REVIEW

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Inhibitors of BTK and ITK: State of the New Drugs for Cancer, Autoimmunity and Inflammatory Diseases

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Abstract

BTK and ITK are cytoplasmic tyrosine kinases of crucial importance for B and T cell development, with loss-of-function mutations causing X-linked agammaglobulinemia and susceptibility to severe, frequently lethal, Epstein–Barr virus infection, respectively. Over the last few years, considerable efforts have been made in order to develop small-molecule inhibitors for these kinases to treat lymphocyte malignancies, autoimmunity or allergy/hypersensitivity. The rationale is that even if complete lack of BTK or ITK during development causes severe immunodeficiency, inactivation after birth may result in a less severe phenotype. Moreover, therapy can be transient or only partially block the activity of BTK or ITK. Furthermore, a drug-induced B cell deficiency is treatable by gamma globulin substitution therapy. The newly developed BTK inhibitor PCI-32765, recently renamed Ibrutinib, has already entered several clinical trials for various forms of non-Hodgkin lymphoma as well as for multiple myeloma. Experimental animal studies have demonstrated highly promising treatment effects also in autoimmunity. ITK inhibitors are still under the early developmental phase, but it can be expected that such drugs will also become very useful. In this study, we present BTK and ITK with their signalling pathways and review the development of the corresponding inhibitors.

Introduction

BTK and ITK are TEC family kinases (TFKs) and loss-of-function mutations cause human disease

Before reviewing the newly developed inhibitors of BTK and ITK, we provide a background to these tyrosine kinases. In the first section, we discuss their identification and the effect of inactivating mutations. In the following section, we describe the intracellular signalling pathway of BTK and ITK and summarize what is known about their regulation. This is followed by the description of the inhibitors.

TFKs, consisting of BTK, BMX (ETK), ITK, TEC and TXK (RLK), form the second largest family of non-receptor kinases in humans, the largest being the SRC family. The TFK ancestor emerged already prior to the evolution of metazoans and shows evidence of differential evolutionary wiring [1–3]. BTK and ITK contain an N-terminal Pleckstrin homology (PH) domain, followed by a Tec homology (TH), Src homology (SH)-3, -2 and -1 (catalytic) domains [4–6]. As depicted in Fig. 1, the TH domain consists of an N-terminal Zn^{2+}-binding BTK motif, and one or two proline-rich motifs [4, 7–9].

All the mammalian TFKs were identified in the 1990s. ITK [10, 11] and BTK [12, 13] were each cloned independently by two groups. This family of kinases soon received wide interest, owing to the fact that *BTK* mutations cause an X-linked form of B-lymphocyte deficiency (X-linked agammaglobulinemia, XLA) in man [13–16]. Today, more than 1000 patients with known mutations exist in the BTKbase registry [17]. In mice, mutations cause the phenotypically milder X-linked immunodeficiency (Xid) [18, 19]. In mice, it seems as if the TEC kinase has a unique compensatory role, because, while the TEC kinase is also expressed in human B lymphocytes, in mice, the double knockout of BTK and TEC causes an XLA-like phenotype. In contrast, TEC single-knockout mice do not have an overt phenotype [20].

ITK deficiency in humans is much less common and was not reported until 16 years after the identification of mutations in *BTK*. Thus, *ITK* is one of the several genes in which loss-of-function mutations cause susceptibility to severe, often fatal, Epstein–Barr virus infections [21].

BTK is constitutively expressed in myeloid and lymphoid cells but absent in T cells and in mature plasma cells [22]. It is found during all stages of the B cell lineage up until the plasma-cell stage, where it is absent in the most

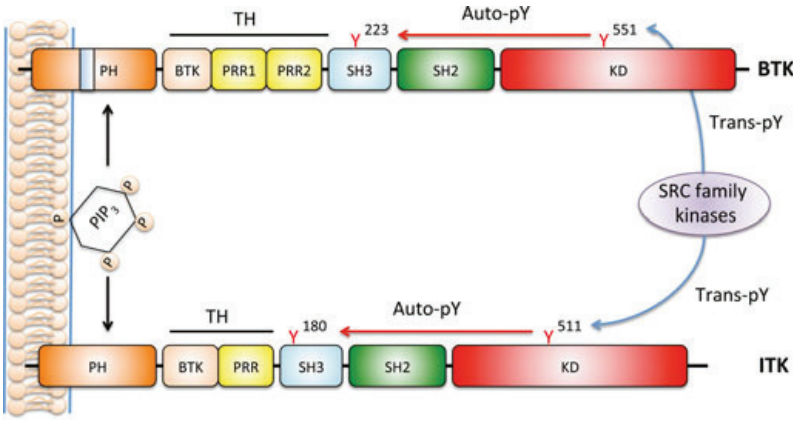

Figure 1 Schematic representation of BTK and ITK showing membrane binding and regulatory tyrosine phosphorylation sites. BTK and ITK have similar domain organization, with the difference that BTK has two proline-rich repeats (PRR) in the Tec homology domain (TH). The highly conserved BTK motif binds a Zn^{2+} ion, which stabilizes the PH domain. The PH domain binds phosphatidylinositol-3,4,5-trisphosphate (PIP3), which is generated by PI3K (Fig. 2). In the PH domain of BTK, there is a 27 amino acid insertion not found in ITK (marked in blue). The SH3 domain binds to proline-rich regions, while the SH2 domain interacts with phosphorylated tyrosine residues forming reversible signalling complexes. The depicted trans-phosphorylated tyrosine residue in the catalytic domain has an activating function, whereas the role of the autophosphorylated tyrosine in the SH3 domain is less defined.

mature form. Mutations cause a differentiation block at the stage of pre-B cells, with mature B cells being very few and non-responsive to foreign antigens, even if rare patients can have close to normal numbers [14, 23–25].

ITK is less widely expressed and is crucial for T-lymphocyte development, as initially shown by knocking out the gene in mice [26]. More in-depth studies revealed that both ITK-deficient mice [5, 6, 27] and the few patients with ITK mutations analysed so far show almost complete absence of invariant natural killer T cells. While several different genetic defects show susceptibility to severe EBV infection, it was recently reported that ITK deficiency is clinically distinct from both signalling lymphocyte activation molecule (SLAM)-associated protein (SAP) and X-linked inhibitor of apoptosis protein (XIAP) deficiency [28]. ITK may also be a crucial host factor needed for the development of an HIV infection [29]. Further studies have shown that ITK-deficient mice have drastically reduced lung inflammation, eosinophil infiltration and mucous production in response to ovalbumin-induced induction of allergic asthma [30]. Therefore, inhibition of T cell activation has been one of the strategies for developing immunosuppressive agents to treat autoimmune disorders and inflammation [31]. Suppression of host immune functions by blocking T cell activation is also a successful modality for preventing organ transplant rejection [32].

In contrast to BTK, ITK is not constitutively expressed. Thus, the corresponding transcript was initially identified from an IL-2-dependent mouse T cell line [11]. This means that even if BTK and ITK frequently are considered to be analogs, selectively expressed in T- and B cells, respectively, their differential expression with regard to the need for inducibility shows that this is not entirely true. This difference is also likely to influence the treatment effect of inhibitors.

Signalling pathways of BTK and ITK and target diseases

TFKs play central, but diverse, modulatory roles in various cellular processes. They participate in signal transduction in response to virtually all types of extracellular stimuli that are transmitted by growth factor receptors, cytokine receptors, G-protein-coupled receptors, antigen receptors and integrins [33, 34]. As illustrated in Fig. 2 (Step 1), following BCR, TCR stimulation, SRC family kinases are activated, leading to the phosphorylation of immunoreceptor tyrosine activation motifs (ITAMs) of the CD79 (Ig-α, Ig-β) and CD3 complex chains. Similarly, PI3K is activated to catalyse the conversion of membrane-associated PIP2 to PIP3 leading to BTK/ITK recruitment to the plasma membrane through the interaction of its PH domain with PIP3 [35]. Concomitantly, the phosphorylation of Ig-α and Ig-β ITAMs leads to the recruitment of SYK/ZAP70 kinase via SYK SH2 domains. Following activation, BTK/ITK ignites multiple downstream signals generating pleiotropic effects (Fig. 2, step 2): (1) PLCγ activation, generation of second messengers, such as inositol [1,4,5]-triphosphate (IP3), diacylglycerol (DAG) and calcium, (2) Cell proliferation, differentiation, apoptosis and survival [16, 36]. However, the molecular basis of many of these pathways is not fully understood, and many interacting molecules remain to be isolated. Using affinity purification combined with tandem mass spectrometry, we have recently characterized the interaction of BTK with an ankyrin-repeat domain protein [37] and the protein 14-3-3,

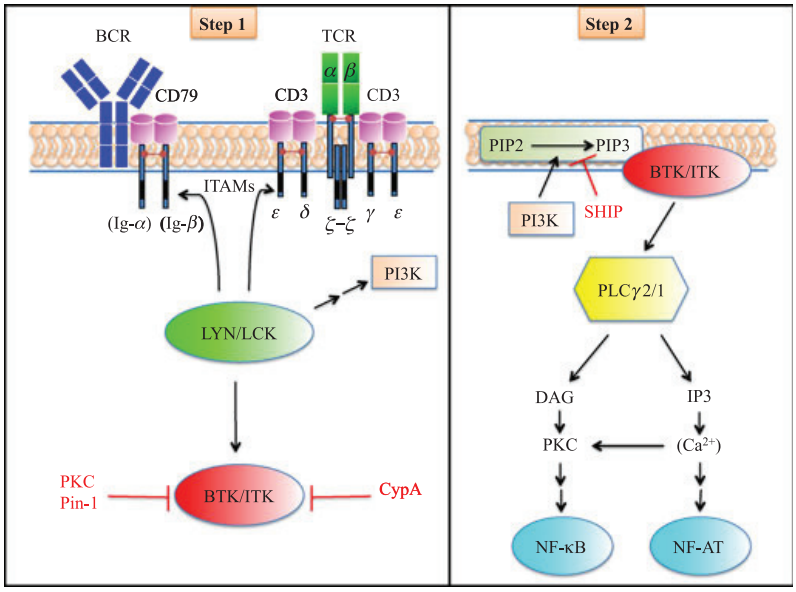

Figure 2 BTK and ITK activation. B cell receptor (BCR) and BTK signalling components are depicted to the left and T cell receptor (TCR) to the right upon engagement of BCR and TCR, SRC family kinases including LYN and LCK are activated leading to phosphorylation of immunoreceptor tyrosine activation motifs (ITAMs) (Step 1). Activated PI3K converts PtdIns-4,5-bisphosphate (PIP2) into PIP3, which tethers BTK/ITK to the membrane (Fig. 1), where they phosphorylate regulatory tyrosine residues in PLCγ2 and PLC, γ1 respectively (Step 2). The increasing PLCγ activity results in the production of the secondary messengers DAG and IP3 inducing activation of the transcription factors NF-κB (active mainly in B cells) and NF-AT (active mainly in T cells). Endogenous BTK/ITK inhibitors (PKC, Pin-1/CypA) regulate their activity affecting cellular responses such as cell survival, apoptosis, adhesion, migration and proliferation.

which regulate nucleo-cytoplasmic shuttling [38] and attenuate signalling [39].

To date, almost only B cell-derived tumours have been treated with the newly developed inhibitor for BTK, Ibrutinib (see below). The rationale is that tumours, similar to non-transformed B cells, may be dependent on BCR signalling for their survival. However, tumours with activating mutations downstream of BTK are likely to be resistant, and this also seems to be the case [40]. Although animal models of autoimmunity show very promising outcomes from Ibrutinib treatment, the drug has not yet been used in clinical studies in man. Allergies and other forms of hypersensitivity and inflammatory diseases with a strong B cell component could also become targets. Target diseases for ITK inhibitors are less defined and will await studies in relevant animal models. As always, potential side effects have to be balanced against the benefit of treatment.

BTK inhibitors

LFM-A13

The leflunomide metabolite analog (LFM-A13) (alpha-cyano-beta-hydroxy-beta-methyl-*N*-(2,5-ibromophenyl)-propen-amide) (Table 2) is one of the first rationally designed antileukemic agents targeting BTK [41, 42]. This small-molecule inhibitor binds non-covalently to the catalytic site of BTK in a reversible manner (half-maximal inhibitory concentration (IC_{50}) = 17.2 μM for human BTK *in vitro* and IC_{50} = 2.5 μM for recombinant BTK). It does not affect the enzymatic activity of other protein tyrosine kinases, including EGFR, HCK, IRK JAK1, JAK3 and IRK at concentrations of 278 μM [41]. However, even if the molecule has been described as a highly specific inhibitor of BTK, it can also efficiently affect the activity of other kinases such as the erythropoietin receptor, JAK2 and downstream molecules [43].

During the last decade, a plethora of *in vitro* and *in vivo* studies have suggested that LFM-A13 could act as a dual-function anticancer drug with apoptosis-promoting and antithrombotic properties [44–46]. In addition, LFM-A13 also exhibits antiproliferative activity against Her2/Neu-overexpressing breast cancer cells [47]. Furthermore, LFM-A13 has been reported to prevent acute fatal graft-versus-host disease in a murine model of allogeneic bone marrow transplantation [48]. In addition, LFM-A13 has been broadly used *in vitro* to inhibit BTK downstream signalling pathways (Fig. 2) and further to elucidate the role of SFKs [49–51]. On neutrophils, for example, it has been shown that LFM-A13 also negatively affects the translocation of Rac-2, RhoA, ADP ribosylation factor-1, TEC, BMX and BTK induced by fMet-Leu-Phe [52]. Moreover, LFM-A13 could block the endogenous phosphorylation of Myd88 adapter-like (Mal) on tyrosine in cells treated with

macrophage-activating lipopeptide-2 or LPS [53] and LFM-A13 inhibited Heme oxygenase (HO-1) induction by the classical TLR4 ligand LPS in cell cultures [54]. Other reports show that LFM-A13 by inhibiting TFKs, rescues the suppression of TCR-induced CD25 expression in Jurkat cells [55]. In primary myeloma-bearing immunodeficient mice, LFM-A13 inhibited osteoclast activity, prevented myeloma-induced bone resorption and moderately suppressed myeloma growth [56]. Administration of LFM-A13 is not toxic to mice, rats or dogs at daily dose levels as high as 100 mg/kg [57]. However, as mentioned, rather high doses are needed for a pharmacological effect, and we are not aware of any ongoing, or planned, clinical studies.

Dasatinib

Dasatinib, BMS-354825 or Sprycel [N-(2-chloro-6-methylphenyl)-2-(6-(4-(2-hydroxyethyl)-piperazin-1-yl)-2-methylpyrimidin-4-ylamino)thiazole-5-carboxamide] (Table 2) is an orally available, dual ABL/SRC tyrosine kinase inhibitor (TKI), which was developed to treat patients with chronic myelogenous leukaemia (CML), who had failed, or were intolerant to, therapy with Imatinib BCR-ABL1 and SFK TKI [58, 59]. Other diseases in which bone metastases are frequent (e.g. breast or prostate tumours) could also benefit from the addition of Dasatinib to standard-of-care treatments [60, 61].

Native targets of Dasatinib in CML cells have been identified using a chemical proteomics approach [62–64]. Besides ABL and SRC kinases, BTK and TEC, but not ITK, were recognized as major binders inhibited by nanomolar concentrations. In addition, the gatekeeper residue as the critical determinant of Dasatinib susceptibility has been detected with the help of structure-based mutagenesis experiments. Mutation of Thr-474 in BTK to Ile and Thr-442 in TEC to Ile conferred resistance to Dasatinib, whereas mutation of the corresponding residue in ITK (Phe-435) to Thr sensitized the otherwise insensitive ITK [64]. Other studies have shown that Dasatinib induces apoptosis in primary chronic lymphocytic leukaemia (CLL) cells blocking LYN kinase activity [65, 66]. Moreover, Dasatinib decreased levels of the activated, phosphorylated forms of AKT, ERK1/2 and p38 and reduced the expression of the antiapoptotic proteins MCL-1 and BCL-X_L [67]. Thus, it seems that Dasatinib as a single agent has activity in relapsed and refractory CLL [66].

In line with the role of TFKs in lymphoid and myeloid cells, Dasatinib inhibited the secretion of several immunomodulators [68, 69]. The observed inhibition of TFKs predicts immunosuppressive (side) effects of this drug and may offer therapeutic opportunities for inflammatory and immunological disorders [64, 70, 71]. However, further experiments are required to describe the exact mechanism of the above-mentioned hypothesis.

In summary, this compound has been approved by the FDA for the treatment of patients with CML in all phases or Ph+-ALL, who were resistant, or intolerant, to therapy, with Imatinib. In Europe, it has been approved for therapy of patients with CML who are resistant, or intolerant, to Imatinib [59]. However, the drug has not been used to clinically interfere with TFKs.

Ibrutinib (PCI-32765)

Ibrutinib, (1-{(3R)-3-[4-amino-3-(4-phenoxyphenyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-1-yl]piperidin-1-yl}prop-2-en-1-one) (Table 2), is a selective and irreversible small-molecule BTK inhibitor that inhibits BCR signalling in human B cells. It was originally named PCI-32765 and re-named Ibrutinib by the World Health Organization (WHO) and the United States Adopted Name (USAN) Council. Orally administered Ibrutinib has demonstrated to be particularly active in different B cell malignancies including CLL, mantle cell lymphoma (MCL), diffuse large B cell lymphoma (DLBCL) and multiple myeloma (MM) [72–75].

Ibrutinib inactivates BTK through covalent binding to the active site (Cys-481) in the ATP-binding domain of BTK with IC_{50} of 0.5 nmol/L [76]. Several TFKs with homology to BTK, including BMX and ITK, have similar cysteine residues that might also be irreversibly inhibited by Ibrutinib. Other kinases that can also be sensitive to Ibrutinib at nanomolar concentrations include BLK, TEC, EGFR, ERBB2, HER2, HER4 and JAK3 [72, 76, 77].

Ibrutinib as a potential drug for B cell malignancies

The use of Ibrutinib in preclinical and clinical trials appears to be a promising new strategy for treatment of B cell malignancies (Table 1). The *in vivo* effect of ibrutinib has been demonstrated in patients with CLL. Recent reports have shown that Ibrutinib inhibits CLL cell survival and proliferation as well as induces CLL apoptosis [77, 78]. In addition, treatment of CD40- or BCR-activated CLL cells with Ibrutinib results in inhibition of BTK tyrosine phosphorylation and also effectively abrogates downstream survival pathways activated by this kinase, including ERK1/2, PI3K and NF-κB [74, 77].

Ibrutinib also acts by modulating the interaction between CLL cells and their microenvironment. For example, it inhibits activation-induced proliferation of CLL cells and effectively blocks survival signals, which are provided externally to CLL cells from the microenvironment (CD40L, BAFF, IL-6, IL-4 and TNFα, fibronectin) engagement and stromal cell contact, as well as migration in response to tissue-homing chemokines (CXCL12, CXCL13) [79]. Moreover, the secretion of BCR-dependent cytokines such as CCL3 and CCL4 is effectively decreased both *in vitro* and in patients with CLL treated with Ibrutinib [78].

Table 1 Clinical trials of Ibrutinib in B cell malignancies.

Disease	Study description	Drugs and doses	Study phase	Estimated patients/age	Objective	Study duration	Clinical trials.gov identifier
CLL and SLL	CLL, SLL	Ibrutinib (420 mg)	II	30 ≥18 years	Impact on leukemia cell trafficking and death	2012–2015	NCT01752426
	ROR CLL,SLL with 17p deletion	Ibrutinib (420 mg)	II	111 ≥18 years	ORR,PFS, OS	2013–2016	NCT01744691
	CLL, SLL in patients older than 65 or have 17p deletion	Ibrutinib (420 mg)	II	86 ≥65 years and ≥ 18 year for 17p deletion	ORR, OS, PFS	2011-2015	NCT01500733
	CLL, SLL	Ibrutinib (420 mg) VS chlorambucil	III	272 ≥65 years	ORR, PFS	2013–2016	NCT01722487
	ROR CLL, SLL	Ibrutinib (420 mg) VS Ofatumumab	III	350 ≥18 years	PFS, OS, ORR	2012–2015	NCT01578707
	ROR CLL,SLL	Ibrutinib (420 mg) + Rituximab+ Bendamustine	III	580 ≥18 years	PFS,OS, ORR	2012–2018	NCT01611090
CLL, SLL, B-PLL	ROR CLL,SLL, B-PLL	Ibrutinib	II	75 ≥18 years	PFS, ORR,OS	2012–2014	NCT01589302
DLBCL	ROR DLBCL	Ibrutinib (560 mg)	II	60 ≥18 years	Efficacy and safety	2011–2014	NCT01325701
FL	Refractory FL	Ibrutinib (560 mg)	II	110 ≥18 years	ORR,OS, FPS	2013–2016	NCT01779791
MCL	MCL	Ibrutinib (560 mg)	II	110 ≥18 years	ORR, PFS, OS	2012–2015	NCT01599949
	ROR MCL	Ibruinib (560 mg) VS Temsirolimus	III	280 ≥18	PFS, OS	2012	NC2012-000601-74
	ROR MCL	Ibrutinib (560 mg) VS Temsirolimus	III	280 ≥18 years	PFS,ORR, OS	2012–2017	NCT01646021
	MCL	Ibrutinib (560 mg) + Rituximab+ Bendamustine	III	520 ≥65 years	PFS,OS, ORR	2013–2019	NCT01776840
B cell neoplasm	Recurrent mature B cell neoplasm	Ibrutinib (420, 560 mg)	I	24 ≥20 years	Safety and pharmacokinetic	2012–2014	NCT01704963
MM	Relapsed or relapsed and refractory MM	Ibrutinib (420, 560, 840 mg)	II	164 ≥18 years	Efficacy and safety	2012–2016	NCT01478581
WM	WM	Ibrutinib	II	33 ≥18 years	ORR, safety	2012–2014	NCT01614821

Follicular lymphoma (FL), overall response rate (ORR), progression-free survival (PFS), overall survival (OS), chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), multiple myeloma (MM), mantle cell lymphoma (MCL), diffused large B cell lymphoma (DLBCL), B cell prolymphocytic leukemia (B-PLL), relapsed or refractory (ROR), Waldenströms macroglobulinemia (WM).

The initial phase I study of Ibrutinib enrolled patients with B cell lymphomas including CLL demonstrated that a dose of 420 mg is as efficient as 840 mg. In fact, the occupancy and inhibition of BTK were similar in both doses, so 420 mg was selected for further studies to minimize adverse effects [77]. Administration of Ibrutinib (420 mg/day) in patients with CLL induces a rapid shrinkage of enlarged lymph nodes and symptomatic improvement within the first few weeks of treatment [73]. The expected pattern of initial rapid nodal response with sometimes marked lymphocytosis was also observed. This increase in lymphocyte count was transient and could be typically resolved after the first few months of therapy [72, 77]. In the 2012 American Society of Hematology meeting, it was reported that Ibrutinib induces an overall response rate (ORR) of 68% in previously untreated CLL patients, aged 65 or older, and an ORR of 71% in previously treated patients [72].

It has been recently reported that BTK is highly expressed in malignant plasma cells from patients with MM [80, 81]. This is in contrast to the most mature normal plasma cells, where BTK is not expressed [22]. In MM models, Ibrutinib reduced osteoclast formation and bone resorption and also inhibited BTK-mediated osteoclastogenesis induced by M-CSF and RANKL [80]. The chemokine and cytokine secretion from bone marrow stromal cells (BMSCs) and osteoclasts was significantly decreased by Ibrutinib, and it blocks SDF-1-induced adhesion and migration [81]. Furthermore, it also inhibited MM cell growth triggered by IL-6 or coculture with

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