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N-4-Pyrimidinyl-1H-indazol-4-amine inhibitors of Lck: Indazoles as phenol isosteres with improved pharmacokinetics

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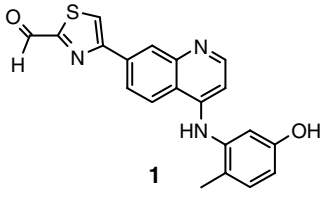
Abstract—2,4-Dianilino pyrimidines are well-known inhibitors of tyrosine kinases including lymphocyte specific kinase (Lck). Structure–activity relationships at the 4-position are discussed and rationalised. Examples bearing a 2-methyl-5-hydroxyaniline substituent at the 4-position were especially potent but showed poor oral pharmacokinetics. Replacement of this substituent by 4-amino(5-methyl-1H-indazole) yielded compounds with comparable enzyme potency and improved pharmacokinetic properties.

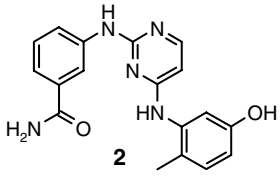
Lck, a member of the Src family of tyrosine kinases, is important for the development and activation of T-cells. Mice which are Lck-deficient or which express a kinase-inactive mutant Lck show T-cell deficiency.[1,2] Antigen response thresholds are elevated in Lck−/− mice and return to normal when Lck is inducibly expressed.[3] T-cell receptor ζ-subunit phosphorylation and signal transduction is also regulated by Lck.[4] A consequence of Lck activation is the induction of cytokines including IL-2 which is capable of supporting T-cell proliferation.[5] Inhibition of Lck kinase activity therefore offers one approach to the treatment of T-cell mediated inflammatory disorders including rheumatoid arthritis, transplant rejection and inflammatory bowel disease.[6]

4-Anilino quinolines and quinazolines are potent inhibitors of tyrosine kinases.[7,8] Of the quinolines, among the most potent against Lck were compounds with a 2-methyl 5-hydroxy aniline as a 4-substituent, for example **1** (Lck IC_{50} = 59 nM).[9]

The 2,4-dianilino pyrimidines are another intensively studied series of potent protein kinase inhibitors.[10,11] 4-Anilino quinolines and the 2,4-dianilino pyrimidines are likely to bind to Lck so that their 4-substituents overlay closely. With this expectation, the 2-methyl 5-hydroxy aniline substituent was taken from **1** and introduced into the 4-position of the pyrimidines (to give e.g. **2**). The routes shown in Scheme 1 were used to synthesise the molecules from readily available 2,4-dichloro pyrimidine or 2-methyl thio-4-hydroxypyrimidine.[12]



1



2

In order to focus on a thorough exploration of SAR at the 4-position, the majority of the early compounds were made with 3-aminobenzamide as the pyrimidinyl 2-substituent. Greater variation at the 2-position was

Keywords: 2,4-Dianilino pyrimidines; Lck inhibitors; p56Lck inhibitors; Protein kinase inhibitors.
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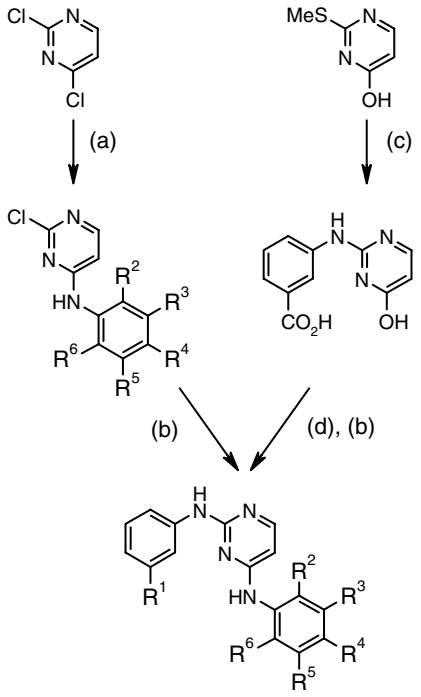


Scheme 1. Synthesis of 2,4-dianilino pyrimidines **2**–**25**. Reagents and conditions: (a) substituted aniline, $NaHCO_3$, *t*-BuOH, reflux; (b) substituted aniline, acetone/water/cHCl (150:100:2), 80 °C; (c) 3-aminobenzoic acid, diglyme, 180 °C; (d) $POCl_3$, reflux; NH_3/dioxane, rt.

incorporated later, but these compounds mostly fall outside the scope of this manuscript. Several analogues with a sulfonamide 2-substituent will be discussed to show that the amide is not crucial for activity and that the SAR at the 4-position is consistent.

Substituents to be used at the 4-position were chosen with the likely binding mode in mind. Compound **2** was docked into the published X-ray structure of Lck (Fig. 1).[13] In the model, the pyrimidine forms two typical hydrogen bonds to the hinge of the Lck ATP-site (between the backbone NH and carbonyl of Met319, and the N1 atom of the pyrimidine and the 2-anilino

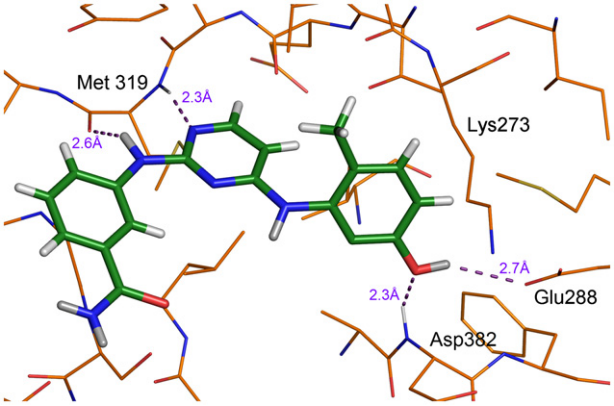


Figure 1. Model of compound **2** docked into the ATP site of Lck.[13] Hydrogen bonds are shown as purple dotted lines.

NH, respectively). This is in agreement with published crystal structures of similar compounds bound to CDK2.[14] In these structures the phenyl ring of the 4-aniline substituent is directed towards the front of the ATP-binding site, but in the model used here it is angled towards the back of the site in a predominantly hydrophobic location. Its 2-methyl substituent binds in a small lipophilic hole formed by sidechains of the Lck N-terminal lobe (Val259, Ala271, and Lys273). The 5-hydroxyl group points towards the backbone of Asp382 and the sidechain of Glu288. These interactions, illustrated in Figure 1, governed the choice of aniline reagents for the pyrimidinyl 4-position.

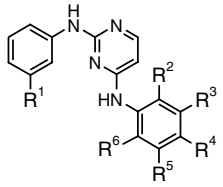
In addition to substituents chosen to present differently shaped hydrophobic groups to the kinase back pocket, anilines were chosen to provide SAR information about the nature of the interactions made by the 2-methyl and the 5-hydroxyl. For the resulting compounds, IC_{50} values were measured for inhibition of Lck activity. Averaged values are shown in Table 1.[9] 3-Aminobenzamide was used as the pyrimidinyl 2-substituent because it is directed towards solvent (Fig. 1) and was expected to be indicative of the activity of 3-aminobenzamides with larger amide substituents.

From these data, it can be seen that the 2-methyl contributes about 10-fold to the activity (compare **3** to **2**). Moving the methyl around the ring to the 3- and 4-positions (**4** and **5**) did not restore full activity. This was predicted by the model: the 2-methyl group not only fills the small lipophilic hole described earlier, but would also affect the orientation of the aromatic ring into the back pocket. In the docking, the NH linker at the 4-position of the pyrimidine lies in the plane of the pyrimidine, while the phenyl sits out of plane. The presence of a 2-substituent would encourage this geometry. Introduction of larger 2-substituents than the methyl (**6** and **7**) was less potent, showing that they are too large for the pocket.

The 5-hydroxyl group was also of great importance. Removing this entirely (**8**) led to a loss of 7-fold in potency, which was only partially restored by moving the hydroxyl to the 4- or 6-positions (**9** and **10**). Replacing the hydroxyl with F or Cl (**11** and **12**) resulted in even lower activity. In an attempt to see whether the acceptor or donor functionality of the hydroxyl was responsible for activity, it was replaced by methoxy (**13**) and amino (**14**) groups. Both showed similar activity to the 5-H compound **8**. It seems that for greatest activity the 5-substituent requires both the acceptor and donor properties of the phenol. Slightly larger groups containing H-bond acceptors and donors were tried (e.g., **15**–**18**) which all led to much reduced activity, suggesting that space at the 5-position is limited. Compounds lacking the 2-Me and the 5-hydroxyl were less potent still, generally having $IC_{50} > 1$ μM (e.g., **19**–**22**). No simple analogues were able to achieve the activity of **2**.

In the model, the oxygen of the 5-hydroxyl is 2.3 Å away from the backbone NH of Asp382. This hydrogen bond explains the importance of the 5-hydroxyl acceptor

Table 1. Lck inhibition by 2,4-dianilino pyrimidines **2**–**25** (nM)[9]



Compound	R^1	R^2	R^3	R^4	R^5	R^6	IC_{50}
2	$CONH_2$	Me	H	H	OH	H	13
3	$CONH_2$	H	H	H	OH	H	130
4	$CONH_2$	H	Me	H	OH	H	73
5	$CONH_2$	H	H	Me	OH	H	190
6	$CONH_2$	Br	H	H	OH	H	41
7	$CONH_2$	iPr	H	H	OH	H	880
8	$CONH_2$	Me	H	H	H	H	89
9	$CONH_2$	Me	H	OH	H	H	40
10	$CONH_2$	Me	H	H	H	OH	100
11	$CONH_2$	Me	H	H	F	H	270
12	$CONH_2$	Me	H	H	Cl	H	380
13	$CONH_2$	Me	H	H	OMe	H	86
14	$CONH_2$	Me	H	H	NH_2	H	97
15	$CONH_2$	Me	H	H	$CONH_2$	H	1300
16	$CONH_2$	Me	H	H	$CONMe_2$	H	9300
17	$CONH_2$	Me	H	H	$NHSO_2Me$	H	7700
18	$CONH_2$	Me	H	H	CH_2NH_2	H	3400
19	$CONH_2$	H	H	H	OMe	H	2700
20	$CONH_2$	H	H	H	F	H	3300
21	$CONH_2$	H	H	H	N(Me)COMe	H	1800
22	$CONH_2$	F	H	F	H	H	1100
23	SO_2NH_2	Me	H	H	OH	H	8.5
24	SO_2NH_2	Me	H	H	OMe	H	54
25	SO_2NH_2	F	H	F	H	H	350

functionality. If the hydroxyl lone pair points towards Asp382, its hydrogen is directed towards the sidechain of Glu288. While the 2.7 Å distance is quite long for a direct H-bond, it seems likely that either Glu288 moves closer when the compound binds or that a water-mediated interaction is present. After this work was completed, a crystal structure of **1** bound to p38 MAP kinase was solved which confirmed these predictions, at least for the quinoline analogue bound to a surrogate kinase.[15] A similar result has been reported for a benzimidazole template, the most potent example of which contained the same 2-methoxy 5-hydroxy aniline. The position of the aniline in the crystal structure of a benzimidazole in Hck agrees with the docking model of **2** and shows that the hydroxyl donates a hydrogen bond to Glu288.[16] This proposed orientation of the bicyclic aniline is flipped relative to that seen in the X-ray structure of unactivated Src with a quinazoline bearing a (5-chloro-1,3-benzodioxol-4-yl)amine 4-substituent.[17] If our binding mode is correct, differences between the active Lck conformation of Asp382 and its equivalent in inactive Src (Asp404) may account for the difference in compound binding.

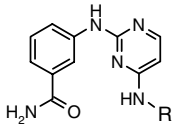
Analogues of many compounds were prepared with 3-aminobenzenesulfonamide instead of 3-aminobenzamide. Their activity was very similar to or slightly greater than their amide analogues. For example, compare **23** to **2**, **24** to **13** and **25** to **22**.

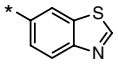
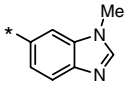
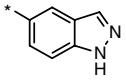
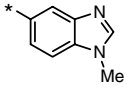
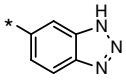
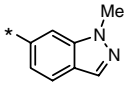
Pharmacokinetic properties of the most potent phenol compounds were determined in mice and found to be poor (see Table 5) making them unsuitable candidates for oral administration. In particular, they exhibited high clearance, short half-life and low oral bioavailability. It was thought likely that the 5-hydroxyl aniline at the pyrimidinyl 4-position was responsible for this, given the susceptibility of phenols to phase II metabolism.[18] The SAR above indicates a preference for a very small group at the 5-hydroxyl position, ideally with H-bond acceptor and donor vectors in the correct directions. Finding a suitable replacement with these properties was a significant challenge. To try to incorporate these features, a second array (**26**–**43**) was prepared using conditions described above in Scheme 1, in which the phenol group was replaced with fused bicyclic heterocycles containing at least some of the H-bonding potential of the 5-hydroxyl group.[19]

Table 2 shows data for selected compounds in which the 4-anilino position of the pyrimidine is a bicyclic ring fused between the 3- and 4-positions relative to the linker. These compounds (**26**–**31**) were only weakly active against Lck, with IC_{50}s in the 1–10 μM range.

Table 3 shows data for compounds where the rings were fused between the 2- and 3-positions relative to the linker. These were more successful than the 3,4-fused rings, with several examples showing $IC_{50} < 1$ μM (**32**–**34**), although

Table 2. Lck enzyme activity of pyrimidines containing 4-substituted amino bicycles, with rings fused between the 3- and 4-positions (nM)[9]



Compound	R	IC_{50}
26		3000
27		16,000
28		3000
29		1800
30		4400
31		8200

some were still weak (**35**). The indazole **36** was the most potent, comparing favourably with the phenol **3**.

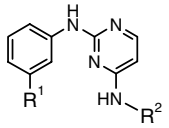
Having established that the indazole ring was an active isostere of the 5-phenol, the possibility of including a methyl ortho to the linker was then explored. This was successfully introduced, giving **37**, which has equivalent Lck activity to **2**. As expected, moving the methyl around the ring led to compounds with lower potency (**38** and **39**).

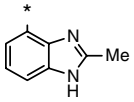
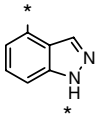
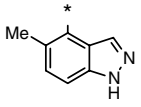
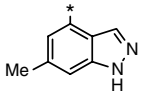
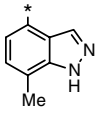
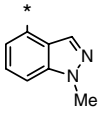
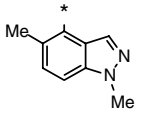
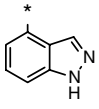
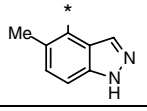
Blocking the indazole H-bond donor capability by N-methylation resulted in over 10-fold loss of activity (compare **40** to **36**). Methylation next to the aniline linker was unable to rescue the activity (**41**). The importance of the H-bond donor NH at the 1-position of the 1H-indazol-4-amine is further shown by the 14-fold loss of activity when the indazole NH is moved around the ring (**34**).

As before, 3-aminobenzenesulfonamide was tolerated as the pyrimidinyl 2-substituent (compare **42** to **36** and **43** to **37**).

These observations were consistent with the modelling predictions. Figure 2 shows the docking of **37** compared to **2**. The hydrogen bonding vectors of the indazole mimic those of the phenol very closely, and maintain the direct interaction with Asp382 and the direct or water-mediated H-bond to Glu288. The loss of these two interactions explains the lower potency of all of the compounds in which they are disrupted.

Table 3. Lck enzyme activity of pyrimidines containing 4-substituted amino bicycles, with rings fused between the 2- and 3-positions (nM)[9]



Compound	R^2	IC_{50}
	$R^1 = CONH_2$	
32		560
33		490
34		1400
35		5600
36		100
37		12
38		520
39		55
40		1600
41		2800
	$R^1 = SO_2NH_2$	
42		33
43		19

The introduction of the indazole group had an unexpectedly beneficial effect on the protein kinase selectivity profile. The more potent phenol and indazole di-anilino pyrimidines did not show submicromolar inhibition of

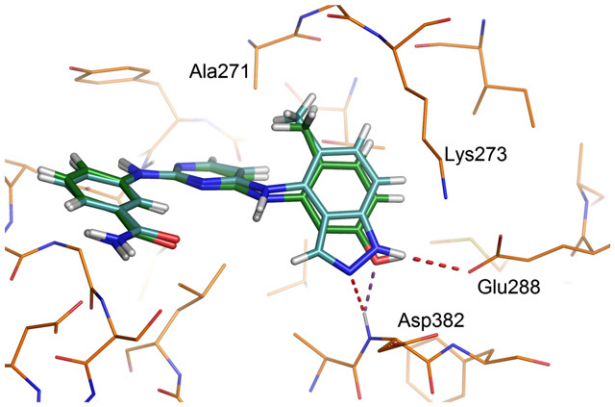


Figure 2. Model of compound **37** (light blue) bound into the ATP site of Lck. Compound **2** (green) is superimposed to show the similar hydrogen bonding vectors.

Ser/Thr kinases tested, including GSK-3β, IKK-β and JNK3. However, the phenols **2** and **23** had appreciable activity against some tyrosine kinases including Btk, Lyn, Syk and Txk (Table 4). The direct indazole analogues **37** and **43** exhibited lower activity against these kinases.

The pharmacokinetic parameters of key compounds were determined in mice.[20] The data shown in Table 5 summarise the pharmacokinetic differences between direct analogues **23** and **43**, where the only structural difference is the replacement of the phenol with the indazole isostere. The indazole has a considerably lower plasma clearance and greater oral bioavailability than the phenol. This suggests that the phenol was metabolically more labile and was the main reason for the poor pharmacokinetic profile of **23**.

In summary, 2,4-dianilino pyrimidines are a class of potent Lck inhibitors. The greatest activity was achieved when the pyrimidyl 4-substituent was 2-methyl 5-hydroxyaniline. However, these compounds had poor pharmacokinetic properties. Simple 4-position alternatives were unable to reach the same level of activity. One of the highest priority isosteric replacements based on modelling predictions was 5-methyl-1H-indazol-4-amine. Pyrimidines containing this group at the pyrimidinyl 4-position retained the high enzyme activity of the phenols (compounds **37** and **43** had Lck IC_{50} of 12 nM and 19 nM, respectively) with improved selectivity. This result provided useful supporting evidence for the binding mode and the role of hydrogen bonding in the inner part of the ATP-binding site for Lck potency. Most significantly, they had much improved pharmacokinetic parameters, including lower plasma clearance, longer half-life and greater oral bioavailability. As well as their significance for the 2,4-dianilino pyrimidine series, these results may be of interest to other medicinal chemistry programmes where phenols are important for activity.

Table 4. Tyrosine kinase selectivity IC_{50} values (nM)

Compound	4-substituent	Lck	Btk	Lyn	Syk	Txk
2	Phenol	13	9	3	26	2
37	Indazole	12	97	40	220	28
23	Phenol	8.5	n.t.	1	4	0.4
43	Indazole	19	69	430	620	220

n.t., not tested.

Table 5. Summary of pharmacokinetic parameters determined in mice[20]

Compound	**23** (phenol)		**43** (indazole)	
Route	IV	PO	IV	PO
Dose (mg/kg)	2.5	10	1	1
Cl_p (ml/min/kg)	65.5	—	22.4	—
V_{ss} (l/kg)	0.3	—	0.7	—
$T_{1/2}$ (h)	0.12	—	0.53	—
AUC_{0-t} (ng h/ml)	635	2	743	190
F (%)	—	<1	—	25

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9. Lck activity was assessed using a TR-FRET (time-resolved fluorescence resonance energy transfer) assay. Murine Lck was overexpressed in insect cells and used as a semi-purified lysate. Lck was pre-activated at room temperature for 30 min in the presence of 10 mM $MgCl_2$, 100 μM ATP, then diluted 100-fold in 100 mM Hepes, pH 7.4. 15 μl of activated Lck was added to wells containing 1 μl of various concentrations of compound or DMSO vehicle (3.3% final) in NUNC 384-well black plates and incubated for 15 min at room temperature. The reaction was initiated by the addition of 15 μl of substrate reagent containing biotinylated peptide, Biotin-EEEEYFELV (0.5 μM final), ATP (120 μM final) and $MgCl_2$ (20 mM final) in 100 mM Hepes, pH 7.4. The reaction mixture was

incubated for 30 min at room temperature, then terminated by the addition of 30 μl of read reagent containing 60 mM EDTA, 150 mM NaCl, 25 nM Streptavidin APC (Perkin-Elmer, Beaconsfield, Bucks, UK), 0.4 nM anti-phosphotyrosine antibody labelled with W-1024 europium chelate (Wallac OY, Turku, Finland) in 40 mM Hepes, pH 7.4, 0.25% BSA. The reaction mixture was further incubated for 15 min at room temperature. The degree of phosphorylation of Biotin-EEEEYFELV was measured using a Packard Discovery plate reader as a ratio of specific 665 nm energy transfer signal to reference Eu 620 nm signal. All values quoted are means of IC_{50} results. The average standard deviation of pIC_{50} retests was 0.3. Human Lck IC_{50} values were found to be in close agreement with mouse Lck, typically within twofold (e.g., **2** had human Lck IC_{50} of 14 nM).

10. See numerous patents, including Davis, P.; Moffat, D.; Davis, J.; Hutchings, M. WO1997019065, 1997.
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12. Compound **14** was formed from the carboxylic acid precursor under standard Curtius rearrangement conditions: (a) diphenylphosphoryl azide, Et_3N, *t*-BuOH, reflux; (b) 4 M HCl in dioxane, CH_2Cl_2, rt. Compounds **15** and **16** were made from the carboxylic acid precursor under standard amide coupling conditions. Phthalimide protection was employed to protect the primary amine functionality in compound **18**.
13. Coordinates of Lck were taken from a complex with AMP-PNP (PDB entry 1qpc) Zhu, X.; Kim, J.; Rose, P.; Stover, D.; Toledo, L.; Zhao, H.; Morgenstern, A. *Structure* **1999**, *7*, 651; Gold version 1.2 (CCDC, Cambridge, UK) Jones, G.; Willet, P.; Glen, R.; Leach, A.; Taylor, R. *J. Mol. Biol.* **1997**, *267*, 727, was used to dock compounds, with H-bonding constraints applied to ensure that the pyrimidine interacted with the hinge.
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19. Compounds in Tables 2 and 3 were prepared as described in Scheme 1, except for: Compound **36**—the second aniline displacement step was performed in a refluxing mixture of *n*-BuOH/MeOH (1:1); 3,4-dimethoxy benzyl N-protection of the indazole nucleophile was employed in the preparation of **37** and **43**.
20. The pharmacokinetic parameters in female BalbC mice were determined following intravenous (IV) and oral (PO) administration at various doses between 1 and 10 mg/kg. Compounds were administered as solutions in formulations typically consisting of 15% DMSO:45% cyclodextrin (25% aq):40% water or 5% DMSO:40% Vit E/EtOH (60:40):40% PEG400:15% mannitol and blood collected over an 8-h time period. Plasma was prepared following centrifugation and compound extracted from 100 μl plasma using protein precipitation with acetonitrile. Samples were then evaporated under nitrogen and re-suspended in 100 μl of 10:90 acetonitrile:water. Analysis was performed using LC–MS/MS on the API365 with a 5.6 min fast gradient comprising 0.1% formic acid in water and 0.1% formic acid in acetonitrile (mobile phases), 40 μl injection volume, flow rate 4 ml/min and ODS3 Prodigy column (5 cm × 2.1 mm, 5 μm). Pharmacokinetic data were generated using PKTools.