

Targeting Bruton's tyrosine kinase in B cell malignancies

Rudi W. Hendriks, Saravanan Yuvaraj and Laurens P. Kil

Abstract | Bruton's tyrosine kinase (BTK) is a key component of B cell receptor (BCR) signalling and functions as an important regulator of cell proliferation and cell survival in various B cell malignancies. Small-molecule inhibitors of BTK have shown antitumour activity in animal models and, recently, in clinical studies. High response rates were reported in patients with chronic lymphocytic leukaemia and mantle cell lymphoma. Remarkably, BTK inhibitors have molecular effects that cannot be explained by the classic role of BTK in BCR signalling. In this Review, we highlight the importance of BTK in various signalling pathways in the context of its therapeutic inhibition.

B cell receptor

(BCR). An antigen receptor that is specifically expressed on the cell surface of B cells and composed of two immunoglobulin heavy (IgH) chains and two immunoglobulin light (IgL) chains.

Pre-BCR

A receptor that consists of an immunoglobulin heavy (IgH) chain, which is associated with two germline-encoded surrogate light chain components and is transiently expressed on the cell surface of pre-B cells after successful IgH chain rearrangement.

Bruton's tyrosine kinase (BTK) was originally identified in 1993 as a non-receptor protein tyrosine kinase that is defective in the inherited immunodeficiency disease X-linked agammaglobulinaemia¹⁻³ (XLA). In affected males, B lymphocytes and immunoglobulins are almost completely absent from the circulation owing to a severe defect in B cell development (BOX 1). This defect was shown to be intrinsic to the B cell lineage: in female carriers of this disease, the X chromosome with the mutant *BTK* gene is inactivated in the B cell lineage, which suggests that B-lineage cells that harbour a defective *BTK* allele on the active X chromosome have a selective disadvantage⁴. Patients with XLA have no significant developmental defects in other immune cells, and this is consistent with the restriction of clinical features to B cell immunity. Likewise, in the CBA/N strain of mice, the less severe X-linked immunodeficiency (XID) phenotype was found to be due to a point mutation in *Btk*^{5,6}, and the effects of this mutation also seem to be limited to the B cell population (BOX 1).

Shortly after the discovery of BTK, it was shown that stimulation of the B cell receptor (BCR) in mature B cells induces tyrosine phosphorylation of BTK and increases its kinase activity, thereby placing BTK in the BCR signal transduction pathway⁷⁻⁹. This is consistent with the defective activation that is observed in BCR-stimulated *Btk*-deficient mouse B cells, which lack progression into cell division, have a high susceptibility to apoptosis and have a limited induction of activation markers on their cell surface¹⁰⁻¹². Moreover, the XLA phenotype in humans can be explained by a parallel function of BTK in signalling downstream of the pre-BCR. The pre-BCR is an immature form of the BCR that monitors for

functional immunoglobulin heavy (IgH) chain rearrangement by deposition of the IgH μ protein on the cell surface, thereby providing signals for survival, proliferation and cellular differentiation¹³. Loss of BTK function disrupts this pre-BCR checkpoint function; therefore, in patients with XLA, clonal expansion and developmental progression of IgH μ chain-expressing pre-B cells is abrogated (BOX 1).

BTK is expressed in many B cell leukaemias and lymphomas^{14,15}. Because of this, targeting BTK to develop new treatment modalities for B cell malignancies became an attractive idea. In 1999, only a few years after the identification of BTK, the first rationally designed BTK small-molecule inhibitor (LFM-A13) was shown to have anti-leukaemic activity *in vitro*¹⁶ (TABLE 1). More selective BTK inhibitors were subsequently developed, including the irreversible inhibitor ibrutinib (also known as PCI-32765) (TABLE 1), which induced objective clinical responses in dogs with spontaneous B cell non-Hodgkin's lymphoma¹⁷. Ibrutinib was also used to show that BTK is involved in oncogenic BCR signalling that controls the survival of a human activated B cell-like subtype of diffuse large B cell lymphoma¹⁸ (ABC-DLBCL). Ibrutinib monotherapy has recently shown encouraging clinical activity in patients with chronic lymphocytic leukaemia (CLL) and mantle cell lymphoma (MCL) in Phase II trials^{19,20}. As a result, ibrutinib received breakthrough designation and was approved for the treatment of relapsed MCL by the US Food and Drug Administration (FDA) in 2013.

Because of the XLA and XID phenotypes and the central role of the pre-BCR and the BCR in B cell development and function, BTK is most intensely studied in the context of pre-BCR and BCR signalling.

Department of Pulmonary Medicine, Room Ee2251a, Erasmus MC Rotterdam, PO Box 2040, NL 3000 CA Rotterdam, the Netherlands. Correspondence to R.W.H. e-mail: r.hendriks@erasmusmc.nl
doi:10.1038/nrc3702

Key points

- Bruton's tyrosine kinase (BTK) was originally identified as a non-receptor protein tyrosine kinase that is defective in the inherited immunodeficiency disease X-linked agammaglobulinaemia (XLA).
- BTK has long been known to be a key component of B cell receptor (BCR) signalling that regulates B cell proliferation and survival.
- Recently, a small-molecule inhibitor of BTK, called ibrutinib, has shown antitumour activity in patients with various B cell malignancies.
- The antitumour activity of BTK inhibition is not solely dependent on the role of BTK in BCR signalling.
- BTK inhibition also targets Toll-like receptor (TLR) signalling, B cell adhesion and migration, as well as cells in the tumour microenvironment.
- Ibrutinib-induced lymphocytosis in patients with chronic lymphocytic leukaemia and mantle cell lymphoma leads to the effective expulsion of malignant B cells out of their nursing microenvironment.
- For many different B cell malignancies, treatment with BTK inhibitors is expected to be more effective and have fewer toxic effects than currently used therapies.

Diffuse large B cell lymphoma

(DLBCL). Numerous heterogeneous subtypes of mostly aggressive B cell lymphomas. The gene expression signatures of activated B cell-like DLBCL (ABC-DLBCL) and germinal centre-like DLBCL (GC-DLBCL) resemble those of activated peripheral blood B cells and germinal centre B cells, respectively.

Chronic lymphocytic leukaemia

(CLL). A leukaemia that mostly affects the elderly and that is characterized by a lymphocytosis ($>5 \times 10^9$ B cells per litre) of monoclonal CD5⁺ B cells.

Mantle cell lymphoma

(MCL). An aggressive form of non-Hodgkin's lymphoma that is characterized by cell cycle deregulation because of cyclin D1 overexpression, which is translocated as part of the t(11;14) translocation. Additional aberrations in genes that control the cell cycle and DNA damage responses have been reported.

Toll-like receptors

(TLRs). Innate receptors that are present on the cell surface or in endosomes and that recognize distinct, highly conserved molecular motifs, including polysaccharides, DNA and RNA, that are shared by pathogens.

However, BTK is also involved in signalling pathways downstream of many other receptors, including G protein-coupled chemokine receptors and Toll-like receptors (TLRs). Moreover, despite the apparently B cell-restricted phenotype in XLA and XID, BTK is expressed in all haematopoietic cells, except in T lymphocytes^{14,21}. Intriguingly, *in vitro* studies, preclinical investigations in animal models and clinical trials have now provided good evidence that the antitumour activity of BTK inhibitors is not solely dependent on the role of BTK in BCR signalling. Rather, BTK inhibition also targets TLR signalling, B cell adhesion and migration, and cells in the tumour microenvironment.

In this Review, we highlight the importance of BTK in multiple signalling pathways in B lymphocytes and other haematopoietic cells. We also discuss the crucial role of BTK in oncogenic signalling that is essential for the survival of leukaemic B cells. In addition, we discuss the promising results of recent clinical trials indicating that although resistance may develop in some cases, for many different B cell malignancies, treatment with BTK inhibitors is expected to be more effective and have fewer toxic effects than currently used therapies.

BTK structure, activity and regulation

Together with four other members (BOX 2), BTK belongs to the TEC family of non-receptor kinases, which have been strongly conserved throughout evolution²². The domain structure of BTK is similar to that of the SRC family kinases, and it contains the SRC homology domains (SH domains) SH2 and SH3, as well as a catalytic domain (FIG. 1). However, unlike SRC family kinases, BTK has an amino-terminal pleckstrin homology domain (PH domain) and a proline-rich TEC homology (TH) domain, which contains a zinc-finger motif that is important for optimal activity and stability of the protein^{22,23}. SRC family kinases are constitutively membrane-associated owing to myristoylation, whereas BTK is cytoplasmic and only transiently recruited to the membrane. This requires binding of the BTK PH domain to phosphatidylinositol-3,4,5-trisphosphate (PIP3), which is generated by PI3K.

BTK activation is initiated by cell membrane association and phosphorylation of Y551 in the kinase domain, either by a SRC family kinase or by spleen tyrosine kinase (SYK). This promotes the catalytic activity of BTK and results in its autophosphorylation at position Y223 in the SH3 domain^{24,25}. Autophosphorylation is increased in the E41K gain-of-function mutation in the PH domain, and expression of this mutant can transform fibroblasts²⁶. However, transgenic expression of BTK-E41K showed that this mutant does not have oncogenic activity in B cells or myeloid cells *in vivo*²⁷, and no constitutively active BTK mutants have been identified in malignancies to date.

Almost 700 unique loss-of-function BTK mutations have currently been described in the [BTKbase mutation registry](#) for XLA²⁸. These include missense, nonsense and splice site mutations, as well as deletions and insertions, and these alterations are more or less equally distributed over the gene. However, missense mutations or in-frame deletions are absent in the SH3 domain — although, based on its size, ~25 of the 251 mutations of this type identified so far should be located in this domain. Therefore, the SH3 domain is either not crucial for BTK function or it has a regulatory role. In particular, the functional importance of SH3 Y223 autophosphorylation remains unclear because phosphorylation of Y223 does not seem to affect BTK activity, and the transgenic expression of BTK-Y223F essentially corrected the XID phenotype²⁹.

Although predominantly cytoplasmic, a small proportion of BTK is detected in the nucleus³⁰. It has been proposed that BTK functions as a transcriptional regulator in B cells by interacting with various nuclear proteins, including BRIGHT (also known as ARID3A) and BAM11 (encoded by *MLLT1*) (FIG. 1), in a nuclear complex that regulates the transcription factor TFII-I^{31,32}. However, the importance of nuclear BTK activity remains elusive.

Upon BCR stimulation, mature B cells increase BTK levels³³, but the post-transcriptional mechanisms involved are complex and only partially resolved. These include regulation of BTK expression by microRNA-185 (miR-185)³⁴, as well as a proteasome-dependent positive autoregulatory feedback loop by which BTK stimulates transcription from its own promoter through a pathway involving nuclear factor-κB (NF-κB)³⁵. Studies in mice have shown that precise regulation of BTK expression levels is essential for normal B cell function. The expression of subphysiological levels of transgenic *Btk* only partially corrects the phenotype of *Btk*-deficient B cells, whereas physiological levels provide a complete rescue^{36,37}. Moreover, transgenic overexpression of wild-type human BTK in mice results in reduced susceptibility to apoptosis and an autoimmune pathology³⁸. Although the overexpression of BTK in B cells did not induce neoplasia in transgenic mice, it did accelerate disease onset and increase mortality in a CLL mouse model³⁹.

This complex regulation of BTK function by membrane association, the induction of enzymatic activity and the modulation of protein expression levels is best studied in the context of BCR signalling, as discussed below.

SRC family kinases

SRC kinase, together with other members of the SRC kinase family, is a crucial regulator of cellular proliferation, survival and migration that is frequently activated in different types of cancers.

SRC homology domains

(SH domains). The SH domains SH2 and SH3 are protein modules that are involved in protein–protein interaction and that bind to phosphorylated tyrosines and proline-rich regions, respectively.

Pleckstrin homology domain

(PH domain). PH domains are protein modules that can bind to phosphatidylinositol lipids and that are involved in the recruitment of proteins to membranes — in particular, the cell membrane.

Wiskott–Aldrich syndrome protein

(WASP). The WASP protein is a cytoplasmic protein that is defective in the X-linked immunodeficiency Wiskott–Aldrich syndrome. It functions as an actin regulator by binding to and activating the ARP2–ARP3 complex.

BTK and BCR signalling

Activation of BTK by BCR signalling. The BCR complex is non-covalently associated with a disulphide-linked Ig α (also known as CD79A)–Ig β (also known as CD79B) heterodimer (FIG. 2). Upon antigen binding to the BCR, a SRC family protein tyrosine kinase, most probably LYN, phosphorylates the Ig α and Ig β immunoreceptor tyrosine-based activation motifs (ITAMs), thereby creating docking sites for SYK⁴⁰. In parallel, LYN phosphorylates tyrosine residues in the cytoplasmic tail of the BCR co-receptor CD19, which enables the binding and activation of PI3K and VAV^{41,42}. Besides CD19, cytoplasmic B cell adapter for PI3K (BCAP) can also recruit PI3K⁴³, which indicates a redundancy in activating PI3K after triggering the BCR. PI3K generates PIP3, which is an important second messenger for activating downstream pathways (FIG. 2). PI3K attracts BTK to the cell membrane through a PIP3–PH domain interaction⁴⁴, which allows SYK and LYN to fully activate BTK by *trans*-phosphorylation at Y551 (REFS 24,45) (discussed above). BTK activation can be regulated by the phosphatases PTEN and SH2 domain-containing inositol-5'-phosphatase-1 (SHIP1; also known as INPP5D), which dephosphorylate PIP3 and thereby inhibit BTK membrane association. Once SYK is activated, signalling is propagated to downstream effectors by the recruitment and phosphorylation of the SH2 domain-containing leukocyte protein of 65 kDa (SLP65; also known as BLNK and BASH), which serves as a scaffold for various signalling molecules, including SYK, BTK and its crucial substrate phospholipase C- γ 2 (PLC γ 2)^{46,47}.

Downstream targets of BTK. The SLP65-mediated recruitment of BTK and PLC γ 2 completes the formation of so-called micro-signalosomes, which comprise VAV, PI3K, BTK, SLP65 and PLC γ 2 (REF. 48) (FIG. 2). BTK is mostly responsible for PLC γ 2 phosphorylation at positions Y753 and Y759, which is essential for the lipase activity of PLC γ 2 (REF. 49). Moreover, independent of its kinase activity, BTK can recruit

phosphatidylinositol-4-phosphate 5-kinase (PIP5K), thereby stimulating a positive-feedback loop that generates phosphatidylinositol 4,5-bisphosphate (PIP2), which serves as a substrate for both PI3K and PLC γ 2 (REF. 50). This feed-forward mechanism allows BTK to stimulate the PIP3 production that is required for its own activation. In agreement with these findings in mature A20 B cells, we noted that during B cell development in mice BTK function is partly independent of its kinase activity²⁹.

Upon activation by BTK, PLC γ 2 cleaves PIP2 to generate two second messengers, inositol triphosphate (IP3) and diacylglycerol (DAG), which activate diverging and partially overlapping signalling pathways. IP3 is involved in regulating intracellular Ca²⁺ levels and thereby activates nuclear factor of activated T cells (NFAT) transcription factors via calmodulin. DAG mediates activation of protein kinase C β (PKC β), which induces RAS signalling-dependent phosphorylation of the extracellular signal-regulated kinases ERK1 and ERK2. Unlike ERK1 and ERK2, other MAPKs, such as p38 and JUN N-terminal kinase (JNK), can be induced by PLC γ 2 without intermediate signalling via RAS⁵¹. Importantly, PKC β also activates the NF- κ B pathway through a scaffold complex that includes caspase recruitment domain-containing protein 11 (CARD11; also known as CARMA1 and BIMP1), BCL-10 and mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1)⁵² (FIG. 2). Whereas BCR stimulation does not induce NF- κ B activation in BTK-deficient B cells, T cell-dependent NF- κ B activation that is mediated by CD40 ligand (CD40L)–CD40 interaction remains unaffected^{53,54}, and this gives an explanation of why B cells in *Btk*-deficient mice can respond to T cell-dependent antigens but not to T cell-independent antigens¹⁰.

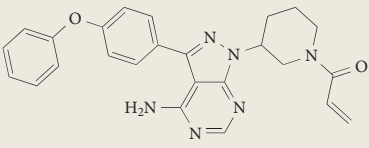
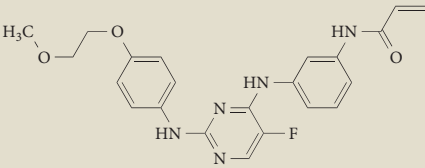
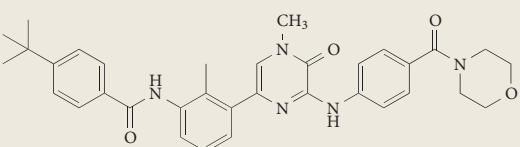
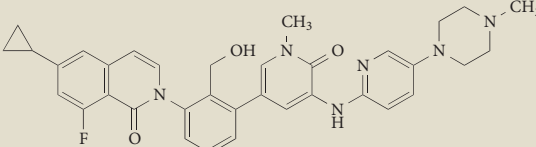
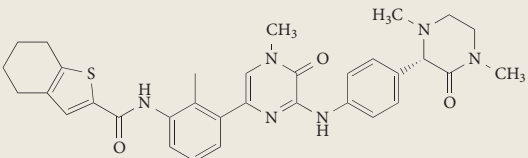
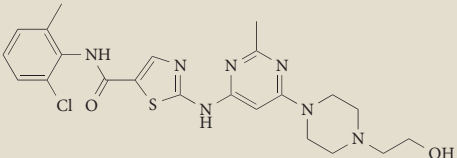
In addition, BTK connects BCR signalling to actin dynamics that are involved in BCR internalization, processing and antigen presentation. BTK directly interacts with Wiskott–Aldrich syndrome protein (WASP) and can activate it by inducing its phosphorylation,

Box 1 | Phenotype of Bruton's tyrosine kinase deficiency in humans and mice

Loss-of-function mutations in the Bruton's tyrosine kinase (*BTK*) gene in humans cause the primary immunodeficiency disease X-linked agammaglobulinaemia (XLA), which was first described by Bruton in 1952 (reviewed in REF. 3). XLA is a rare disease (with an incidence of 1 per 380,000 live births in the United States)¹⁵² that is characterized by an almost complete block of B cell development at the pre-B cell stage. In the bone marrow, the number of pre-B cells expressing the immunoglobulin- μ heavy (IgH μ) chain in their cytoplasm is variable but often reduced. Pre-B cells in patients with XLA are small in size, which suggests that BTK is essential for their proliferation¹⁵³. Patients have few circulating B cells, and these cells have an aberrant immature IgM^{high} phenotype, as well as a distinct antibody repertoire that is enriched in autoreactive B cell receptors (BCRs)^{3,154}. Serum immunoglobulin levels of all subclasses are very low. Affected boys usually present with severe or recurrent infections with encapsulated bacteria — predominantly otitis media, sinusitis and pneumonia. In addition, gastrointestinal infections that are caused by *Giardia* parasites and skin infections are frequently found, but meningitis and osteomyelitis are less common¹⁵². Current treatments involve antibiotics and intravenous or subcutaneous immunoglobulin substitution therapy. Although BTK is abundantly expressed in myeloid cells, patients with XLA do not show substantial defects in innate immune responses. Moreover, patients who are maintained on sufficient Ig therapy are generally quite healthy, which suggests that BTK is dispensable outside the B cell compartment.

By contrast, *Btk*-deficient mice — either the X-linked immunodeficiency (XID) CBA/N strain that harbours the R28C *Btk* mutation, or mice with a targeted disruption of the *Btk* gene — show a mild phenotype^{5,6,10,155}. Although minor defects in pre-BCR checkpoint function have been observed¹⁵⁶, *Btk* deficiency is mainly characterized by impaired differentiation and survival of mature B cells. B cell numbers in the spleen and the lymph nodes are reduced by ~50%, serum levels of IgM and IgG3 are low, but other immunoglobulin subclasses are normal.

Table 1 | Bruton's tyrosine kinase inhibitors in preclinical development and clinical trials

Name and structure	IC ₅₀	Mode of action	Selectivity	Clinical trials	Refs
Ibrutinib (also known as PCI-32765) (Pharmacyclics) 	0.5 nM	<ul style="list-style-type: none"> Irreversible, covalent binding to C481 Inhibition of BTK kinase activity Does not prevent BTK Y551 phosphorylation 	<ul style="list-style-type: none"> High level of cross-reactivity with BLK and BMX (IC₅₀ = 0.5 nM), which contain a cysteine that aligns with C481 in BTK Cross-reactivity with ITK (IC₅₀ = 11 nM) affects T cell-mediated immunity in infectious disease models¹⁶² Low cross-reactivity with TEC (IC₅₀ = 78 nM), LYN (IC₅₀ = 200 nM) and SYK (IC₅₀ >10,000 nM) 	Of 48 studies: <ul style="list-style-type: none"> Reported: Phase I in CLL, DLBCL, follicular lymphoma, MCL and WM; high response rates in Phase II in CLL and MCL Ongoing: CLL (Phases I, II and III); MCL (Phases II, III and IV); DLBCL (Phases II and III); multiple myeloma (Phase II); WM (Phase II); follicular lymphoma (Phases I and II) and HCL (Phase II) 	17, 19, 20
CC-292 (also known as AVL-292) (Celgene) 	<0.5 nM	<ul style="list-style-type: none"> Irreversible, covalent binding to C481 Inhibition of BTK kinase activity Does not prevent BTK Y551 phosphorylation 	<ul style="list-style-type: none"> High cross-reactivity with BMX (IC₅₀ = 0.7 nM), which contains a cysteine that aligns with C481 in BTK Limited cross-reactivity with TEC (IC₅₀ = 6.2 nM) and ITK (IC₅₀ = 36 nM) Low cross-reactivity with LYN (IC₅₀ = 4,400 nM) and SYK (IC₅₀ >10,000 nM) 	Of four studies: <ul style="list-style-type: none"> Safety studies in CLL Escalating dose study in CLL and WM Phase IB study in B cell lymphoma 	163
CGI-1746 (CGI Pharmaceuticals) 	1.9 nM	<ul style="list-style-type: none"> Reversible binding to unphosphorylated BTK in SH3 domain, stabilizing BTK in an inactive conformation Blocks BTK phosphorylation at Y551 and Y223 	<ul style="list-style-type: none"> Superior BTK selectivity No or minimal cross-reactivity with TEC family kinases (ITK IC₅₀ = 4,270 nM; TEC IC₅₀ >10,000 nM and BMX IC₅₀ = 1,870 nM) or SRC family kinases (LYN IC₅₀ = 10,000 nM; SRC IC₅₀ = 3,650 nM) 	Preclinical	146
RN486 (F. Hoffmann-La Roche) 	0.3 nM	<ul style="list-style-type: none"> Reversible binding to BTK Blocks BTK phosphorylation at Y551 	<ul style="list-style-type: none"> Minimal cross-reactivity SLK (IC₅₀ = 43 nM) TEC (IC₅₀ = 64 nM) ITK (IC₅₀ = 240 nM) 	Preclinical	148, 164
GDC-0834 (Genentech/Gilead) 	5.9 nM	Highly selective, reversible and ATP-competitive small-molecule inhibitor of BTK, being developed as a therapeutic agent for rheumatoid arthritis	Unknown	Has shown efficacy in mouse autoimmune models, but its use in treatment of human disease is challenged by the rapid hydrolysis of the active form to an inactive metabolite in healthy human volunteers; no clinical studies	147, 165
Dasatinib (also known as BMS-354825) (Bristol-Myers Squibb) 	5.0 nM	Binds to the ATP-binding site of the active and inactive conformation of the ABL kinase domain	Reversible multi-kinase inhibitor, originally developed as a SRC and ABL inhibitor; high cross-reactivity with the TEC family kinases TEC (IC ₅₀ = 297 nM) and the SRC family kinases LYN and SRC	A total of 230 studies (completed and ongoing): Phase I-IV studies in BCR-ABL-positive patients with ALL, CML, CLL and multiple myeloma	116

Box 2 | The TEC kinase family

The TEC family of non-receptor tyrosine kinases consists of Bruton's tyrosine kinase (BTK), as well as four additional members: TEC, inducible T cell kinase (ITK), resting lymphocyte kinase (RLK) and bone marrow-expressed kinase (BMX)²².

Three TEC kinase family members, ITK, TEC and RLK, are expressed in the T cell lineage. In thymocytes and naive T cells, ITK is the most highly expressed family member and, parallel to BTK, it is activated in response to T cell receptor (TCR) engagement and is required for downstream phosphorylation of phospholipase C γ 1 (PLC γ 1) and for Ca²⁺ mobilization¹⁵⁷. Moreover, ITK contributes to TCR-induced cytoskeleton regulation, integrin activation, chemokine signalling and cytokine production. Although ITK and RLK show partial compensatory activity during T cell development, they have differential effects in CD4⁺ T helper cell polarization¹⁵⁷. ITK loss-of-function mutations cause a rare immunodeficiency syndrome that is characterized by the inability to control Epstein–Barr virus (EBV) infection, resulting in EBV-associated B cell lymphoproliferative disorders¹⁵⁸.

Although TEC is expressed throughout B cell and T cell development, *Tec*-deficient mice do not show an immunological phenotype. *Btk;Tec* double-deficient mice, however, manifest a severe arrest of B cell development at the pre-B cell stage. Therefore, phenotypic differences between X-linked immunodeficiency (XID) in mice and X-linked agammaglobulinaemia (XLA) in humans might partly be explained by the ability of TEC to compensate for BTK in mice¹⁵⁹. Moreover, *Btk;Tec* double-deficient mice show severe osteopetrosis that is caused by defective osteoclast function¹²⁶.

BMX is expressed in myeloid cells, where it regulates the secretion of pro-inflammatory cytokines, epithelial cells, endothelial cells and fibroblasts¹⁶⁰. Like BTK, it interacts with the Toll-like receptor (TLR) pathway adaptors myeloid differentiation primary response 88 (MYD88) and MYD88 adaptor-like protein (MAL). Most importantly, in arterial endothelial cells, BMX is involved in transduction of vascular endothelial growth factor (VEGF) signals, and it thereby actively contributes to tumour angiogenesis and growth¹⁶¹. BMX is expressed in several cancer types and was shown to activate signal transducer and activator of transcription 3 (STAT3) signalling in glioblastoma cancer stem cells.

Pre-B cell acute lymphoblastic leukaemia (Pre-B ALL). ALL is the most common childhood malignancy, derived from the precursor B cell compartment in the bone marrow.

however, independent of its kinase activity, BTK does cooperate with SLP65 to regulate IL-7-mediated pre-B cell proliferation and survival, which suggests that BTK could function as a tumour suppressor^{69,70}. Somatic *BTK* or *SLP65* mutations have not been observed in human childhood pre-B cell acute lymphoblastic leukaemia (pre-B ALL), whereas kinase-deficient *BTK* splice variants and a deficiency of SLP65 protein owing to aberrant splicing have been documented^{71–73}. Further studies are required to determine whether defective SLP65 or BTK expression has a role in the oncogenic transformation of pre-B cells in ALL.

Role of BTK in other signalling pathways

BTK is expressed in many haematopoietic cell types^{14,21}, where its involvement in various pathways has been defined³. Likewise, in B cells, BTK participates in multiple pathways, including chemokine receptor and TLR signalling.

BTK and chemokine receptor signalling. Biochemical analyses and *in vitro* adhesion and migration assays have established the involvement of BTK in chemokine receptor pathways that are essential for B cell trafficking, tissue homing and homeostasis. BTK is a key signalling molecule for the chemokine receptors CXCR4 and CXCR5 (REF. 74). CXC-chemokine ligand 12 (CXCL12; also known as SDF1), which is highly expressed by stromal cells in the bone marrow and in germinal centres, induces BTK activation⁷⁴, most probably by direct interactions

between BTK and the CXCR4-linked heterotrimeric G protein subunits (FIG. 3a). Both G α and G β subunits can directly bind to the PH domain and the adjacent TH domain of BTK^{75,76}. G β subunits might also bind to the catalytic domain⁷⁷. Although G β subunits stimulate membrane translocation, the membrane anchorage of BTK is dependent on PIP3 (REF. 77). Because a reduction of CXCL12-controlled migration was found in B cells that lacked LYN or SYK, these kinases were postulated to activate BTK after CXCR4 ligation⁷⁴. This was confirmed by the finding that CXCL12-induced phosphorylation of BTK at Y551 is reduced in the presence of a SYK inhibitor⁷⁸. In agreement with the idea that BTK has a key role in chemokine receptor signalling, treatment of MCL and CLL cells with ibrutinib inhibited CXCL12-induced and CXCL13-induced phosphorylation of PLC γ 2, ERK1, ERK2, JNK and AKT, as well as cell adhesion and migration, *in vitro*^{78–80}. Ibrutinib also reduced CC-chemokine ligand 19 (CCL19)-induced adhesion and migration, which is mediated by CC-chemokine receptor 7 (CCR7) — a receptor that is highly expressed on CLL cells.

A role for BTK in chemokine signalling *in vivo* was first demonstrated by adoptive transfer experiments with *Btk*-deficient B cells in mice, which showed that B cell homing to lymph nodes was particularly affected⁷⁴. Recent data from clinical trials show that ibrutinib treatment results in an egress of malignant cells into the circulation^{19,20}, which indicates that BTK function is essential for the homing of B cells into lymphoid tissues in humans (discussed below).

BTK and TLR signalling. A role for BTK in TLR signalling was first shown by the finding that the proliferation of *Btk*-deficient B cells is reduced in response to the TLR4 ligand bacterial lipopolysaccharide (LPS)¹⁰. Upon activation, most TLRs recruit the adaptor myeloid differentiation primary response 88 (MYD88). Exceptions to this include TLR3 (a receptor that is specific for detecting viral double-stranded RNA), which uses TIR domain-containing adaptor protein inducing interferon- β (TRIF; also known as TICAM1), and TLR4, which can signal both via a MYD88-dependent pathway (FIG. 3b) and via a MYD88-independent pathway⁸¹. TLR signalling induces the downstream transcription factors NF- κ B, activator protein 1 (AP1) and interferon regulatory factor 3 (IRF3), which, in B cells, results in upregulation of activation markers, proliferation, antibody secretion, class switch recombination and the production of pro-inflammatory cytokines. BTK can directly interact with cytoplasmic Toll/IL-1 receptor (TIR) domains of most TLRs, as well as with the downstream adaptors MYD88, TRIF and MYD88 adaptor-like protein (MAL; also known as TIRAP), and IL-1R-associated kinase 1 (IRAK1)^{82–85} (FIG. 3b). How B cells integrate adaptive BCR and innate TLR activation is an area of intensive research. Interestingly, TLR9 and BCR stimulation can synergistically induce the production of IL-6, whereby BTK is required for colocalization of TLR9 and BCR in an autophagosomal-like compartment⁸⁶. Given that BCR signalling is initiated at the cell surface and continues to activate MAPK

Anergic response

A reversible programme that is characterized by low levels of B cell receptor (BCR) expression and limited BCR-induced activation of signalling molecules, which serves to silence autoreactive B cells.

Nurse-like cells

(NLCs). Peripheral blood monocyte-derived adherent cells to which chronic lymphocytic leukaemia (CLL) B cells become attached. This interaction can protect CLL cells from spontaneous apoptosis *in vitro*.

as it traffics to intracellular compartments⁸⁷, it is conceivable that in endosomes, TLR and BCR signalling are interconnected by BTK.

BTK signalling in B cell malignancies

Evidence is accumulating that BTK activity is crucial for the survival or proliferation of leukaemic B cells, either in a B cell-intrinsic manner or in the context of the tumour microenvironment. Here, we discuss the role of BTK and the effects of BTK inhibition for individual B cell malignancies (summarized in TABLE 2).

CLL. CLL is characterized by the accumulation of non-proliferating monoclonal CD5⁺ mature B cells in the blood. CLL B cells generally have low surface IgM expression and show an anergic response to BCR ligation, which indicates chronic BCR internalization and signalling⁸⁸. Continuous antigen binding in lymphoid tissues is thought to be essential to anergize CLL cells, because IgM-BCR unresponsiveness can be restored following *in vitro* culture⁸⁹. Expansion of malignant CLL cells occurs in proliferation centres in lymph nodes where antigens, chemokines, TLR ligands and T cell co-stimulation provide important signals for survival, proliferation and migration⁹⁰. Patients who have CLL with hypermutated IgH chain V (IGHV) genes (M-CLL) have a more favourable prognosis than those with unmutated IGHV genes (U-CLL). Together with the highly restricted and biased IGHV gene repertoire in CLL, which is referred to as ‘stereotyped BCRs’, and the recent identification of the antigen specificity of particular CLL B cells, these findings strongly indicate that B cell proliferation that is driven by specific autoantigens or antigens derived from apoptotic

cells or pathogens is essential for CLL pathogenesis^{91,92}. In contrast to this ligand-dependent BCR signalling, CLL BCRs showed Ca²⁺ signalling in the apparent absence of a ligand, which resulted from self-recognition through an internal BCR epitope in the second framework region of the IgH chain⁹³. The two models of BCR signalling, one dependent on external ligands and the other dependent on an internal BCR epitope, might not be mutually exclusive, because cell-autonomous signalling could be augmented by an antigen-driven response.

Several findings show that BTK signalling substantially contributes to the initiation or maintenance of CLL. BTK is overexpressed in CLL B cells and is constitutively phosphorylated in a proportion of CLL samples⁹⁴. In a CLL mouse model, *Btk* deficiency abrogated tumour formation, whereas transgenic BTK overexpression increased tumour incidence and overall mortality^{39,95}. The treatment of CLL cells with ibrutinib *in vitro* diminished cell survival and proliferation^{80,94}, and it abolished BCR-stimulated AKT and ERK phosphorylation^{78,80}, as well as VCAM1-mediated adhesion⁷⁸ and the expression of lymphocyte cytosolic protein 1 (LCP1; also known as plastein 2), which is a filamentous actin (F-actin) crosslinking molecule that is essential for CXCL12-mediated migration⁹⁶.

BTK signalling might support CLL cell migration to proliferative centres in lymph nodes, because ibrutinib treatment of CLL cells *in vitro* effectively blocked CXCL12-induced and CXCL13-induced migration^{78,80}. In addition, ibrutinib-treated CLL cells showed reduced viability *in vitro* when cultured with B cell-activating factor (BAFF; also known as TNFSF13B), tumour necrosis factor (TNF), IL-6, IL-4 and CD40L⁹⁷, which suggests that BTK inhibition might counteract the effects of pro-survival factors in the CLL microenvironment. The potential for disruption of co-stimulatory feedback in lymph node microenvironments was also shown by reduced CLL cell survival, proliferation and CCL3 (also known as MIP1α) and CCL4 production when CLL cells were co-cultured with nurse-like cells (NLCs)⁸⁰. The finding that ibrutinib-treated patients with CLL have a decline in serum CCL3 and CCL4 levels⁸⁰ is particularly important because CCL3 is a strong marker of disease progression⁹⁸.

Inhibition of BTK interferes with multiple pathways that are potentially important for CLL cell survival, proliferation and migration *in vivo*. However, the effects on cell migration may be decisive in CLL. The ibrutinib-induced lymphocytosis that is observed in both patients with CLL and mouse CLL adoptive transfer models^{19,80} shows the effective expulsion of CLL cells out of the nursing environment of lymph nodes, thereby depriving CLL cells from multiple activating signals, including antigenic BCR stimulation⁹⁰.

MCL. The remarkably biased BCR repertoire of MCL suggests a crucial role for antigenic stimulation in its pathogenesis⁹⁹. The antigens that are involved are unknown, but they probably differ from those in CLL, because the stereotypical BCR subsets are different in MCL and CLL⁹⁹. A pro-survival role of BCR signalling is suggested by the observation of constitutive phosphorylation of LYN,

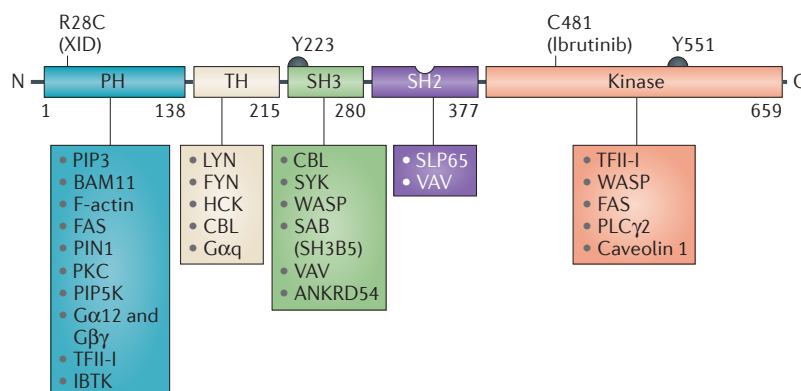


Figure 1 | Structure of Bruton's tyrosine kinase and interactions. The structure of Bruton's tyrosine kinase (BTK) is shown, with the five different domains. The position of the R28C loss-of-function mutation that is present in X-linked immunodeficiency (XID) mice, the position of two tyrosine (Y) phosphorylation sites and the binding site of ibrutinib are shown. For each domain, interacting signalling molecules are shown. In addition, myeloid differentiation primary response 88 (MYD88), IL-1R-associated kinase (IRAK) and Toll/IL-1 receptor (TIR) domains of various Toll-like receptors (TLRs) have been shown to bind to BTK, but the interacting domain is unknown. ANKRD54, ankyrin repeat domain-containing protein 54; F-actin, filamentous actin; IBTK, inhibitor of Bruton's agammaglobulinemia kinase; PH, pleckstrin homology; PIP3, phosphatidylinositol-3,4,5,-triphosphate; PIP5K, phosphatidylinositol-4-phosphate 5-kinase; PIN1, peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1; PKC, protein kinase C; PLCγ2, phospholipase Cγ2; SH, SRC homology; SLP65, SH2 domain-containing leukocyte protein of 65 kDa; SYK, spleen tyrosine kinase; TH, TEC homology; WASP, Wiskott-Aldrich syndrome protein.

SLP65, SYK and PKC β in a limited panel of patients^{100,101}. In addition, it was found that amplification of the SYK gene and SYK protein overexpression occurred¹⁰². Furthermore, MCL cells show constitutive activation of NF- κ B and AKT, which might reflect BCR or TLR signalling¹⁰³.

BTK is strongly expressed in MCL¹⁰⁴, and increased BTK autophosphorylation at Y223 was observed in unstimulated primary MCL cells⁷⁹. Parallel to

observations in CLL, ibrutinib treatment of primary MCL cells or cell lines resulted in reduced viability, as well as impaired adhesion and migration upon activation of the BCR or in response to CXCR4 or CXCR5 (REF. 79). Patients with MCL who receive intermittent or continuous ibrutinib treatment have recurrent lymphocytosis or a single episode of lymphocytosis, respectively⁷⁹.

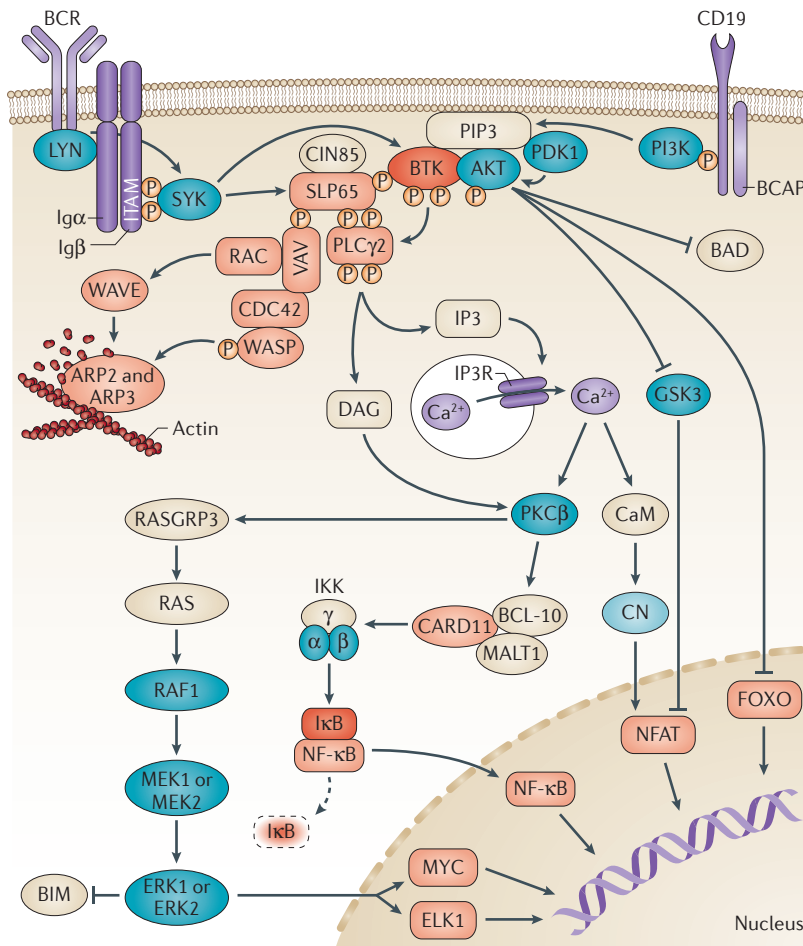


Figure 2 | Involvement of Bruton's tyrosine kinase in B cell receptor signalling.

B cell receptor (BCR) signalling results in the formation of a micro-signalosome that is composed of VAV, PI3K, Bruton's tyrosine kinase (BTK), SH2 domain-containing leukocyte protein of 65 kDa (SLP65) and phospholipase C γ 2 (PLC γ 2). BTK is mostly responsible for the activation of PLC γ 2, which leads to an influx of Ca²⁺ and the subsequent activation of the transcription factors nuclear receptor of activated T cells (NFAT) and nuclear factor- κ B (NF- κ B), as well as ERK1 or ERK2 activation. In addition, BTK activation leads to the activation of Wiskott–Aldrich syndrome protein (WASP), which induces cytoskeleton changes that are associated with BCR activation. Independently of Ca²⁺, AKT is activated via PI3K, which phosphorylates (P) forkhead box O (FOXO) transcription factors and thereby inactivates them. AKT also blocks pro-apoptotic proteins, including the BH3-only protein BCL-2 antagonist of cell death (BAD), thereby releasing it from BCL-X_L and stabilizing MCL1. ARP, actin-related protein; BCAP, B cell adaptor for PI3K; BIM, BCL-2 interacting mediator of cell death; CaM, calmodulin; CARD11, caspase recruitment domain-containing protein 11; CIN85, CBL-interacting protein of 85 kDa; CN, calcineurin; DAG, diacylglycerol; GSK, glycogen synthase kinase; Ig, immunoglobulin; I κ B, inhibitor of κ B; IKK, inhibitor of NF- κ B kinase; IP3, inositol trisphosphate; IP3R, IP3 receptor; ITAM, immunoreceptor tyrosine-based activation motif; MALT1, mucosa-associated lymphoid tissue lymphoma translocation protein 1; PDK1, 3-phosphoinositide-dependent protein kinase 1; PIP3, phosphatidylinositol-3,4,5,-trisphosphate; PKC, protein kinase C; SYK, spleen tyrosine kinase; RASGRP3, RAS guanyl-releasing protein 3.

ABC-DLBCL. ABC-DLBCL cells are dependent on NF- κ B signalling for survival and proliferation, and this is reflected by a high expression level of NF- κ B target genes¹⁰⁵ and high sensitivity to inhibition of NF- κ B signalling^{106–109}. This NF- κ B dependency can be explained by chronic activation of TLR or BCR signalling. Mutations in CARD11, as well as other mediators and regulators of NF- κ B signalling, occur in ~50% of patients with ABC-DLBCL. In ~29% of patients with this disease, constitutive NF- κ B activation is mediated by an L265P gain-of-function mutation in MYD88 (REF. 110).

Importantly, knockdown of BCR signalling molecules induced the death of ABC-DLBCL cell lines that expressed unmutated CARD11 (REF. 18). Further support for a disease-promoting role of BCR signalling is derived from transcriptome analyses that show a BCR signalling signature¹¹¹ and from various genetic alterations in DLBCL, including a mutation of CD79B in ~18% of ABC-DLBCL cases. SYK amplification and downregulation of protein tyrosine phosphatase receptor-type O truncated (PTPROt), which can dephosphorylate SYK, additionally contribute to increased chronic BCR signalling^{112–115}. Blocking chronic BCR signalling in ABC-DLBCL cell lines by dasatinib — a BCR-ABL inhibitor that can also inhibit the SRC family kinases and TEC family kinases, including BTK¹¹⁶ — effectively compromised the survival of CARD11 wild-type cell lines only¹⁸. Consistent with a crucial role for BTK in TLR and BCR signalling, ibrutinib treatment and RNA interference experiments showed that the survival of ABC-DLBCL cell lines with wild-type CARD11 relied on BTK activity¹⁸.

Waldenström's macroglobulinaemia. A commonly recurring L265P point mutation in MYD88 in patients with Waldenström's macroglobulinaemia (WM) results in a constitutively active form of MYD88 (REF. 117). In WM cell lines with this mutation, BTK is also constitutively active, although the mechanisms involved remain unclear. MYD88^{L265P} was recently shown to bind to phosphorylated BTK in WM cells, which indicates that BTK might have a direct role in MYD88^{L265P} signalling in these cells. Ibrutinib treatment of WM cells abrogated MYD88^{L265P}-BTK association and reduced NF- κ B activation¹¹⁸. In both WM cell lines and primary WM cells, ibrutinib induced apoptosis and increased the levels of apoptosis when IRAK1 and IRAK4 were also inhibited¹¹⁸. The efficacy of ibrutinib treatment in MYD88^{L265P}-mutant WM cells might therefore fully rely on the abrogation of NF- κ B activation. Nevertheless, it is conceivable that BTK inhibition might also affect BCR-mediated survival signals or homing,

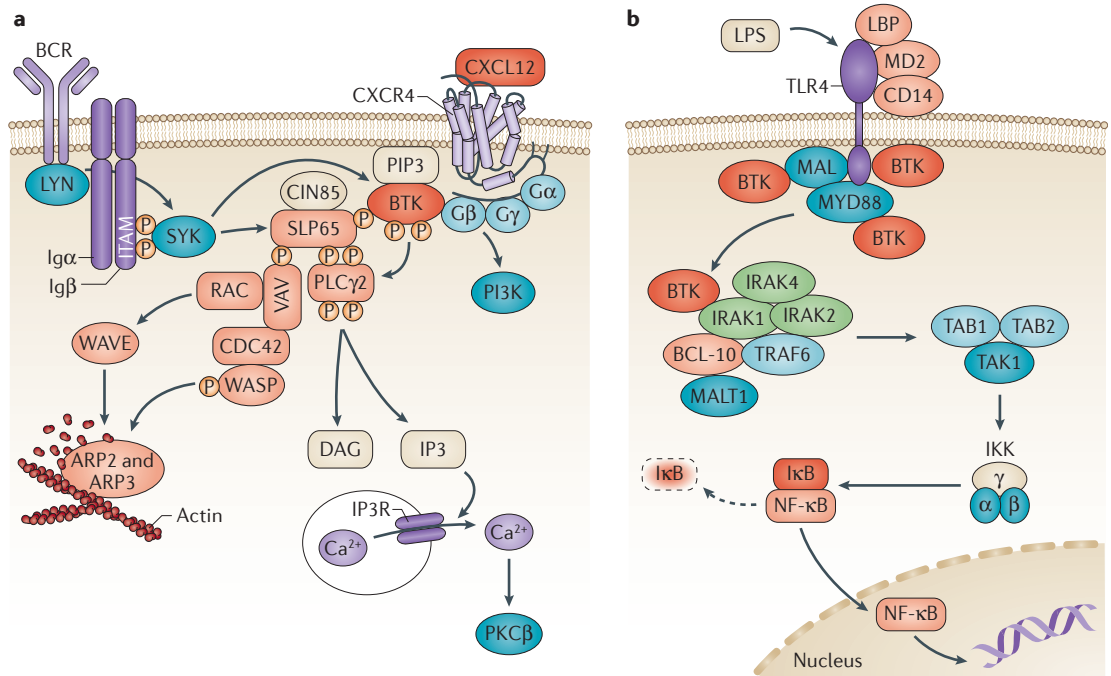


Figure 3 | Involvement of Bruton's tyrosine kinase in chemokine and Toll-like receptor signalling. **a** | A schematic representation of the signalling pathways that underlie the dual role of Bruton's tyrosine kinase (BTK) in B cell receptor (BCR) and chemokine receptor signalling. BTK is a key signalling molecule for CXC-chemokine receptor 4 (CXCR4) and CXCR5. CXC-chemokine ligand 12 (CXCL12) induces BTK activation, most probably involving heterotrimeric G protein subunits, which are known to interact with BTK. **b** | A schematic representation of downstream signalling induced by lipopolysaccharide (LPS), as an example of myeloid differentiation primary response 88 (MYD88)-dependent Toll-like receptor (TLR) signalling. TLR signalling induces various downstream transcription factors, including nuclear factor-κB (NF-κB), which contributes to cellular proliferation. BTK has been shown to contribute to TLR signalling by interacting with five different molecules: the intercellular domains of most TLRs, the downstream adaptors MYD88 and MYD88 adaptor-like protein (MAL), IL-1R-associated kinase 1 (IRAK1) and TIR domain-containing adaptor protein inducing interferon-β (TRIF (not shown) part of the MYD88-independent pathway). ARP, actin-related protein of 85 kDa; DAG, diacylglycerol; Ig, immunoglobulin; IκB, inhibitor of κB; IKK, inhibitor of NF-κB kinase; IP3, inositol trisphosphate; IP3R, IP3 receptor; ITAM, immunoreceptor tyrosine-based activation motif; LBP, lipopolysaccharide-binding protein; MALT1, mucosa-associated lymphoid tissue lymphoma translocation protein 1; P, phosphorylation; PIP3, phosphatidylinositol-3,4,5,-trisphosphate; PKC, protein kinase C; PLCγ2, phospholipase Cy2; SLP65, SH2 domain-containing leukocyte protein of 65 kDa; SYK, spleen tyrosine kinase; TAB, TGFβ-activated kinase 1 and MAP3K7-binding protein; TAK1, TGFβ-activated kinase 1; TRAF, TNF receptor-associated factor; WASP, Wiskott–Aldrich syndrome protein.

Waldenström's macroglobulinaemia

(WM). An uncommon B cell malignancy with heterogeneous clinical presentation that is characterized by high levels of monoclonal immunoglobulin M, secreted by lymphoplasmacytic lymphoma cells with bone marrow infiltration.

Multiple myeloma

(Also known as Kahler's disease). A malignancy of plasma cells that accumulate in the bone marrow. In contrast to resting healthy human plasma cells, multiple myeloma cells show a low rate of proliferation, which results from cell cycle deregulation.

because gene expression profiling of WM samples showed aberrant BCR signalling, including increased CD79B expression¹¹⁹, and because WM cells depend on CXCR4 signalling for homing to the bone marrow¹²⁰.

Multiple myeloma. Multiple myeloma cells originate from plasma cells that no longer express a BCR on their cell surface. For survival and proliferation, multiple myeloma cells seem to rely on signals that are produced as a result of increased osteoclastic activity and bone remodelling^{121,122}. Bone marrow stromal cells, osteoclasts and osteoblasts give crucial activation and homing signals to multiple myeloma cells, such as a proliferation inducing ligand (APRIL; also known as TNFSF13)¹²³, IL-6 and CXCL12 (REF. 124); therefore, therapies are being developed that interfere with the pro-survival signals that are derived from this bone marrow niche¹²⁵.

In mice, BTK and TEC are indispensable for osteoclast formation, which is induced by receptor activator of NF-κB ligand (RANKL; also known as TNFSF11)^{126,127}.

In line with this observation, ibrutinib blocked RANKL-induced phosphorylation of BTK and downstream PLCγ2, and inhibited human osteoclast function *in vitro*, as measured by bone resorption activity. In osteoclasts or bone marrow stromal cells from patients with multiple myeloma, ibrutinib downregulated the production of tumour-supporting factors¹²⁸, including CCL3, transforming growth factor-β, APRIL and CXCL12. Ibrutinib blocked CXCL12-induced adhesion and migration of multiple myeloma cells and reduced multiple myeloma cell growth and survival that was triggered by IL-6 (REF. 128). *In vivo* ibrutinib treatment in a mouse multiple myeloma transplantation model strongly abrogated tumour progression. Because ibrutinib also suppressed the potential of multiple myeloma stem-like cells to form colonies *in vitro*, it is conceivable that this drug might also disrupt BTK signalling in multiple myeloma cells¹²⁸. BTK inhibition in multiple myeloma cells might also block additional pathways involved in disease progression, as TLR signalling in multiple myeloma might

Table 2 | The role of Bruton's tyrosine kinase in B cell malignancies

Malignancy	Incidence	Presumed B cell of origin	Evidence for a role of BCR or TLR signalling	Effects of BTK inhibition
CLL	4.2 per 100,000 per year	<ul style="list-style-type: none"> U-CLL: germinal centre-independent mature CD5⁺ B cell (IgM⁺; low SHM) M-CLL: post-germinal centre memory CD5⁺CD27⁺ B cell (IgM⁺; high SHM) 	<ul style="list-style-type: none"> Poor prognosis for ZAP70⁺ CLL and U-CLL, versus M-CLL Stereotypical BCRs BCR with known antigens (and autoantigens) Increased basal Ca²⁺ influx; MYD88^{L265P} mutation in ~3% of cases 	<ul style="list-style-type: none"> <i>In vitro</i>: inhibition of CXCL12-mediated and CXCL13-mediated migration; BCR-induced VCAM1-mediated adhesion; reduced CLL viability <i>In vivo</i>: lymphocytosis; decline in the concentration of CCL3 and CCL4 in the serum
MCL	4–8 per 1,000,000 per year (~6% of NHL cases)	<ul style="list-style-type: none"> Pre-germinal centre mature CD5⁺ B cell IgM⁺ with low SHM A small proportion carry BCRs with SHM 	<ul style="list-style-type: none"> Biased and stereotypical BCRs Phosphorylation signature of BCR signalling molecules SYK amplification 	<ul style="list-style-type: none"> <i>In vitro</i>: inhibition of phosphorylation of the BCR-downstream molecules PLCγ2, AKT, ERK and JNK; inhibition of CXCL12- and CXCL13-mediated migration and adhesion <i>In vivo</i>: lymphocytosis
ABC-DLBCL	~3 per 100,000 per year	<ul style="list-style-type: none"> Post-germinal centre B cell arrested during plasmacytic differentiation IgM⁺ with high levels of SHM 	<ul style="list-style-type: none"> Constitutive NF-κB signalling Mutation in NF-κB pathway in ~50% of patients Activating CD79B mutation in ~18% of patients BCR signature in transcriptome MYD88^{L265P} mutation in ~29% of cases 	<i>In vitro</i> : inhibition or knockdown hampers cell survival
WM	~0.3 per 100,000 per year (~2% of NHL cases)	<ul style="list-style-type: none"> Post-germinal centre B cell IgM⁺ with high levels of SHM 	<ul style="list-style-type: none"> Limited evidence for a role of BCR signalling MYD88^{L265P} mutation in >90% of cases 	Shown in WM cell lines: <ul style="list-style-type: none"> Inhibition of MYD88–BTK association Reduction of NF-κB activation Induction of apoptosis
Multiple myeloma	~5.6 per 100,000 per year	Long-lived post-germinal centre plasma cell, which is class switched, with high levels of SHM	BCR is not expressed on the cell surface	<ul style="list-style-type: none"> A reduction in the concentration of CCL3 and APRIL in bone marrow stromal cells and osteoclasts Inhibition of CXCL12-induced adhesion and migration in multiple myeloma cells Reduced IL-6-driven proliferation and survival of multiple myeloma cells

ABC-DLBCL, activated B cell-like diffuse large B cell lymphoma; BCR, B cell receptor; BTK, Bruton's tyrosine kinase; CCL, CC-chemokine ligand; CLL, chronic lymphocytic leukaemia; CXCL, CXC-chemokine ligand; Ig, immunoglobulin; IL-6, interleukin-6; JNK, JUN N-terminal kinase; MCL, mantle cell lymphoma; M-CLL, mutated CLL; MYD88, myeloid differentiation primary response 88; NF-κB, nuclear factor-κB; NHL, non-Hodgkin's lymphoma; PLCγ2, phospholipase Cγ2; SHM, somatic hypermutation; SYK, spleen tyrosine kinase; TLR, Toll-like receptor; U-CLL, unmutated CLL; VCAM1, vascular cell adhesion molecule 1; WM, Waldenström's macroglobulinaemia; ZAP70, ζ-associated protein of 70 kDa.

increase disease progression¹²⁹ and mutations in many genes that are involved in NF-κB signalling^{130–132} have been identified in multiple myeloma.

BTK inhibitors in clinical trials

In a Phase I open-label study of 56 patients with refractory or relapsed B cell malignancies (including CLL, small lymphocytic lymphoma (SLL), MCL, DLBCL, WM and follicular lymphoma) who failed at least one previous therapy, the safety and tolerability of ibrutinib (TABLE 1) was assessed¹³³. Adverse events were mostly limited to grade 1 or 2 in severity, and grade 3 or 4 events were mostly haematological, including neutropenia, thrombocytopenia and anaemia. Pneumonia and bacteraemia were also among the most frequent severe adverse events reported in ibrutinib-treated patients with CLL or SLL¹⁹. Six patients discontinued study participation because of adverse events. Thus, ibrutinib is mostly well tolerated and safe.

None of the 14 patients with CLL or SLL included in this Phase I study showed progressive disease, and the overall response rate (ORR) was 79%¹³³. In a follow-up Phase II study, 85 patients with refractory or relapsed CLL or SLL received continuous ibrutinib treatment¹⁹.

An ORR of 71% was reported, without significant differences between subgroups. Ibrutinib caused a transient increase in blood lymphocyte levels, concurrent with a reduction in lymph node or spleen size. This asymptomatic lymphocytosis is explained by the role of BTK in chemokine receptor signalling. Approximately 77% of patients with U-CLL and only ~33% of patients with M-CLL showed a response, but this difference was due to a more rapid and more frequent resolution of lymphocytosis in patients with U-CLL. Another ~42% of patients with M-CLL showed a partial response with lymphocytosis, and if these patients were included, the ORR of patients with M-CLL and patients with U-CLL was not different. Similar results were obtained in an open-label Phase IB/II trial in 31 previously untreated patients with CLL or SLL¹³⁴. Moreover, progression-free survival was not reduced in patients with prolonged lymphocytosis during ibrutinib therapy, which indicates that persistent lymphocytosis does not signify a suboptimal response to ibrutinib¹³⁵. The estimated progression-free survival and ORR at 26 months in the Phase II study¹⁹ were ~75% and ~83%, respectively, which indicates that ibrutinib is efficacious in heavily pretreated patients with CLL or SLL.

Germinal centre B cell
An antigen-stimulated, proliferating B cell that is in contact with T helper cells and follicular dendritic cells in the microenvironment of lymphoid tissues.

Small lymphocytic lymphoma
(SLL). A different clinical manifestation of chronic lymphocytic leukaemia, in which most of the malignant cells are not in the bloodstream and bone marrow, but are in the lymph nodes and the spleen.

These findings have encouraged the initiation of additional clinical studies to assess the effectiveness of other BTK inhibitors in patients with CLL and SLL (TABLE 1). Moreover, interim analyses of ibrutinib in combination regimens show good ORRs^{136,137}. It is expected that a substantial proportion of patients will reach long-term progression-free survival with ibrutinib, but specific subgroups may experience relatively early relapses: that is, patients with deletions of 11q or 17p¹⁹ who, owing to genomic instability, might be highly likely to acquire resistance mutations¹³⁸. The presence of BTK^{C481S} and PLCγ2^{R665W} mutations in patients treated with ibrutinib indicates possible mechanisms of ibrutinib resistance, although the rates of the occurrence of these mutations might be low, as few cases have been documented so far¹³⁸.

In the ibrutinib Phase I study¹³³, seven of the nine patients with MCL responded to treatment. In a subsequent Phase II study in which 111 patients with refractory or relapsed MCL were treated, an impressive ORR of ~68% (including 21% complete responses) was reported²⁰. Paralleling the Phase II study in CLL¹⁹, the response to ibrutinib treatment was independent of disease severity, risk factors and patient characteristics²⁰. Interestingly, recent transcriptome sequencing showed that insensitivity to ibrutinib is associated with activation of the alternative NF-κB pathway and mutations in TNF receptor-associated factor 2 (*TRAF2*) and baculoviral IAP repeat-containing 3 (*BIRC3*), in contrast to the chronic activation of the BCR-driven classical NF-κB pathway that is found in highly ibrutinib-sensitive MCL lines¹³⁹.

Although grade 3 or grade 4 adverse events were infrequently seen, two patients died of pneumonia and one patient died of bacterial sepsis in this trial²⁰. The recurrence of bacterial complications in both Phase II trials may, to some extent, mimic the XLA phenotype (BOX 1), but there is no evidence that ibrutinib treatment is associated with infection due to blockade of BTK signalling in functional B cells. Serum IgM, IgG and IgA levels did not decrease during treatment^{19,20,134}, and increased susceptibility to infection is one of the characteristics of the clinical course of B cell malignancies.

In a Phase I study¹³³, two of the seven patients with DLBCL had a partial response. The lower ORR in DLBCL compared with CLL and MCL might be attributed to differences in the BTK dependency of DLBCL subtypes¹⁸. Indeed, interim results of a Phase II study showed that ibrutinib treatment induced an ORR of ~41% and ~5% in ABC-DLBCL and germinal centre-DLBCL, respectively¹⁴⁰. Importantly, ibrutinib also induced responses in patients carrying mutant *CD79B* or *MYD88* alleles, which indicates that BTK inhibition can override pathogenic constitutive signalling of these molecules. Clinical trials addressing the effectiveness of different BTK inhibitors in DLBCL are currently ongoing (TABLE 1).

The finding that three of the four patients with refractory or relapsed WM showed a partial response in the Phase I study¹³³ prompted further investigation of BTK inhibition in WM. Recent interim results of a Phase II trial with ibrutinib showed an ORR of ~83% and markedly reduced serum IgM levels in patients¹⁴¹. Likewise, in an ongoing Phase II trial of ibrutinib treatment in

refractory or relapsed multiple myeloma, reduced values for various markers in the blood were reported¹⁴². These markers included RANKL and CCL3, which indicates that ibrutinib has a biological effect on the microenvironment in multiple myeloma.

Perspectives

Targeting BTK function shows promise as a therapy for various B cell malignancies: BTK inhibition is mostly well tolerated, and ibrutinib induced a durable objective response in many patients. Numerous Phase II and Phase III clinical trials are currently ongoing to evaluate BTK inhibition in patients with CLL, MCL, DLBCL, WM and multiple myeloma (TABLE 1). Moreover, it will be interesting to investigate BTK inhibition in several other B cell malignancies, such as splenic marginal zone lymphoma, in which mutations in *MYD88* and components of the NF-κB pathway have recently been found¹⁴³, and follicular lymphoma, in which BCR antigen (and autoantigen) recognition has been implicated in survival¹⁴⁴. In this context, it is of note that the incidence of DLBCL and marginal zone lymphoma is increased in patients with systemic autoimmune diseases¹⁴⁵, in which BTK activation is thought to be a key pathogenic event and for which preclinical models show strong *in vivo* effects of BTK inhibition^{17,146–149}. The recent finding of a novel BTK isoform that protects breast cancer cells from apoptosis¹⁵⁰ should prompt analyses of BTK expression in other tumours, as well as experiments that address how BTK becomes involved in epithelial cell signal transduction pathways.

Much progress has been made in recent years in defining the mechanisms of action of BTK inhibitors. BTK is involved in different pathological mechanisms, and it now seems likely that in many cases the effects of BTK inhibition on tumour progression are a result of a complex interplay of signals that are derived from various receptors on B cells and responses to micro-environmental stimuli. Therefore, novel insights into the oncogenic role of BTK signalling in the context of genomic aberrations in malignancies are crucial to optimize the use of BTK-targeting therapeutics. Development, as well as clinical and preclinical investigation, of BTK inhibitors with greater or different specificity might be beneficial for patient groups that do not show a significant response to ibrutinib. In some cases, it may be advantageous to use BTK inhibitors that show additional specificity for related kinases, as exemplified by the synergy between BTK and TEC in osteoclast function in multiple myeloma cells. More studies are needed to learn which patients benefit the most from which specific compound. In this context, the application of new technology, including transcriptome analysis by next-generation sequencing, will be beneficial. High-throughput combinatorial screening strategies should help to identify ibrutinib combinations that can be prioritized for clinical investigation¹⁵¹. Such combination regimens are expected to result in long-lasting responses by preventing the development of resistance to BTK inhibition and avoiding lifelong treatment with inhibitors.

Follicular lymphoma

The most common type of indolent non-Hodgkin's lymphoma, which originates from germinal centre B cells. It is characterized by an overexpression of the anti-apoptotic BCL-2 protein, which is caused by translocation of the *BCL2* gene near the site of the immunoglobulin heavy chain enhancer element.

Overall response rate

(ORR). The proportion of patients whose best overall response to a therapy is either complete or partial.

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Acknowledgements

Part of these studies were supported by the Association for International Cancer Research (to R.W.H.; 10–562) and the Dutch Arthritis Foundation (9-01-302).

Competing interests statement

The authors declare no competing interests.

DATABASES

BTKbase mutation registry: <http://bioinf.uta.fi/BTKbase>

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