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Hit-to-lead studies on benzimidazole inhibitors of ITK: Discovery of a novel class of kinase inhibitors

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Abstract—Benzimidazole **1** was identified as a selective inhibitor of ITK by high throughput screening. Hit-to-lead studies defined the SAR at all three substituents. Reversing the amide linkage at C6 led to **16**, with a fivefold improvement of potency. This enhancement is rationalized by the conformational preference of the substituent. A model for the binding of the benzimidazoles to the ATP-binding site of ITK is proposed.

Interleukin-2-inducible T cell kinase (ITK), also known as T cell-specific kinase (TSK) and, expressed mainly in T-lymphocytes (EMT) is a member of the Tec family of protein tyrosine kinases.[1–3] ITK is expressed in T cells, mast cells, and natural killer cells. It is activated in T cells upon stimulation of the T cell receptor (TCR), and in mast cells upon activation of the high affinity IgE receptor. Following stimulation of the TCR, ITK is phosphorylated in the kinase domain activation loop by the kinase LCK.[4] Activated ITK is required for phosphorylation of PLC-γ[5] which in turn leads to calcium mobilization and activation of downstream pathways.[6] $CD4^+$-T cells from ITK knockout mice have a diminished proliferative response in a mixed lymphocyte reaction[7] or upon anti-CD3 stimulation. These T cells produce little IL-2 upon TCR stimulation, resulting in reduced proliferation. Diminished production of IL-4, IL-5, and IL-13 upon stimulation of the TCR has also been observed in ITK deficient $CD4^+$-T cells.[8] ITK deficient mice show a greatly diminished inflammatory response in a model of allergic asthma induced by OVA.[9] These studies support a key role for ITK in the activation of T cells, thus inhibitors of ITK should be useful as immunosuppressive or anti-inflammatory agents.

Recently Das et al.[10,11] disclosed a series of thiazole inhibitors of ITK. We now report our initial studies in this area which led to the discovery of a novel class of kinase inhibitors active against ITK. Ultra high throughput screening (UHTS) of the BI sample collection against ITK was carried out using a DELFIA assay.[12] Initial hits were retested in dose response against ITK, and also against BTK and LYN, both of which are highly homologous with ITK, to focus on selective inhibitors. One class of hits that did not inhibit in either of the counterscreens was a series of benzimidazoles from a general screening library. The initial hit **1** had ITK IC_{50} 0.11 μM and showed activity in a cellular assay, at 3.0 μM. This series was particularly interesting because its structure was not related to any known kinase inhibitor.

The original screening library contained 1760 members ($11R^1 \times 20R^2 \times 8R^3$) (Fig. 1). At the R^1 amide position, active hits included the thiophene, benzene, and

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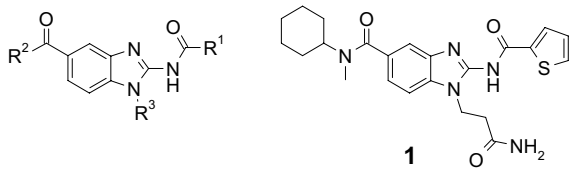


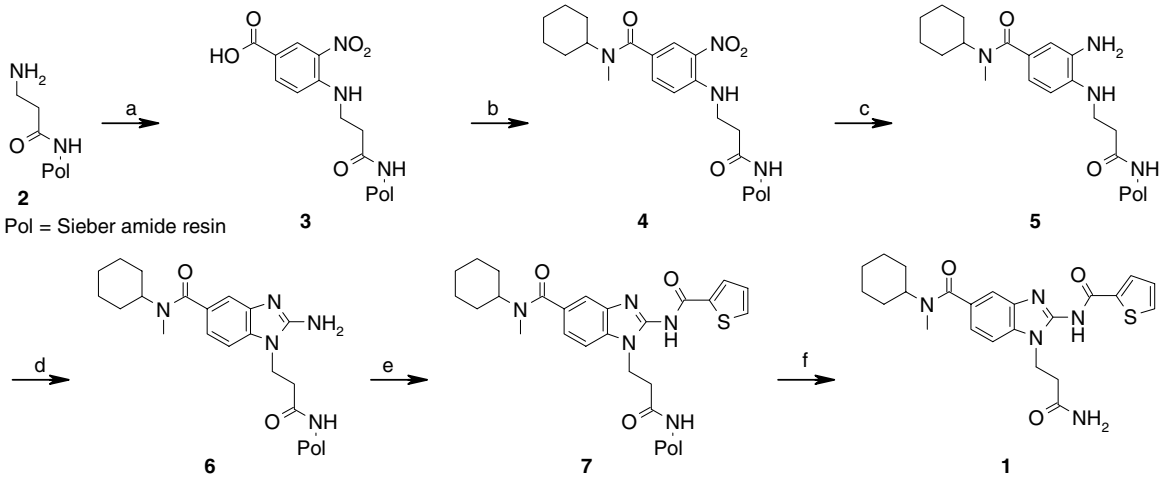
Figure 1. General structure of the screening library.

4-methoxyphenyl. At R^2 the preferred substituents were the *N*-cyclohexyl-*N*-methyl and *N*-benzyl-*N*-methyl amides. For the R^3 group on the benzimidazole nitrogen, the amide side chain derived from β-alanine was the only member of the library that showed appreciable activity. Many of the compounds in the library had R^3 groups derived from α-amino acid amides, none of which hit in the screen.

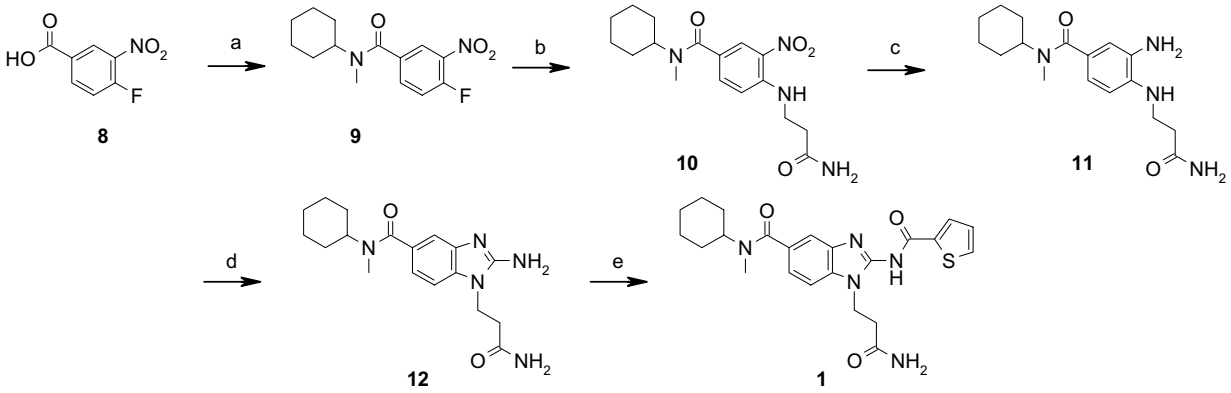
To further define the SAR, exploration of the hit was carried out by a combination of solid-phase library synthesis and single point synthesis using solution-phase chemistry. To this end two focused libraries were synthesized, in one case varying R^1, and keeping R^2 constant as *N*-cyclohexyl-*N*-methyl, (77 compounds) and in the other varying R^2 and keeping R^1 constant as phenyl (99 compounds). In both cases the β-alanine amide side chain at R^3 was kept constant. The libraries were synthesized using solid-phase methodology reported by Lee[13] (Scheme 1). Sieber amide resin loaded with β-alanine **2** was treated with the 4-fluoro-3-nitro benzoic acid to effect the S_NAr reaction. The R^2 amide was then introduced, by coupling the acid with *N*-methyl cyclohexylamine in the presence of TBTU to give **4**. Reduction of the nitro group with tin chloride worked well on solid phase, to form the diamine **5**, which was converted to the 2-aminobenzimidazole with cyanogen bromide. The amine was acylated with the appropriate acid chloride, and the product was cleaved from the resin with TFA to leave the primary amide at the terminus of the R^3 side chain. Compounds that showed activity were resynthesized and purified.

Initial exploration of other side chains at R^3 was carried out using a solution-phase method (Scheme 2). For this study R^1 was kept constant as the thiophene and R^2 as *N*-cyclohexyl-*N*-methyl amine. The same general approach was followed, but in this case the R^2 amide was introduced first from 4-fluoro-3-nitro benzoic acid, yielding **9**. This intermediate was reacted with a variety of R^3 amines to give **10**. For the solution-phase synthesis, reduction of the nitro group by transfer hydrogenation was preferred over tin chloride reduction. The 2-aminobenzimidazole **12** was formed with cyanogen bromide, and acylation yielded **1**. In the final step impurities presumably resulting from acylation on the ring nitrogen and double acylation were observed. Pure product was obtained by chromatography on silica. An analogous route was used to obtain the cyclohexyl ester **50** at R^2. The analog **47** where the amide has been moved to the 6-position of the benzimidazole was prepared in a similar way from 3-fluoro-4-nitrobenzoic acid. The reverse amide analogs of **1** were obtained as shown in Scheme 3, by acylation of 4-fluoro-3-nitroaniline with cyclohexylcarbonyl chloride. For the tertiary amides, the product was methylated on nitrogen to give **14** before performing the S_NAr reaction. Completion of the synthesis followed the same steps of reduction, benzimidazole formation, and acylation as before to give reverse amide **16**.

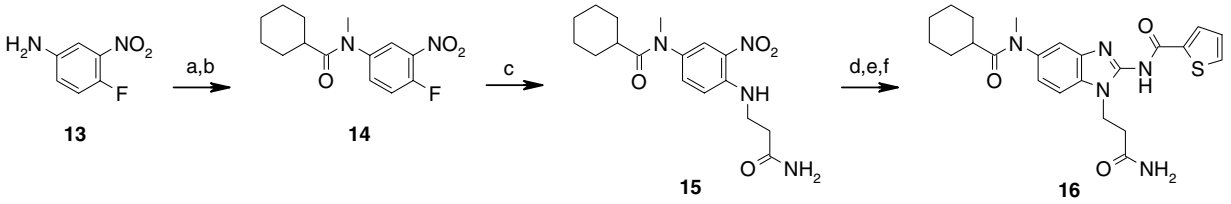
Compounds were assayed for ITK inhibitory activity in a DELFIA format.[12] Cellular activity was assessed by measuring calcium influx following B cell receptor stimulation of BTK deficient DT40 cells[14] stably transfected to express human ITK, using a FLIPR instrument. During the hit-to-lead phase the cellular assay was used to rule out inactive compounds, rather than to rank activity. Work to improve cellular potency will be detailed in subsequent papers. Results for selected compounds illustrating the SAR at R^1 and R^2 are shown in Table 1. At R^1 an aromatic amide is required. No inhibition was observed with aliphatic amides (data not shown). Phenyl can take the place of thiophene with only a slight loss in potency (**17**). Electron withdrawing groups at C3 or



Scheme 1. Solid-phase synthesis of compound **1**. Reagents: (a) 4-fluoro-3-nitrobenzoic acid, DMF, DIPEA; (b) *N*-methyl cyclohexylamine, TBTU, DMF/DCM; (c) $SnCl_2{\cdot}2H_2O$; (d) BrCN, EtOH; (e) thiophene-2-carbonylchloride, pyridine; (f) 20% TFA, DCM.

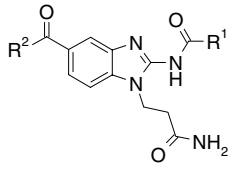


Scheme 2. Solution-phase synthesis of compound **1**. Reagents and condition: (a) *N*-methyl cyclohexylamine, EDC, HOBT, DCM; (b) β-alanineamide, Et_3N, DMSO, 90 °C; (c) NH_4HCO_2, Pd/C, EtOH; (d) BrCN, EtOH; (e) thiophene-2-carbonylchloride, pyridine.



Scheme 3. Solution-phase synthesis of compound **16**. Reagents and conditions: (a) cyclohexanecarbonylchloride THF, reflux; (b) NaHMDS, MeI, THF; (c) β-alanineamide, Et_3N, DMSO, 90 °C; (d) NH_4 HCO_2, Pd/C, EtOH; (e) BrCN, EtOH; (f) thiophene-2-carbonylchloride, pyridine.

Table 1. Representative SAR of the R^1 and R^2 groups



Compound	R^1	R^2	ITK IC_{50}[a] (μM)	DT40 cell IC_{50}[b] (μM)
1	2-Thienyl	*c*-HexNMe	0.11	3.0
17	Ph	*c*-HexNMe	0.37	11
18	4-MeOPh	*c*-HexNMe	1.1	17
19	4-BrPh	*c*-HexNMe	0.26	1.2
20	4-FPh	*c*-HexNMe	0.25	4.1
21	4-NO_2Ph	*c*-HexNMe	0.076	6.9
22	3-FPh	*c*-HexNMe	0.11	7.4
23	3-CNPh	*c*-HexNMe	0.20	7.2
24	2-MePh	*c*-HexNMe	2.7	nt
25	5-Isoxazolyl	*c*-HexNMe	0.062	4.1
26	3-Pyridyl	*c*-HexNMe	0.069	2.7
27	2-Thienyl	*c*-HexNH	>20	nt
28	Ph	*c*-HexNH	>20	nt
29	Ph	(*c*-Hex)$_2$N	>20	nt
30	Ph	*i*-PrNMe	11	nt
31	Ph	*c*-HexNEt	0.39	8.1
32	Ph	*c*-HexNallyl	0.73	7.2
33	4-BrPh	*c*-HexNEt	0.081	1.2
34	4-BrPh	*c*-HexNallyl	0.23	1.2
35	Ph	PhNMe	0.33	2.6

nt, not tested.

[a] Values are geometric means of at least two experiments. In most cases the individual results vary by less than a factor of 2.

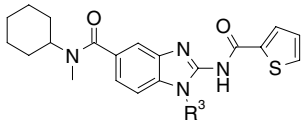
[b] Mean of at least two experiments.

C4 of the phenyl ring (**20**–**23**) are tolerated, the 4-nitro analog **21** showing a fourfold improvement, whereas electron donating substituents such as methoxy (**18**) decrease activity. Substitution at C2 in **24** is detrimental. Isoxazole **25** and pyridine **26** show somewhat improved potency in the enzyme assay, though this is not reflected in the cellular assay. At R^2 there is a requirement for a tertiary amide with one cyclic substituent and one small alkyl substituent. Removing the *N*-methyl to give a secondary amide in **27** or **28** greatly reduces activity. The methyl can be replaced with small alkyl groups **31**–**34**, but not with a more bulky group such as a second cyclohexyl ring **29**. The cyclohexyl ring can be replaced with phenyl **35**, but potency is lost on replacing it with a smaller alkyl group in **30**. This intriguing SAR observation is discussed in more detail below.

At the R^3 position more variation is possible (Table 2). While the unsubstituted benzimidazole **41** is only weakly active, a simple alkyl substituent **42** restores much of the potency. Polar substituents, either as the amide or alcohol, are preferred, with **40** and **45** showing the best potency. Substitution of the amide (**37**,**38**) maintains binding, but diminishes cellular activity. Replacing the hydroxyl in **45** with an amine (**46**) resulted in a 10-fold decrease in enzyme activity, and loss of cellular activity. Substitution or branching at the carbon next to the benzimidazole in the R^3 side chain is not tolerated (data not shown).

To further investigate the requirements of the R^2 substituent, additional variations at the 5- and 6-positions of the benzimidazole were investigated (Table 3). Moving the amide to the 6-position (**47**) causes a sixfold decrease in activity, and removing the amide altogether (**48**) still retains micromolar potency. Replacing the amide with a methyl (**49**) or a cyclohexyl ester (**50**) greatly reduces potency, even though the cyclohexyl ester might be viewed as isosteric with the corresponding amide. The most exciting result was observed with the reverse amide **16**, IC_{50} 25 nM, a significant improvement over the screening hit. Removal of the *N*-methyl from **16** again gives a substantial loss in affinity, though in this case the activity of the secondary amide **51** is measurable. The SAR at this position clearly involved a hydrophobic interaction with the cyclohexyl ring. The large difference in potency between the secondary and tertiary amides seemed to argue against a hydrogen bonding interaction with the carbonyl. We hypothesized that this difference in affinity is due to changes in the conformation of the side chain. Tertiary amides can more readily adopt the cis amide conformation than secondary ones, and the activity may be linked to the ability to achieve the cis amide conformer. To determine the conformational preference, molecular modeling was carried out on a simplified fragment of the molecule, with methyl groups at R^1 and R^3. In the cis amide conformation, the cyclohexyl ring is forced out of the plane of the benzimidazole. The energy difference between the two conformers is shown in Table 4. In all cases the trans conformation is preferred, but for the reverse amide **16** the energy difference is much lower (0.3 kcal/mol) than for the other linkers. The NH version of the reverse amide **51** and the original tertiary amide **1** are in a similar range of 2–3 kcal/mol, while the inactive secondary amide **27** and the ester **50** have values over 3 kcal/mol, corresponding to <1% cis conformer at equilibrium. This analysis provides support for the hypothesis that the R^2 side chain binds in a cis amide conformation, which would allow the cyclohexyl ring to access a putative hydrophobic interaction out of the plane of the benzimidazole ring.

Table 2. Representative R^3 groups



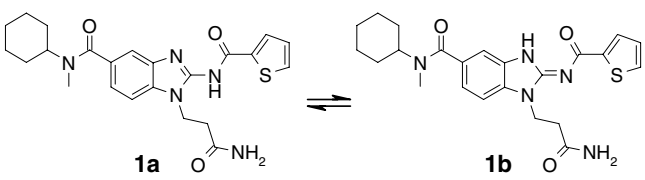
Compound	R^3	ITK IC_{50}[a] (μM)	DT40 cell IC_{50}[b] (μM)
1	$(CH_2)_2CONH_2$	0.11	2.7
36	$(CH_2)_2CO_2Et$	0.27	1.1
37	$(CH_2)_2CONHMe$	0.23	5.4
38	$(CH_2)_2CONMe_2$	0.16	24
39	$(CH_2)_3CONH_2$	0.14	4.6
40	$(CH_2)_4CONH_2$	0.06	3.1
41	H	4.8	nt
42	*n*-Pr	0.76	1.6
43	$(CH_2)_2Ph$	1.3	1.8
44	$(CH_2)_2OH$	0.19	4.6
45	$(CH_2)_3OH$	0.092	2.0
46	$(CH_2)_3NH_2$	0.95	>20

nt, not tested.

[a] Values are geometric means of at least two experiments. In most cases the individual results vary by less than a factor of 2.

[b] Mean of at least two experiments.

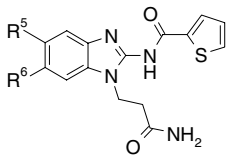
With the initial SAR established, we sought to understand how these novel inhibitors bound to the ITK active site. The majority of kinase inhibitors bind to the ATP site via an essential H-bonding interaction. In many cases, including ATP itself, this involves a bidentate interaction with a donor–acceptor pair. Compound **1** contains several possible H-bonding sites, but apparently none that would give a bidentate interaction. A clue to this came during structure confirmation by NMR, when an NOE was observed between the exchangeable NH and the proton at the 4-position of the benzimidazole. This can only be consistent with the exocyclic imine tautomer, **1b**, where the proton is on the ring nitrogen. This tautomerism has been observed previously in 2-acylaminobenzimidazoles.[15]



1a **1b**

In this tautomer a potential H-bonding interaction is evident, and this led us to propose a binding interaction with ITK outlined in Figure 2. By analogy with the structure of HCK,[16] ATP binds to ITK by way of a donor interaction with the carbonyl of Glu436, and an

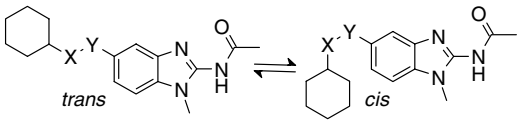
Table 3. Variation of C5 and C6 substitution



Compound	R^5	R^6	ITK IC_{50}[a] (μM)	DT40 cell IC_{50}[b] (μM)
1	*c*-HexN(Me)CO	H	0.11	2.7
27	*c*-HexNHCO	H	>20	nt
47	H	*c*-HexN(Me)CO	0.71	20
48	H	H	1.5	12
49	MeO_2C	H	11	nt
50	*c*-HexO_2C	H	>20	nt
51	*c*-HexCONH	H	2.5	1.7
16	*c*-HexCONMe	H	0.025	2.4

nt, not tested.
[a] Values are geometric means of at least two experiments. In most cases the individual results vary by less than a factor of 2.
[b] Mean of at least two experiments.

Table 4. Energy difference between the cis and trans conformers corresponding to compounds in Table 3



Compound	X	Y	Energy difference[a] (kcal/mol)
1	N(Me)	C=O	2.8
27	NH	C=O	3.8
50	O	C=O	9.3
16	C=O	N(Me)	0.3
51	C=O	NH	2.2

[a] Difference between the minimum energy conformers of the cis and trans isomers of the above structure, calculated using Cerius 2 (Accelerys Inc.).

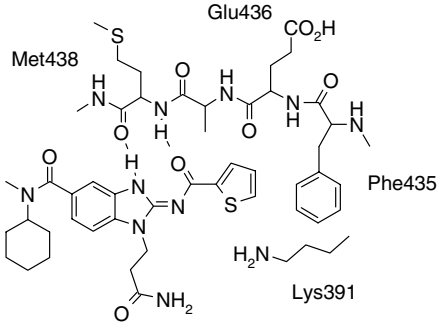


Figure 2. Proposed binding interaction of compound **1**, as the exocyclic imine tautomer, with the hinge region of ITK.

acceptor interaction with the NH of Met438. This interaction is observed with many kinase inhibitors, but a second binding mode has also been observed in which both donor and acceptor interactions are with a single residue,[17–19] corresponding to Met438 in ITK. This seems much more likely for these benzimidazoles than an alternative with the ATP mode, which would reverse the orientation of the core.

Table 5. Kinase selectivity of compound **16**

Kinase	IC_{50}[a] (μM)	Kinase[b]	% inhibition[c]
BMX	>22	CDK2	7
BTK	>22	CDK5	−4
ECK	>10	CHK1	8
EGFR	>6	GSK3b	−18
FGFR3	>6	JAK3	−7
HGFR	>10	MKK1	−2
IRK	0.2	MAPKAPk2	1
LYN	>10	PDGFR	10
TEC	22	PKCb	21
TXK	1.4	ROCKII	−1
VEGFR1	>10	SYK	6

[a] Assays run in dose response.
[b] Screening at a single concentration.
[c] % inhibition at 10 μM.

To provide support for the involvement of tautomer **1b**, an analog of **16** was subjected to methylation, which occurred on the ring nitrogen, and resulted in loss of activity.[20]

The improved potency of the reverse amide **16** resulted in this compound becoming the program lead. To confirm that these compounds are ATP-competitive the enzyme assay was run at four ATP concentrations: 3, 20, 100, and 500 μM. IC_{50} values of 0.11, 0.32, 1.6, and >5 μM, respectively, were observed for **1**. The corresponding results for **16** were 0.025, 0.08, 0.24, and 1.1 μM. **16** was profiled against more than 100 other kinases. Selected data are shown in Table 5. The main cross-reactivity was with insulin receptor kinase (IRK), and to a lesser extent with TXK, also a member of the Tec family.

Optimization of all three substituents of compound **16** to improve molecular and cellular potency and evaluate in vivo activity will be reported in subsequent papers.

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