Discovery of potent inhibitors of interleukin-2 inducible T-cell kinase (ITK) through structure-based drug design

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ABSTRACT
Interleukin-2 inducible T-cell kinase (ITK) is a member of the Tec kinase family and is involved with T-cell activation and proliferation. Due to its critical role in acting as a modulator of T-cells, ITK inhibitors could provide a novel route to anti-inflammatory therapy. This work describes the discovery of ITK inhibitors through structure-based design where high-resolution crystal structural information was used to optimize interactions within the kinase specificity pocket of the enzyme to improve both potency and selectivity.

Interleukin-2 inducible T-cell kinase (ITK) is a member of the Tec family of non-receptor tyrosine kinases which has been found to play an important role in T-cell activation and proliferation.1 ITK is expressed primarily in T-cells, NK cells, and mast cells. Upon stimulation of the T-cell receptor (TCR), ITK is activated and serves to amplify the TCR signaling cascade.2 Murine CD4+ T-cells lacking Itk showed reduced IL-2, IL-4, IL-5, and IL-13 production upon T-cell receptor stimulation.3–5 ITK therefore represents a novel potential target for anti-inflammatory therapy in a variety of indications such as psoriasis and allergic asthma.6

We have recently reported on the identification of a structurally new lead series7 and initial optimization studies8 that culminated in a number of acylated 2-aminobenzimidazoles, represented here by compound 1 shown in Figure 1. Compound 1 displayed good potency in a DELFIA-based molecular assay7 (IC50 = 180 nM) and moderate cellular potency (IC50 = 2.3 μM), as measured in a stably transfected, itk-expressing DT40 cell line assay.7 This series of compounds was tested for selectivity against insulin receptor tyrosine kinase (IRK), an enzyme with close structural homology to ITK. Unsubstituted analogs such as compound 1 displayed poor selectivity with an IRK IC50 of 560 nM. A series of substituted phenyl analogs showed good selectivity and improved potency, yet still displayed modest cellular potency.8 Therefore, we focused our optimization effort on increasing the molecular and cellular potency of the lead series, while concomitantly maintaining or improving its selectivity over IRK and other related kinases.

Figure 1. The structure and binding mode of 1 in ITK.
Structure-based drug design offered an appealing opportunity towards that end. Indeed, crystallographic information revealed a number of new potential interactions that could be gained. Of particular interest is a vacant kinase specificity pocket (KSP) adjacent to the ATP binding site. This pocket is analogous to other reported KSPs, which have been successfully exploited to yield a substantial increase in both potency and selectivity with other kinase inhibitors. Access to this pocket is restricted by Phe435 which serves as a gatekeeper residue. Previous reports suggest that gatekeeper residue interactions are a route to increase both potency and selectivity, as this residue often varies between disparate kinases.

Within the pocket, there exists a variety of functional groups that could be exploited to increase potency, such as the conformationally flexible Lys391, Glu406 (which makes up part of the distal surface of the pocket), and DGF motif loop at the bottom portion of the pocket (Asp500, Gly501, Phe502). This is in stark contrast to the lipophilic nature of the majority of KSPs described in the literature. Based on the available crystallographic information, it appeared that placement of the appropriate functionality within the KSP should significantly increase binding potency.

The acylated 2-aminobenzimidazole lead series displays a somewhat unique binding motif to the hinge region of ITK. The double bond of the benzimidazole core exists as the exo-tautomer as supported by both NMR NOE studies in solution and X-ray crystallography of the molecule in complex with the ITK protein. Two hydrogen bonds are observed between Met438 in the hinge region of the peptide backbone and the benzimidazole ring N–H groups that could be exploited to increase potency, such as the conformationally flexible Lys391, Glu406 (which makes up part of the distal surface of the pocket), and DGF motif loop at the bottom portion of the pocket (Asp500, Gly501, Phe502). This is in stark contrast to the lipophilic nature of the majority of KSPs described in the literature. Based on the available crystallographic information, it appeared that placement of the appropriate functionality within the KSP should significantly increase binding potency.

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Among several approaches devised to access the vacant KSP, this work describes a lead optimization strategy specifically aimed at achieving this goal via a biaryl binding motif.

The general synthetic route is shown in Scheme 1. The commercially available 4-methyl-3-nitro-aniline (2) was acylated with benzoyl chloride and then N-alkylated through treatment with NaH and methyl iodide. Amine containing side-chains were introduced through SnAr displacement of the activated aromatic fluoride 3, and the nitro group was then reduced quantitatively to yield diamino compound 4. Dianiline 4 was treated with cyanogen bromide to provide the 2-aminobenzimidazole 5, which was acylated using standard conditions to provide the desired ITK inhibitors. The various carboxylic acids used were synthesized through standard methods.

![Scheme 1. General method for synthesis of 2-aminobenzimidazoles. Reagents and conditions: (a) benzoyl chloride, pyridine, CH2Cl2, 0 °C–rt; (b) CH3I, NaH, THF rt, 24 h; (c) 3-aminopropanamide, DIEA, CH3CN, 50 °C, 24 h; (d) Pd/C, H2, CH3OH; (e) BrCN, EtOH; rt, 24 h; (f) benzoic acid, EDCI, DIEA, DMF, rt, 24 h.](image-url)
As observed previously, the thiophene group served as a good replacement of the phenyl group, resulting in a compound of increased potency. The other heterocycles tested showed a relatively flat SAR in terms of ITK potency, although increased IRK selectivity was usually observed. We hypothesized that this could again result from unfavorable interactions (perhaps electrostatic in nature) with the IRK Met gatekeeper residue. Since substitution of the thiophene would allow for presentation of other groups at angles distinct from the corresponding phenyl analogs, the 2,5-disubstituted thiophene was chosen as a scaffold for further optimization as shown in Table 3.

Substitution at the 5-position of the thiophene ring was found to increase potency dramatically. Simple halogen substitution, as in 22 and 23, increased molecular potency 4-fold, with similar gains observed in cellular potency. Cyano substitution at the 5-position also enhanced potency, while providing a 250-fold selectivity for ITK over IRK (see compound 24). Appending a 5-formyl group provided the highly potent compound 25, likely though formation of a covalent intermediate with Lys391. In follow-up experiments, incubation of the enzyme with compound 25 in the presence of a reducing agent showed formation of the irreversible Lys391 adduct, supporting the formation of a Schiff’s base interme-

diate. To a lesser extent, the ketone containing compounds 26–28 may also interact with the Lys391 residue. These compounds show improved selectivity against IRK, however, likely due to the distinct steric requirements of the gatekeeper residues in the different active sites.

Aryl substitution (examples 31–34) was tolerated at the 5-position, confirming that the 5-position of the thiophene offered a beneficial presentation of functionality which avoided the Phe435 gatekeeper residue. Of particular significance was the dramatic increase in molecular and cellular potency of the 3- and 4-pyridine functionalized compounds. Introduction of the 2-pyridyl motif [32] did not result in a substantial increase in potency as compared to the phenyl analogue 31. However, both the 3- and 4-pyridyl analogues 33 and 34 (respectively) showed much improved molecular potency, with similar gains observed in the cellular assay for 33. In addition, these compounds displayed very good selectivity against IRK.

A co-crystal structure of compound 32 with ITK was obtained, providing insights about the interactions within the KSP. As shown in Figure 2, the 2-pyridyl moiety of 32 may benefit from a hydrophobic π-stacking interaction with the gatekeeper Phe435, which is absent in the IRK active site. In addition, the pyridine ring points directly into the vacant selectivity pocket. From the structure, the 2-pyridyl does not appear to pick up any additional interactions. However, compounds 33 and 34 may be able to engage in hydrogen bonding interactions with either Lys391 or Asp500, resulting in the increased potencies observed.

Based on these results, the thiophene biaryl inhibitors were further modified through the introduction of pendant heterocyclic replacements of the pyridine motif of 32–34 (Table 4). The various oxazole analogues showed good potency when assayed against ITK, with the 5-oxazole being the most potent. This compound overlaps nicely with the 3-pyridyl analogue 33 which was previously observed to impart high potency. The 2- and 4-oxazoles displayed reduced activity, similar to 2-pyridyl analogue 32, again suggesting that opportunities for H-bonding interactions exist deep within the KSP. All other nitrogen containing aromatic heterocycles were potent ITK inhibitors, while saturated analogues 43–46 were weaker. This loss of potency was attributed to a lack of a π-stacking interaction with Phe435, or unfavorable electro-
static repulsion between Lys391 and the protonated basic amine (or oxygen heteroatom) in the saturated ring system.

Interestingly, pyrazole analogue 41 was the most potent compound tested in the cellular assay, with an IC50 of 2 nM. It was 25-fold more potent than 5-oxazole analogue 37, even though they displayed similar molecular potency under the assay conditions. Based on the conditions used for the molecular assay, the predicted assay range does not extend below 2 nM. Therefore, we developed an assay with a higher concentration of ATP to extend the dynamic range of the assay, thereby allowing for the direct comparison of the most potent of inhibitors. Additional analogues containing the pyrazole and 5-oxazole moieties were synthesized with various groups appended from the 1-position of the benzimidazole to improve physicochemical properties of the molecules. When these analogues were tested in the higher ATP concentration assay, the pyrazole analogues were found to be consistently 10-fold more potent than the corresponding oxazole analogues. This finding supports the cellular potency increase observed in the case of pyrazole analogue 41.

Molecular modeling suggests that the pyrazole is able to form two distinct hydrogen bonds within the KSP: one with Lys391 and the other with Asp500. In addition, the T-shaped orientation of the heterocycle and the aryl ring of Phe435 can provide a favorable interaction (Fig. 3 below). In contrast, the oxazole analogue occupies a similar space, but only benefits from a single hydrogen bond interaction with Lys391.

Both the oxazole and pyrazole derivatives showed greater than 1000-fold selectivity over IRK. This is in stark contrast to lead compounds 1 and 14, which each showed approximately 3-fold selectivity. In addition, the cellular potency of the biaryl compounds was increased more than 1000-fold over the unsubstituted thiophene inhibitor 14.

In summary, a series of biaryl thiophene-containing ITK inhibitors was identified. These compounds were designed to avoid the gatekeeper Phe residue and occupy the vacant KSP of ITK to increase potency and selectivity against IRK. Molecular modeling as well as X-ray crystallography guided molecule design and led to ITK inhibitors that showed a greater than 100-fold increase in molecular potency and a coincident 1000-fold increase in cellular potency. In addition, selectivity for ITK inhibition over IRK was increased from 3-fold to greater than 1000-fold. Notably, compound 41 is the most potent ITK inhibitor reported to date. Further details regarding the in vivo efficacy of these biaryl-containing ITK inhibitors will be presented in forthcoming publications.

References and notes


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Figure 2. The X-ray crystal structure of 32 and interactions in the KSP of ITK.

Figure 3. The molecular modelling structure and binding mode of pyrazole compound 41 in the KSP. Two possible H-bond interactions are observed to K391 and D500.


