (1 M in THF, 0.25 mL, 0.25 mmol). The mixture was stirred for 30 min at room temperature. EtOAc was added, followed by water, and then the mixture was acidified with 1 N HCl. The crude product was applied to a Varian SCX cartridge and washed in turn with 1 N HCl, water, acetone, MeOH, and MeOH/CH_2Cl_2 (1:1). The product was then eluted with MeOH/CH_2Cl_2/NH_4OH (49:49:2). Evaporation of the eluent provided 15 mg (72%) of the title compound. Mp >300 °C. ^{1}H NMR (TFA-d_6, 400 MHz): δ 2.76 (s, 3H), 3.49 (br, 2H), 4.22 (br, 2H), 4.45 (s, 3H), 7.55 (t, 1H, $J = 8$ Hz), 7.68 (d, 2H, $J = 8$ Hz), 8.04 (d, 1H, $J = 9$ Hz), 8.19 (d, 1H, $J = 9$ Hz). ESMS *m*/*z* 417, 419 (MH^+). Anal. ($C_{20}H_{18}Cl_2N_4O_3 \cdot 1.5H_2O$) C, H, N.

Tyrosine Kinase Inhibition Assay. The kinase activity is measured using DELFIA (dissociation enhanced lanthanide fluoroimmunoassay), which utilizes europium chelate-labeled anti-phosphotyrosine antibodies to detect phosphate transfer to a random polymer, poly-Glu_4-Tyr_1 (PGTYR). The kinase assay is performed in a neutravidin-coated 96-well white plate (PIERCE) in kinase assay buffer (50 mM HEPES, pH 7.0, 25 mM $MgCl_2$, 5 mM $MnCl_2$, 50 mM KCl, 100 μM Na_3VO_4, 0.2% BSA, 0.01% CHAPS). Test samples initially dissolved in DMSO at 1 mg/mL are prediluted for dose response (10 doses with starting final concentration of 1 μg/mL, 1−3.5 serial dilutions) with the assay buffer. A 25 μL aliquot of this diluted sample and a 25 μL aliquot of diluted enzyme (0.8 nM final concentration) are sequentially added to each well. The reaction is started with a 50 μL/well of a mixture of substrates containing 2 μM ATP (final ATP concentration is 1 μM) and 7.2 ng/μL PGTYR-biotin (CIS Biointernational) in kinase buffer. Background wells are incubated with buffer and substrates only. Following 45 min of incubation at room temperature, the assay plate is washed three times with 300 μL/well DELFIA wash buffer. A 100 μL/well aliquot of europium-labeled anti-phosphotyrosine (Eu^{3+}-PT66, 1 nM, Wallac CR04-100) diluted in DELFIA assay buffer is added to each well and incubated for 30 min at room temperature. Upon completion of the incubation, the plate is washed four times with 300 μL/well of wash buffer and 100 μL/well of DELFIA wash buffer. Enhancement solution (Wallac) is added to each well. After 15 min, time-resolved fluorescence is measured on the LJL's analyst (excitation at 360 nm, emission at 620 nm, EU 400 dichroic mirror) after a delay time of 250 μs.

Calcium Release in Jurkat Cells. 1. Loading of Jurkat Cells with Fluo-3. Jurkat cells were pelleted, washed 2 times with RPMI, 10 mM HEPES, and 10% fetal bovine serum (complete media), and then resuspended at 2×10^7 cells/mL in the above. Fluo-3 solution was prepared from 40 μL of Fluo-3 stock AM (Molecular Probes catalog no. F-1242) (4 μM), 180 μL of pluronic F127 detergent (Molecular Probes catalog no. P-3000), and 9.8 mL of complete media. An equal volume of Fluo-3 solution was added to give a 1×10^7 cells/mL solution and 2 μM Fluo-3. The tube was wrapped in foil and incubated at room temperature with very gentle rocking for 45 min. Additional media to fill a 50 mL tube was added, and it was incubated another 15 min at room temperature.

Cells were pelleted and washed three times with Hanks' balanced salt solution (HBSS) (GibcoBRL no. 14175-095), containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 2 mM probenecid, and 1% fetal bovine serum (FBS), pH 7.4. Finally, cells were resuspended at 2×10^7 cells/mL and kept on ice and in the dark until ready for use.

Compounds were made up as 5 mg/mL stocks in DMSO and diluted as appropriate with the following diluent: HBSS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4, 2 mM probenecid, 1% FBS. A 96-well V-bottom plate is used for the compound dilutions.

2. Stimulus Plate. Anti-CD3 (Immunotech, clone X35, catalog no. 0178) was prepared by taking 69 μL of a 200 μg/mL stock into 11 mL of diluent to give a 5× stock for a final test concentration of 0.25 μg/mL. Then 100 μL was added per well of V-bottom 96-well plate.

3. Cell Plate Preparation. An amount of 2 mL of loaded cell suspension was mixed with 18 mL of diluent, and 150 μL of this mixture was then added per well of a 96-well Black Packard viewplate for a final cell number of 3×10^5/mL. The plate was then centrifuged at 1500 rpm for 5 min at room temperature and immediately placed in the center position in the drawer of an FLIPR (fluorometric imaging plate reader).

4. Assay. The assay was performed on the FLIPR system (Molecular Devices). All experiments were run at room temperature. The cell plate was placed in the center position in the drawer. The compound dilution plate was placed in the left position and the stimulus plate in the right. An amount of 50 μL of compound was added to the cells, and this was incubated at room temperature for 15 min. An amount of 50 μL of anti-CD3 was added to the cell plate, and fluorescence intensity was monitored for an additional 13 min.

5. Analysis. Calcium release was measured by fluorescence intensity via a SAS program that measures the difference between baseline values and peak height. These values are plotted versus compound concentration to obtain an EC_{50} value. A decrease in fluorescence intensity indicated inhibition of the release of calcium by the compound being tested.

Inhibition of IL-2 Production. The 96-well flat bottom plates were coated with anti-CD3 and clone UCHT1 (Immunotech catalog no. 1304) at 4 μg/mL in phosphate-buffered saline (PBS), 100 μL/well. The solution was prepared by taking 200 μL of 200 μg/mL anti-CD3 stock/10 mL PBS. The plate was then incubated at 37 °C for 2 h. Jurkat cells were pelleted and counted. The cells were resuspended at 2.5×10^6 cells/mL in RPMI and 10% FBS (complete media). Test compounds were diluted from a 5 mg/mL DMSO stock directly into complete media.

An amount of 10 μL of 20 X compound per well was added to a separate plate, followed by 100 μL of cell suspension in triplicate, and this plate was preincubated at 37 °C for 30 min. The 96-well plate containing anti-CD3 was aspirated, and the cells and compound were transferred to this plate. An amount of 100 μL of PMA (phorbol 12-myristate 13-acetate, Sigma catalog no. P-8139) at 20 ng/mL was added, and the plate was incubated overnight at 37 °C. (PMA stock at 1 mg/mL in ethanol, diluted 10 μL/mL in complete media, then 20 μL/10 mL in complete media; 100 μL/well = 10 ng/mL, final concentration). The next day, the plate was centrifuged at 1500 rpm for 5 min at room temperature and the supernatants were removed. The supernatants were tested using R&D Systems Quantikine Human IL-2 Kit (catalog no. 2050). Samples were diluted 1:5 in RPMI 1640, and 100 μL/well was used in the ELISA. The optical density of each well was determined using a microplate reader set to 450 nm. EC_{50} values were determined using Origin (nonlinear regression) or SAS by plotting absorbance vs concentration of compound.

Inhibition of IL-2 Production in Human Whole Blood. Human whole blood was obtained from in-house donors by venipuncture collected into 25 mL heparinized Vacu-tainer tubes.

Test compounds were dissolved in DMSO to yield 5 mg/mL stock solutions. Stock solutions were freshly diluted in RPMI 1640 and 10% fetal bovine serum (complete media) to yield solutions that were 10 times the final assay concentration. Compounds were diluted serially into complete media.

SEB Solution. Staphylococcal enterotoxin B was dissolved in PBS to yield a 2.5 mg/mL stock. This is further diluted in complete media to yield a 3000 ng/mL solution (10 times the final assay concentration). SEB stock solution is stored at 4 °C.

Assay Procedure. Add 20 μL of 10× compound dilutions, in triplicate, to wells of a U bottom 96-well plate. For "no compound" samples, add 20 μL of complete media. Also, dilute DMSO to the highest concentration used in the assay and use 20 μL/well as controls. Add 160 μL of whole human blood to each well and incubate plate for 20−30 min at 37 °C. Add 20 μL of 10x SEB solution to all test wells and to stimulated control wells. Add 20 μL of complete media to nonstimulated wells as controls. Incubate plate overnight at 37 °C. The next day, centrifuge plate at 2000 rpm for 10 min at room temperature and transfer supernatants to a fresh plate. Dilute supernatants 1:5 in RPMI 1640 and then use 100 μL/well in R&D Systems Quantikine Human IL-2 Kit.

Follow the ELISA procedure as outlined in the section "Inhibition of IL-2 Production" in Jurkat cells protocol.

1. Analysis. EC_{50} values are determined using Origin (nonlinear regression) or SAS (Secondary Screening Program, EC_{50} calculation) by plotting absorbance vs actual concentration of the compound.

2. In Vivo Anti-CD3 Assay. Female BALB/c mice (Charles River, The Jackson Laboratories) were used for anti-CD3 studies. Animals were numbered by tail tattoo and were 6−8 weeks old at the initiation of experiments. For the induction of IL-2 in vivo by anti-CD3, 1 μg of anti-CD3 (monoclonal hamster−antimouse 145-2C11, lot M03283) in 200 μL of PBS was injected intraperitoneally (ip) into experimental mice to stimulate the polyclonal activation of T cells. Upon activation, these T cells produced the T cell cytokine IL-2 that was detected in the plasma.

For the measurement of plasma cytokine levels, 3 h after anti-CD3 injection, each mouse was anesthetized with isoflurane, and approximately 0.5 mL of whole blood was collected in heparinized tubes following cardiac puncture. Plasma IL-2 levels were determined by ELISA (R&D Systems, Minneapolis MN) of 1:10 dilutions of samples. Levels were quantitated by linear regression analysis of samples in comparison to a recombinant cytokine IL-2 curve, and plasma levels in vehicle-treated groups were 1900−2800 pg/mL in each experiment.

3. Treatment Groups. All doses of compounds and controls are represented as milligram of compound per kilogram of body mass and were administered orally in 100 μL of vehicle, 30% Cremophor. The compound was administered to mice ($n = 8$ per group) 1 h prior to anti-CD3 injection. Control mice received either 100 μL of vehicle alone (negative control) or 30 mg/kg cyclosporin A (positive control).

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