DOI: 10.1002/cmdc.200600221

Discovery of Selective Irreversible Inhibitors for Bruton's Tyrosine Kinase

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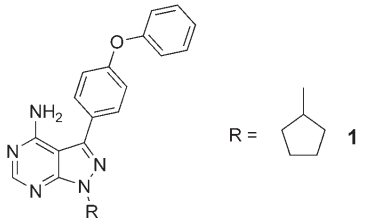
The importance of B cells in rheumatoid arthritis (RA) pathogenesis has been recently demonstrated in several clinical studies using the anti-CD20 antibody rituximab, which selectively depletes B cells. A recent phase III clinical trial led to the FDA approval of rituximab for a subset of RA patients. Bruton's tyrosine kinase (Btk), a member of Tec family kinases, is a key component in the B-cell receptor signal pathway (BCR).[1] Upon activation by upstream kinases (for example, Lyn and Syk), Btk phosphorylates and thereby activates phospholipase-Cγ (PLC-γ), leading to several important downstream events including calcium ion transportation, NF-κB activation, and (auto)antibody generation. Previous biological studies (genetic loss of function[2] and siRNA knockdown[3]) strongly suggest that Btk is also a mediator of proinflammatory signals. Taken together, these studies indicate Btk may be a potential target for the treatment of RA. However, despite the previous discovery of LFM-A13 as a selective Btk inhibitor,[4] there is no published study that has demonstrated that inhibition of Btk activity leads to in vivo efficacy in an animal model of rheumatoid arthritis.

As ATP binding sites in kinases are highly conserved, it is a formidable task to develop selective ATP competitive kinase inhibitors. Among several approaches, the use of electrophilic inhibitors has been shown as a viable method to achieve selectivity.[5] Considering the relative scarcity of knowledge on "chemical knockdown" of Btk activity, it is crucial to discover a potent and selective tool compound for this kinase. Herein, we describe the discovery of a selective, irreversible Btk inhibitor and its efficacy in a mouse RA model.

An initial campaign to scan for scaffolds capable of inhibiting Btk's kinase activity identified compound **1** as having



8.2 nM potency against Btk in a FRET based biochemical enzymology assay. In agreement with a previous study,[6] screening against more than 100 kinases at Ambit Biosciences[7] showed that **1** inhibited activity of certain Tec and Src family kinases (Table 1). Interestingly, **1** shows only modest inhibitory activity against Itk, another Tec family kinase, probably due to the difference at the "gatekeeper" residue.[8]

Table 1. Selectivity screening results of compound **1**.[a]

Kinase	Activity remaining [%]	Kinase	Activity remaining [%]
Abl	5.1	Btk	0
Aurora A	>30	Fgr	0
CSK	1.4	Fyn	0.2
EGFR	3.5	Hck	0.3
INSR	>30	Lck	0.1
JAK1	>30	Lyn	0
JNK3	>30	Src	0.1
Kit	0.7	Yes	0
p38α	>30	Bmx	0
Syk	>30	Itk	10

[a] Binding assay at Ambit Biosciences, concentration of **1** is 10 μM.

We realized that these two kinases could have SAR homology[9] because of compound **1**'s high potency towards Btk and Lck, and constructed a homology model based on the known X-ray structures of Btk kinase domain 1K2P[10] and Lck 1QPE[11] (Figure 1). The amino acids that were within contact range of compound **1** were identified in this homology model (Table 2). Alignment of Btk and Lck sequences revealed Cys 481 in Btk as a nucleophilic site, which could potentially form a covalent complex between Btk and an electrophilic inhibitor. Further sequence analysis indicated that this cysteine was at the same position as Cys 773 in EGFR family kinases that were the target of several irreversible kinase inhibitors in clinical trials, for example, CI-1033[12] and HKI-272.[13] Based on sequence alignments of 491 kinases,[14] there are only 10 kinases with a cysteine at this position: Blk, Btk, Bmx, EGFR, ErbB2, ErbB4, Itk,

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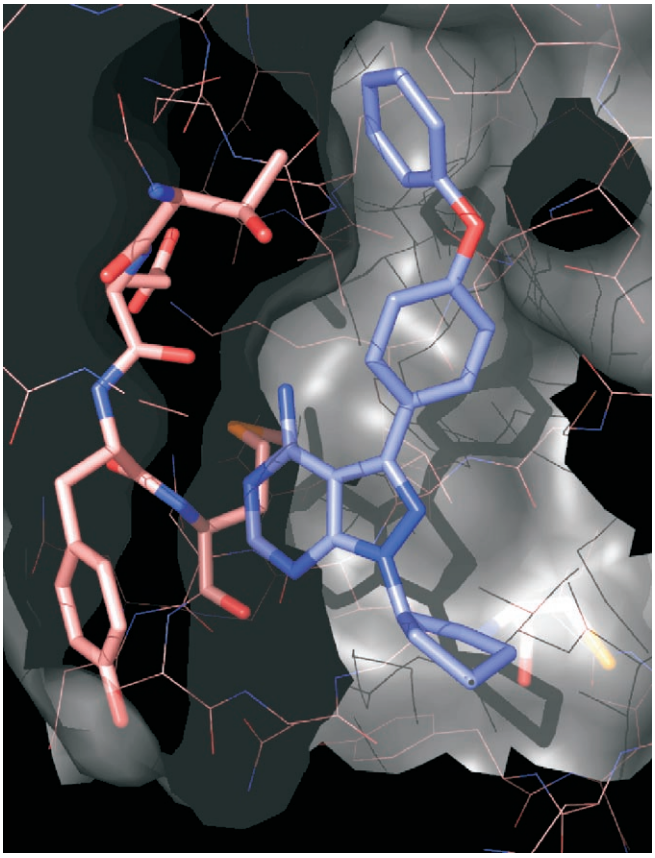


Figure 1. The proposed binding mode of compound **1** in a homology model of Btk: the highlighted residues on the left side are Thr-Glu-Tyr-Met; the residue at the bottom right corner is the irreversibility handle Cys 481.

maining recombinant Btk activity after 60 min incubation of the kinase and various concentrations of inhibitors in buffer (Table 3). We confirmed the irreversibility of these compounds using two methods: 1) after recombinant Btk was pretreated with compounds, its activity was not recovered by multiple washings with inhibitor-free medium;[15] and 2) a major peak was observed by mass spectrometry corresponding to the molecular weight of a 1:1 covalent complex between compound **4** and Btk.[16]

All these compounds were highly potent inhibitors with IC_{50} values in the subnanomolar to single digit nanomolar range except compound **5**. Their cellular (Ramos cell) inhibitory potencies in a Ca^{2+} flux assay[17] ranged from 3 to 92 nM. Three types of Michael acceptors, acrylamide, vinyl sulfonamide, and propiolamide, exhibited strong interactions with Btk. Adding a trans-oriented methyl group to the vinyl group decreased potency as shown by compound **5**, which was 28-fold less potent than **4**. This presumably relates to the reduced electrophilicity of the more substituted olefin. Consistent with a previous report,[18] compound **15** with a tertiary amine group regained some potency compared to **5**, even though it still suffered a potency drop compared to **13**. It was interesting to observe that compound **10** was about 6-fold more potent than **9**, presumably because of the difference in the electrophile orientation. Finally, *R* configuration was determined as the slightly preferred absolute stereochemistry configuration by two sets of enantiomers (**11** versus **12** and **13** versus **14**).

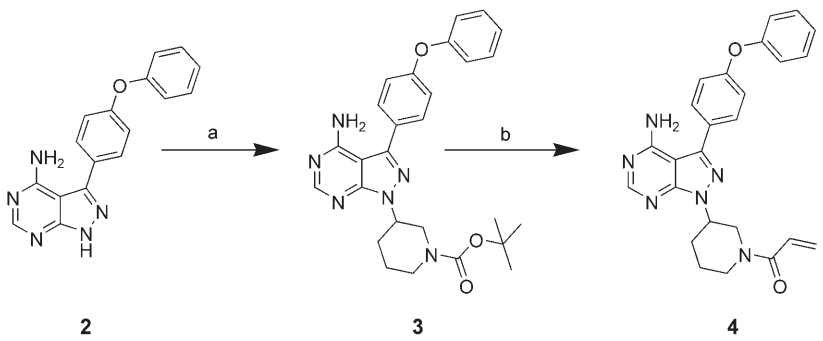
Kinases are low abundance proteins, which are tightly regulated in cells. It is well-known that biochemical assays with recombinant kinases in simplified conditions may not fully reflect an inhibitor's capability of modulating kinase activities in live cells.[19] Therefore we further characterized properties of these compounds in cellular systems (Table 4). Even though compounds **1** and **4** showed limited selectivity in biochemical enzymology experiments with purified kinases, it was very encouraging to observe that **4** exhibited significant selectivity improvements in cellular assays. In anti-IgM stimulated Ramos

Table 2. Selected residues of Lck and Btk.

Kinase	Sequence Number in Btk								
	427	428	474	475	476	477	480	481	528
Btk	V	A	T	E	Y	M	G	**C**	L
Lck	V	A	T	E	Y	M	G	**S**	L

Jak3, Tec, and Txk. This result reinforced our initial proposal to develop a selective inhibitor for Btk through an electrophilic center capable of irreversibly inactivating the target.

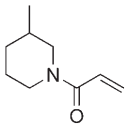
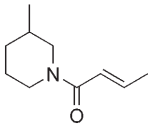
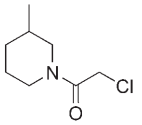
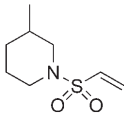
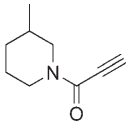
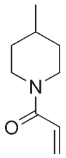
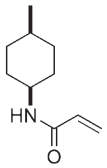
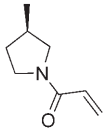
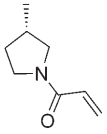
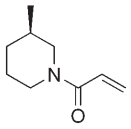
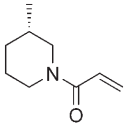
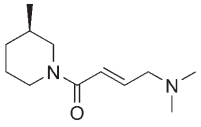
A series of irreversible inhibitors were synthesized through steps outlined in Scheme 1 with compound **4** shown as the example. Intermediate **2** was coupled with *N*-Boc-3-hydroxypiperidine by Mitsunobu reaction to give the Boc protected intermediate **3**. After deprotection with acid, coupling with acid chlorides completed the synthesis. Their IC_{50} values were measured by determination of re-



2 3 4

Scheme 1. Synthesis of irreversible Btk inhibitor **4**: a) polymer-bound TPP, DIAD, THF; b) HCl/dioxane; then acryloyl chloride, TEA.

Table 3. Assay data for irreversible inhibitors.

Compd	R	Btk IC_{50} [nM]	Ramos Cell Ca Flux IC_{50} [nM][a]
4		0.72	10
5		20	89
6		0.52	92
7		0.58	9
8		0.72	9
9		3.6	48
10		0.58	3
11		1.6	24
12		1.9	90
13		< 0.5	10
14		1.4	7
15		2.5	36

[a] A wider range of deviation (±50%) was observed for potent compounds including **4**, **13**, and **14** due to their high potency and relatively low solubility in aqueous cell culture media.

cells, a human B cell line, compound **4** greatly reduced the phosphorylation of Btk's substrate PLC-γ1 with an IC_{50} = 0.014 μM, while the Lyn and Syk dependent phosphorylation of tyrosine 551 on Btk was inhibited more weakly (IC_{50} > 7.5 μM). Thus, compound **4** exhibits a > 500-fold selectivity between Btk and Lyn or Syk in cells. In contrast, compound **1** exhibits only a 4-fold difference in the IC_{50} values for the same assays, suggesting the advantages that irreversibility can provide. We also measured the effect of compound **4** on Jurkat cells, a human T cell line, in which Lck and Itk are required for T cell receptor mediated Ca^{2+} flux. Here, compound **4** was 11-fold less active in inhibiting Ca^{2+} flux than in Ramos cells, supporting the expected selectivity for B versus T cells.

The in vivo efficacy of compound **4** was evaluated in a mouse model of rheumatoid arthritis. Arthritis was induced in Balb/c mice by administration of anticollagen antibodies and LPS.[20] Compound **4** was administrated orally once a day in an aqueous suspension for 10 consecutive days, starting one day after LPS administration. As shown in Figure 2, compound **4** inhibited arthritis development in a dose dependent manner, with a maximum effect (> 95% inhibition) at dose levels of 10 and 30 mg kg^{-1} ($p < 0.05$). The plasma concentrations of compound **4** that induced this maximum effect were in the 0.6–1.7 μM range at T_{max} (2 hours) and did not need to be sustained at high levels for 24 h to achieve efficacy, which is not surprising for an irreversible inhibitor.[21]

In summary, we have discovered a series of irreversible inhibitors for Bruton's tyrosine kinase. These inhibitors were highly potent in both in vitro and

Table 4. Enzymology and cellular assay data for compounds **1** and **4**.								
Compd	Btk[a] [nM]	Lck[a] [nM]	Lyn[a] [nM]	Itk[a] [μM]	Btk p551[b] [μM]	pPLC-γ1[b] [μM]	Ramos Ca^{2+} Flux[b] [μM]	Jurkat Ca^{2+} Flux[b] [μM]
1	8.2	4.6	2.5	>3.0	1.4	0.33	0.53	n/a
4	0.72[b]	97	14	1.0	>7.5	0.014	0.0405	0.466

[a] K_i (app) values. [b] IC_{50} values.

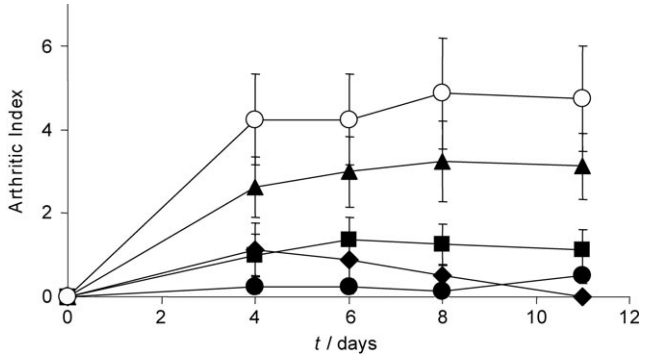


Figure 2. Efficacy study of compound **4** in a mouse arthritis model. ○: vehicle; ▲: 1 mg kg^{-1}; ■: 3 mg kg^{-1}; ◆: 10 mg kg^{-1}; ●: 30 mg kg^{-1}.

in vivo assays. Compound **4** showed preference towards Btk over closely related kinases. This compound inhibited B cell function, and within the BCR pathway it selectively acted to inhibit Btk-dependent processes. Finally, compound **4** demonstrated clear dose-dependent efficacy in a mouse arthritis model. This study not only presents a strategy to discover selective kinase inhibitors but also provides convincing evidence that Btk is a very attractive target for the treatment of rheumatoid arthritis.[22]

Keywords: biological activity · drug design · enzymes · medicinal chemistry · structural bioinformatics

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Received: September 8, 2006
Published online on December 12, 2006

