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4-Pyridin-5-yl-2-(3,4,5-trimethoxyphenylamino)pyrimidines: Potent and Selective Inhibitors of ZAP 70.

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Abstract: Activation of the tyrosine kinase ZAP 70 has been shown to be crucial to the transduction of the T-cell receptor signalling pathway, which leads ultimately to proliferation, cytokine gene expression and T-cell effector functions. A series of 2-phenylaminopyrimidines have been identified as potent and selective inhibitors of ZAP 70[1].

Occupancy of the T-cell antigen receptor (TCR) initiates an intracellular signalling cascade leading ultimately to cytokine gene expression, proliferation and the execution of T-cell effector functions. Crucial to the transduction of TCR signalling is the phosphorylation of a number of intracellular substrates, mediated by sequential activation of two distinct families of cytoplasmic protein tyrosine kinases (PTKs)[2]. The src kinases p56lck and p59fyn phosphorylate tyrosine residues on the TCR/CD3ζ-chain, contained within conserved sequences known as immunoreceptor tyrosine-based activation motifs (ITAMs). The phosphorylated ITAMs serve to recruit the T-cell specific syk family PTK, ZAP 70, to the activated TCR complex. Several reports have demonstrated that ZAP 70 plays an important role in T-cell activation. A familial form of severe combined immunodeficiency in humans through loss of functional ZAP 70 has been documented[3]. Targeted disruption of the ZAP 70 gene in mice leads to defects in thymic development and T-cell activation[4]. A T-cell line (P116) lacking ZAP 70 displays severe defects in TCR induced signaling functions, including tyrosine phosphorylation, intracellular Ca^{2+} mobilisation and IL-2 transcription[5]. Inhibitors of ZAP 70 may therefore represent potential therapies for autoimmune disease and transplantation. Given that the tyrosine kinases represent a large family of structurally related proteins involved in many signal transduction pathways[6], the design of high selectivity into such inhibitors is crucial to the development of therapeutically useful agents.

To date, although no specific inhibitors of ZAP 70 have been reported, several compound classes have been reported as tyrosine kinase inhibitors and have been extensively reviewed[6]. Efforts from our laboratories to generate lead structures involved the design of compounds containing known inhibitor motifs, derived from kinase inhibitors such as **1-3**[7] which compete for the ATP-binding site at the catalytic domain of their target enzyme. This approach led us to our lead compound **4,** a 2-phenylaminopyrimidine.

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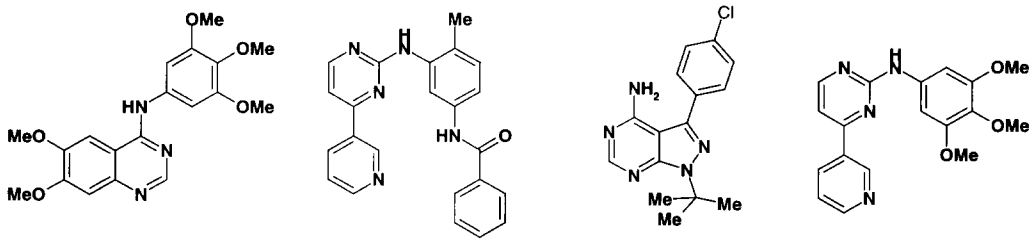
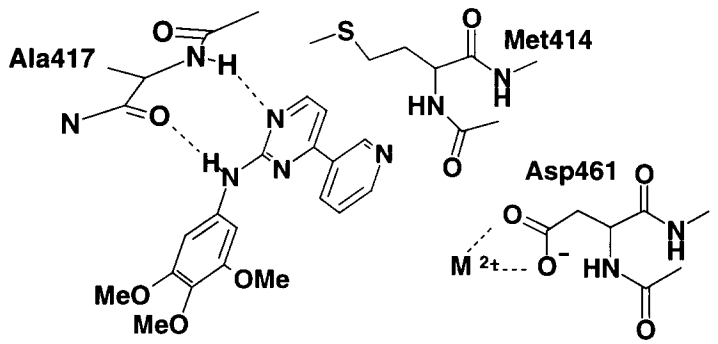


1 2 3 4

Consideration of the interaction of **4** with a homology model of the ATP-binding site of ZAP 70, constructed from the crystal structure of cyclic AMP-dependent kinase[8], led us to propose the mode of binding shown in Figure 1 on the basis that residue Met 414 prevented access of the inhibitor to space not occupied by ATP.

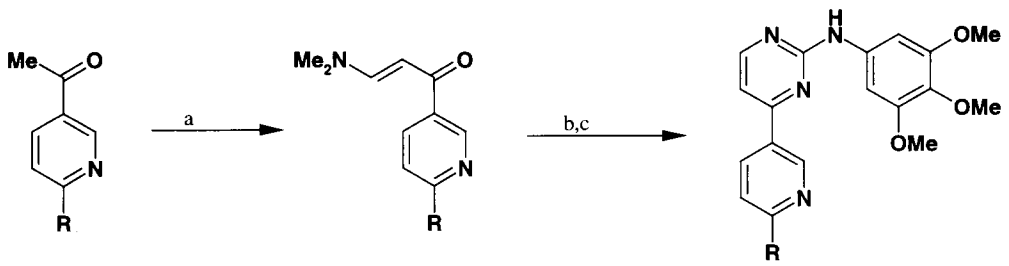
Figure 1



The anilinopyrimidine function of **4** provides a dual hydrogen bonding acceptor-donor function which can interact with the protein backbone at Ala 417. The orientation of the inhibitor also suggests that a putative cation binding site involving Asp 461 (Asp 184 in PKA[8]) could be accessed by a positively charged substituent appended to the C4-pyridyl group.

The synthesis shown in Scheme 1 allowed us to prepare both **4** and analogues **10-16**, enabling exploration of the pharmacophore described above. Thus the enaminones **7** and **8** were obtained from their respective acetylpyridines **5** and **6,** by heating at reflux in dimethylformamide diethylacetal[9], as crystalline solids which 2-2-phenylaminopyrimidines **4** and **9** respectively, and **9** was converted into final products **10-16** through reaction with neat alkylamines at elevated temperature[10,11].

Scheme 1



5 (R=H)
6 (R=Cl)

7 (R=H)
8 (R=Cl)

4 (R=H)
9 (R=Cl)
10-16 (R=alkylamino)

Reagents and conditions: (a) dimethylformamide diethylacetal, reflux; (b) 3,4,5-trimethoxyphenylguanidinium nitrate, NaOH, propan-2-ol, reflux; (c) alkylamine, 120°.

The compounds were assayed for their ability to inhibit ZAP 70 phosphorylation of polyGluTyr substrate under conditions previously described by us[12], and the activities are summarised in Table 1. Our lead molecule **4** was found to inhibit ZAP 70 with an IC_{50} value of 1.9μM. Introduction of the 2-aminoethylamino substituent in **10** led to a ten-fold increase in potency, supporting our hypothesis that the amine function may occupy a cation binding site. Investigation of the effects of conformational restriction led to a further increase in potency to 54nM, observed for the piperazine **11**. A small lipophilic substituent can be tolerated on N-4 of the piperazine ring, although this interaction appears size-limited as inhibition is clearly abrogated by the introduction of the ethyl moiety in **13**. The contribution of the charged ammonium species towards potency is underlined by the comparitively weak inhibitory properties of compounds **15** and **16**. Addition of a methyl substituent to the piperazine C-3 position in **17** gave an IC_{50} of 26nM. Preparation of both enantiomers indicated that activity resided in the (S)-enantiomer **18**. The loss of activity for the isopropyl analogue **21** suggested that this was also a size-limited hydrophobic interaction, when measured against our most potent compound **20**.

Table 1

Compound	R	ZAP70 IC50(nM)	m.p. °C
4	**H**	**1900**	**155**
10	**2-aminoethylamino**	**125**	**117-118**
11	**piperazin-1-yl**	**54**	**134-135**
12	**4-methylpiperazin-1-yl**	**46**	**178-179**
13	**4-ethylpiperazin-1-yl**	**4300**	**139**
14	**homopiperazin-1-yl**	**31**	**144-145**
15	**morpholino**	**424**	**157-158**
16	**piperidin-1-yl**	**332**	**150**
17	**3(RS)-methylpiperazin-1-yl**	**26**	**138-139**
18	**3(S)-methylpiperazin-1-yl**	**11**	**139-140**
19	**3(R)-methylpiperazin-1-yl**	**396**	**138-139**
20	**3(S)-ethylpiperazin-1-yl**	**8**	**66-67**
21	**3(S)-isopropylpiperazin-1-yl**	**188**	**91**

As can be seen from Table 2, selectivity was also optimised during the process of improving potency. Our lead compound **4,** a relatively weak inhibitor of ZAP 70, also demonstrates little selectivity across a panel of kinases. The introduction of the 2-aminoethylamino substituent to **10** provides a compound which shows an improved profile with only weak inhibitory activity against p56lck , EGFr and csk. However a major concern to us, was that this compound showed equipotent inhibition of the ubiquitously expressed PKC, a serine/threonine kinase known to play a crucial role in many signal transduction pathways associated with important physiological functions[13]. Presumably the alkylamino functionality of this flexible substituent can also access the corresponding cation binding site of PKC, but not that of the other members of our selectivity panel. We were able to circumvent this potential problem with the observation that conformational restriction of this pharmacophore, as in the piperazines **11** and **20,** furnished us with compounds which showed excellent selectivity over PKC.

Table 2

	IC_{50} (nM)				
Compound	**ZAP 70**	**PKC**	**p56Lck**	**EGFr**	**csk**
4	**1900**	**1400**	**1100**	**>10000**	**>10000**
10	**125**	**150**	**1200**	**5100**	**>10000**
11	**54**	**1300**	**3300**	**1704**	**>10000**
20	**8**	**2874**	**2200**	**>10000**	**>10000**

In conclusion, we have identified compounds such as **20** which show potent and selective inhibition of ZAP 70, relative to a number of other tyrosine and serine/threonine kinases, and have demonstrated that the observed selectivity depends largely upon conformational restriction of a basic substituent appended to the 4-pyridyl-2-phenylaminopyrimidine template. Further work towards the replacement of the 3,4,5-trimethoxyphenyl substituent and establishing the physiological relevance of these results to will be reported separately.

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11. The final compounds were determined as being analytically pure by CHN analysis and 1H nmr.
12. The tyrosine kinase activity of ZAP 70 was determined using a capture assay performed in 20mM HEPES pH 7.5 containing 10mM $MgCl_2$, 10mM $MnCl_2$, 0.05% brij, 1μM ATP (0.5 μ Ci[γ-^{33}P]ATP) and 17 μg/mL polyGlu-Tyr (Sigma; Poole, Dorset, U.K.). Inhibitors in DMSO were added such that the final concentration of DMSO did not exceed 1%, and the enzyme such that the consumption of ATP was less than 10%. After incubation at 30° for 15min, the reaction was terminated by the addition of one-third volume of stop reagent (0.25mM EDTA and 33mM ATP in dH_20). A 15 mL aliquot was removed, spotted onto a P-30 filtermat (Wallac, Milton Keynes, Bucks, UK) and washed sequentially with 10% (w/v) chloroacetic acid and dH_20 to remove ATP. The bound ^{33}P-polyGlu-Tyr was quantified by scintillation counting of the filtermat in a Betaplate scintillation counter (Wallac, Milton Keynes, UK) after addition of Meltilex scintillant. The dpm obtained, being directly proportional to the amount of ^{33}P-polyGlu-Tyr produced by ZAP 70, were used to determine the IC_{50} for each compound.
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