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RESEARCH ARTICLE

Burgeoning Data on BTK Inactivating Mutations in Lymphomagenesis and Therapeutic Resistance

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ABSTRACT

The B-cell antigen receptor signaling pathway has been a primary focus in the targeted treatment of B-cell malignancies for the past decade. When aberrantly activated, this pathway initiates a cascade of phosphorylation mediated by several tyrosine kinases, with Bruton's tyrosine kinase (BTK) being essential among them. Multiple generations of covalent and non-covalent BTK inhibitors have revolutionized therapeutic options for several B-cell lymphomas. However, the use of continuous BTK inhibition is limited by development of resistance resulting from acquired point mutations that allow persistent B-cell receptor signaling. Genomic sequencing of patient samples at disease progression has recently led to the discovery of novel resistance mutations in BTK that result in diminished or absent BTK kinase activity (termed kinase-deficient). However, the mechanisms underlying the potential advantage of kinase-deficient BTK mutations are incompletely understood and still under investigation. In this review, we provide a background of the pathway leading to the development of current therapies that target BTK and review the literature describing kinase-deficient BTK mutations. We propose that BTK inactivating mutations provide an advantage to neoplastic B-lymphocytes in patients with BTK inhibitor resistant B-cell malignancies and highlight potential mechanisms through which BTK kinase-deficient mutations could be acting, either due to differential protein conformation or by behaving as a scaffold for other signaling molecules. Due to the novelty of these mutations and their increasing rate of incidence over the last decade, it is imperative to continue studying BTK kinase deficient mutations across B-cell malignancies and to propose alternate therapies that could target them.

Keywords: Kinase deficient, Bruton's tyrosine kinase, B-cell receptor signaling, B-cell lymphomas, BTK inhibitors

Introduction

The B-cell antigen receptor (BCR) is a transmembrane receptor complex present in both normal and malignant B-lymphocytes that is essential for survival, development, and antibody production[1]. Antigen binding of the BCR in healthy B-cells leads to the formation of a 'signalosome', a complex of kinases and structural proteins, that initiates a cascade of phosphorylation mediated by several tyrosine kinases. This phosphorylation cascade triggers activation of Bruton's tyrosine kinase (BTK), phospholipase C gamma 2 (PLCγ2) and Ca2+ mobilization. This ultimately leads to activation of neighboring pathways such as the NF-κB and mitogen-activated protein kinase (MAPK) pathways, leading to increased proliferation, survival, and migration of B-cells.[2]

BTK is early in the BCR signaling cascade and is a non-receptor, cytoplasmic tyrosine kinase that is specifically expressed in B-cells. It is part of the Src subfamily of proteins and is encoded by the *BTK* gene on the X chromosome[3,4]. Mutations in BTK that disrupt BCR signaling confer B-cell developmental deficiencies. Loss-of-function mutations in BTK are implicated in immunodeficiency diseases such as X-linked agammaglobulinemia, where B-cells become incompetent in their antibody-producing function[5,6]. On the other hand, BTK mutations are also expressed in various B-cell lymphoproliferative disorders, including chronic lymphocytic leukemia (CLL), diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), amongst others with proven active BCR signaling[7–10]. In fact, B-lymphocytes in these neoplasms are considered addicted to BTK and its kinase function, resulting in a convenient target for B-cell lymphomas[11].

BTK has five domains that are ordered as follows: pleckstrin homology (PH) domain, proline-rich TEC homology (TH) domain, SRC homology (SH) domains SH3 and SH2, and the catalytic domain. Phosphatidylinositol triphosphate (PIP3) binds to the PH domain and mobilizes proteins to the cell membrane, while the TH domain is important for integrity and stability of the molecule. Both SH domains are involved in protein-protein interactions. The catalytic kinase domain contains the Y551 site that when phosphorylated, leads to the autophosphorylation of Y223 in the SH3 domain, initiating activation of downstream effectors in BCR[12]. In this review, we discuss how mutations in the BTK catalytic domain that diminish its kinase activity might be favored when developing resistance to BTK inhibitor therapy. Additionally, we will explore different B-cell lymphomas that benefit from BTK kinase-deficiency and suggest potential functions that BTK could fulfill as an alternative to acting as a kinase.

BTK inhibitors and resistance mutations

The development of BTK inhibitors has provided an efficacious avenue for B-cell lymphoma patients. Ibrutinib, a first generation irreversible BTK inhibitor, was developed to covalently bind to BTK residue C481 in its active kinase domain thus blocking ATP phosphorylation. This results in the inhibition of BCR downstream signaling and a halt in cell survival and proliferation[13,14]. Ibrutinib became a frontline therapy for chronic lymphocytic leukemia (CLL) after randomized phase 3 trials of ibrutinib in both relapsed/refractory (R/R) and untreated CLL patients proved the inhibitor confers improved progression-free survival and overall survival compared to ofatumumab in the relapse setting and chlorambucil, bendamustine, and rituximab in the frontline setting[2]. Ibrutinib, however, demonstrates off-target binding with at least 19 other kinases including BLK, BMX, ITK, TEC, EGFR, ERBB2, and JAK3. Second generation covalent BTK inhibitors include acalabrutinib and zanubrutinib, which both bind to BTK residue C481 with greater potency and selectivity for BTK than ibrutinib. Both acalabrutinib and ibrutinib have demonstrated efficacy and safety superior to conventional chemoimmunotherapy in patients with CLL and relapsed mantle cell lymphoma (MCL), leading to their use as standard of care[15–18]. Ibrutinib has also been approved by the FDA as a first-line therapy for Waldenstrom's macroglobulinemia (WM), after the iNNOVATE study (NCT02165397) showed that ibrutinib plus rituximab resulted in improved progression-free survival and overall survival compared to placebo plus rituximab in both untreated and R/R WM patients[12]. A phase II trial conducted in all subtypes of marginal zone lymphoma (MZL) (NCT01980628) identified ibrutinib as a single active agent with a favorable toxicity profile, accelerating its FDA approval for R/R MZL[19]. All covalent inhibitors face resistance and disease progression, mediated by mutations to the C481 residue in BTK which disrupt inhibitor binding[20–22]. The mutation profiling of B-cell malignancies on progression while on treatment with a BTK inhibitor, has uncovered BTK residue site C481 as the most frequent resistance mutation, with the most common substitution being cysteine for

serine[20,23]. In a cohort of CLL patients treated with ibrutinib for three years, next generation sequencing revealed a minimum of one BTK mutation in 57% of the patients. There were a total of 20 mutations identified, all of them at residue C481 with 14 being a C481S point mutation[23]. The subsequent most commonly occurring mutations are substitution of cysteine 481 with arginine (C481R), phenylalanine (C481F), tyrosine (C481Y) and threonine (C481T).

In addition to being susceptible to BTK C481 resistance mutations, the 3 aforementioned first- and second-generation inhibitors also have short half-lives, with serum levels dropping as BTK undergoes turnover[2]. Additionally, these inhibitors demonstrate relatively high levels of toxicity, likely due to inhibition of off-target proteins[24]. For example, off-target TEC inhibition is attributed to increased anti-platelet and bleeding activity in patients treated with ibrutinib[25]. For these reasons, reversible BTK inhibitors that target the ATP binding site in a non-covalent manner through hydrogen bonding[26] were developed. Non-covalent BTK inhibitors include vecabtrutinib, fenebrutinib, nemtabrutinib and pirtobrutinib. Vecabrutinib was tested in a phase 1/2 dose escalation trial for Non-Hodgkin Lymphomas (NCT03037645), however, the trial was terminated because of suboptimal activity[27]. Fenebrutinib is being tested in autoimmune diseases with no active clinical trial in hematologic malignancies[28,29]. A phase 2 clinical trial of Nemtabrutinib in B-cell malignancies (NCT03162536) was recently presented, demonstrating a 57.9% overall response rate in CLL/SLL patients who received prior BTK inhibitor therapy, but unfortunately treatment emergent adverse events in 68% of the patients[30].

Pirtobrutinib is a novel, non-covalent BTK inhibitor developed to give a treatment avenue for patients that have developed resistance to covalent BTK inhibitors. Similar to other non-covalent BTK inhibitors, pirtobrutinib inhibits BTK independently of the commonly mutated C481 residue and has been characterized by improved selectivity for BTK and decreased toxicity. The first human clinical trial for Pirtobrutinib is a multicenter phase 1/2 study in patients with CLL, mantle cell lymphoma (MCL), Waldenstrom's macroglobulinemia (WM), and several other B-cell lymphomas (NCT03740529) that has demonstrated efficacious results[31]. Pirtobrutinib was tolerated at all doses (no dose-limiting toxicities) with 87% of adverse events having grades $\leq$ 2. Overall response rates were 63% in CLL/SLL, 52% in MCL, and 68% in WM with similar response rates seen in patients with BTK C481S mutations. Response rates were also unaffected by TP53 and 17p deletions[2,31]. As with any targeted monotherapy, resistance may occur and indeed a small number of patients developed resistance to pirtobrutinib due to acquired BTK mutations outside of C481. In the following text, we will examine these novel BTK mutations and their role in BTK inhibitor resistance.

B-cell neoplasms with kinase-deficient BTK mutants

In nine CLL patients included in the pirtobrutinib phase 1/2 trial, progression of the disease occurred, indicating the need to define mechanisms of resistance to noncovalent BTK inhibition [32]. Using genomic analysis of patient samples from both pretreatment and time-of-progression time points, Wang et al. found that 7 of those 9 patients acquired new BTK kinase domain mutations outside of the C481 residue that were not present at the start of treatment. These mutations included V416L, A428D, M437R, T474I, and L528W[32]. Of these, BTK L528W mutations have also been recently found to be enriched in CLL patients progressing while on zanubrutinib[33]. Functional studies of lymphoma cell lines expressing BTK V416L, A428D, M437R and L528W mutations showed a decreased BTK autophosphorylation of Y223, a marker of its kinase activity. These catalytically inactive mutants still enabled activation of AKT, ERK and NF-κB upon IgM stimulation, even after treatment with non-covalent and covalent BTK inhibitors[32]. Other kinase dead/deficient BTK mutations found in CLL occur at the C481 residue exhibiting C481R/F/YTF substitutions, which preclude covalent bond formation by ibrutinib and result in the loss of kinase activity (**Figure 1**)[27,34]. Based on prior knowledge, mutations that disable BTK catalytic activity should halt BCR signaling and be disadvantageous for cancer progression. However, these kinase deficient mutations possibly undergo positive selection because they permit BCR signaling despite loss of catalytic activity. This suggests BTK has a nonenzymatic, or tertiary scaffolding, function that continues to promote BCR signaling in the absence of enzymatic activity.

Beyond CLL, follicular lymphoma, a B-cell lymphoma where ibrutinib has shown less efficacy in its treatment, has recently been associated with BTK mutations that destabilize the protein and render it catalytically inactive[35,36]. Hu et al. tested BTK variants in HEK293T cells and four lymphoid

cell lines and found that following inhibition of protein translation, serial pBTK immunoblotting revealed that BTK mutations R77S, Y315N, P566L, P597S, E90K, Y361H, K433T, and V568I led to destabilization of pBTK itself. Additionally, BTK mutations K433T, P566L, V568I, P597S, and K430R (**Figure 1**) resulted in kinase deficient BTK mutants, with the impaired ability to auto-phosphorylate at residue Y223. They found that signaling through downstream targets of the B-Cell Receptor decreased or were unchanged, while AKT signaling increased across all mutants following anti-Ig stimulation[36]. The instability of BTK in the 8 mutations mentioned previously proposes that the absence of BTK protein, not specifically its kinase function, might mediate compensatory AKT activation[37]. However, there is still a mechanistic linkage to be made between decreased BTK levels and upregulation of AKT pathway.

Mantle cell lymphoma (MCL), a mature B-cell non-Hodgkin lymphoma with an aggressive course, has previously been shown to overexpress BTK. The use of BTK inhibitor ibrutinib in relapsed and/or refractory (R/R) MCL patients was revolutionary in its management, as it brought Progression-Free Survival (PFS) from 4-9 months up to 13-14.6 months[2,38]. Jain et al performed deep targeted next generation sequencing on 15 MCL patients before and after progression on ibrutinib. BTK mutations were observed on 17% of patients, one of the identified variants being the kinase dead C481R[39]. Unfortunately, the treatment options after progression while on BTKi therapy remains limited and with a poor outcome in overall survival. A preferred combination therapy for patients at this stage is yet to be established[2].

Waldenstrom's macroglobulinemia (WM) patients have also developed ibrutinib resistance, associated with poor prognosis, independent of BTK C481S mutations[2,40]. An important somatic activating mutation of MYD88, L265P, found in around 90% of WM patients promotes activation of toll-like receptor (TLR) via BTK interaction and signaling of interleukin 1, IRAK4/IRAK1 and NF-kB[41]. In *MYD88^{L265P}* mutated WM cells derived from patients progressing on ibrutinib, mutations with a variable clonal distribution in the BTK C481 binding site were identified[2,42].Similar to what occurs in follicular lymphoma, continuous use of ibrutinib in WM activates compensatory AKT signaling. Additionally, there is also activation of the ERK 1/2 pathway that could explain progression despite BTK inhibition. It is also important to note that activating MYD88 mutations trigger Hematopoietic cell kinase (HCK), another tyrosine kinase relevant to pro-survival pathways and an off-target kinase inhibited by ibrutinib[2,43]. Xu et al. performed Sanger sequencing of lymphoplasmacytic cells from 6 WM patients who had progressed after an initial response to ibrutinib and found 3 patients had variants BTK C481S and kinase-dead BTK C481R. An additional screening of 38 WM patients on ibrutinib without clinical progression identified 2 patients that subsequently progressed and in which targeted deep-sequencing identified kinase dead variant BTK C481Y[44].

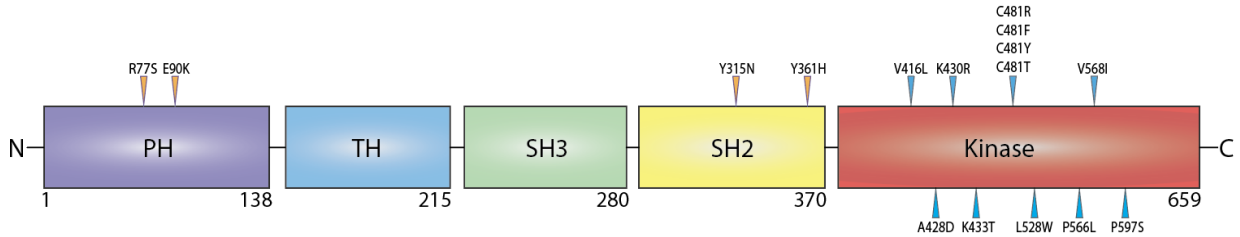

Figure 1. BTK mutations seen in patients with acquired resistance to BTK inhibitors. BTK resistance mutations that are either kinase deficient (blue arrowhead) or have pBTK destabilization (orange arrowhead).

Increased recognition of BTK kinase-deficient mutations

Precision medicine allows for tailored therapies depending on each patient's induvial mutation profile[45]. Therefore, understanding the resistance mechanisms that develop as patients progress through different therapies will help us find better ways to target these mutations. Unfortunately, the variability in acquired BTKi resistance in each patient gives us a wide range of mutations. For example, regarding CLL first-line treatment with ibrutinib, mutations frequently arise in BTK at the C481 residue. A recent study by Dhami et al. showed that ibrutinib-resistant mutations BTK C481F and BTK C481Y displayed a lack of kinase activity, when compared to BTK wild type (WT) and BTK C481S[34]. These mutations have been found in patients that relapse or are refractory to BTK inhibition and although these mutations arise less frequently, they are two among other BTK mutants that are termed kinase-deficient. Additionally, it was noted that phosphorylation of tyrosine 223 in

BTK (the residue required for BTK activation) was not detectable in C481F/Y, but downstream calcium flux assays and analysis of downstream BCR targets determined BCR signaling to be active. The same study tested the hypothesis that in the absence of BTK kinase function, HCK was able to activate BTK and promote BCR signal transduction[34]. Given that dual HCK/BTK inhibitor KIN-8194 has already been tested in preclinical studies and proven to overcome ibrutinib resistance in MD88-driven B-cell lymphomas (such as WM and diffuse large B-cell lymphoma)[46], a detailed mechanistic link between HCK and kinase-dead BTK might push forward the application of HCK/BTK dual inhibition of patients with BTK inhibitor-resistant lymphomas. A different study created *in vitro* and *in vivo* ibrutinib-resistant CLL models of BTK mutation C481R and through both IgM stimulation and ibrutinib treatment showed decreased phosphorylation at Y223 residue of BTK when compared to cells overexpressing BTK WT or C481S. Although cell proliferation was slightly lower in BTK C481R, it was similar to WT and the other mutants, and there was no noted difference in BCR signaling between the cell lines[47]. Thus, we might consider the kinase deficient character of BTK C481R and evaluate if there is an interaction occurring with HCK that permits BCR signaling.

Wang et al. performed mutagenesis of the BTK coding sequence (CDS) and screened the resulting library for mutant BTKs that confer resistance to ibrutinib and noncovalent BTK inhibitors. This analysis found a new L528W mutation in 88 out of 350 clones[48]. Intriguingly, L528W mutations have been seen in ibrutinib-naïve lymphoma patients, though at low frequency, and in 1 CLL patient that relapsed after ibrutinib[49,50]. It is important to note that this same CLL patient also had the BTK mutations C481R and C481Y, both of which we have mentioned have a kinase-dead characteristic.[51] To understand how BTK L528W impairs covalent BTK inhibition specifically, a computational model of its structure and its interaction with ibrutinib was developed and showed that the L528W substitution was capable of sterically hindering C481, which we know as the ibrutinib binding site, thus increasing the free energy required for binding[48]. Whether this collection of mutations was selected for simultaneously (or in a manner) to activate the alternative, non-catalytic, functions of BTK remains to be understood. However, the fact that these mutations do not interrupt BCR signaling can prompt us to ask how we could target them given that they do not respond to covalent or non-covalent BTK inhibitors.

Another BTK kinase-deficient mutation K430R that has been previously generated in preclinical models was recently identified in follicular lymphoma[36]. The K430R BTK mutant was demonstrated by Middendorp et al. to be present in a BTK/SLP-65 double-deficient transgenic mouse model that had reversal of developmental arrest at the pre-B-cell stage. However, this research portrayed the BTK mutant as a tumor suppressor that exerted its function in pre-B cells as an adaptor protein[52,53]. Finally, phospholipase C gamma 2 (PLCγ2), a substrate for BTK in the BCR pathway has also been observed in BTK inhibitor resistant patients. When the BCR is constitutively activated in a malignancy such as CLL, the interplay of BTK and PLCγ2 is implicated in intracellular calcium release and downstream pathway activation[54]. A study on the PLCγ2 variant S707Y found in CLL patients that progressed on ibrutinib, reported that in the presence of catalytically inactive BTK K430R, BTK-mediated stimulation of PLCγ2 was reduced by 50%, as measured through inositol phosphate formation after incubation with *myo*-[2-^{3}H]inositol[55]. Therefore, when present simultaneously as certain PLCγ2 mutations, kinase deficient BTK can both overcome inhibition of its active site and continue activating the BCR cascade.

Potential therapeutic strategies to overcome kinase deficient BTK mutations

We have thus far discussed acquired BTK kinase deficient mutations in the context of potential B-cell lymphomagenesis and in the context of BTK inhibitor resistance. However, because the mechanistic implications of kinase deficient mutants are not clear and have only recently been gaining recognition in the scientific and clinical community, management of patients that relapse with kinase deficient mutations while on BTKi therapy has not been standardized. One of the potential combination therapies is that of dual BTK and phosphoinositide-3-kinase (PI3K) inhibition, as both kinases are part of the essential BCR pathway, and it is expected that targeting them simultaneously might prevent adaptive crosstalk[56]. In an ongoing clinical trial (NCT02328014), acalabrutinib, a

selective and covalent BTK inhibitor, is being evaluated in combination with the PI3Kδ inhibitor ACP-319 in patients with R/R B-cell malignancies. This trial was supported by previously published data demonstrating the two agents showed activity independently in B-cell lymphoma cell lines and synergistically when used in combination[57]. Investigators have thus far reported an overall response rate (ORR) of 100% [$n = 8/8$; complete response (CR) rate 13%] for patients with CLL/SLL; CR in 2/3 MCL patients; a partial response in one WM patient; and no response in FL patients. In patients with non-germinal center B-cell like DLBCL, the ORR was 63% ($n = 10/16$; CR rate 25%) with a median duration of response of 8.2 months. Unfortunately, there was a significant level of hepatotoxicity reported, with 33% of patients having grade 3/4 liver enzyme elevations[58]. The trial investigators instead suggest a combination of acalabrutinib and umbralisib, a potentially more selective PI3K-δi that was previously shown to be well tolerated and active when combined with ibrutinib in relapsed or refractory CLL and MCL[58,59].

A novel approach for R/R B-cell lymphoma patients who have previously been exposed to BTK inhibition is the use of targeted BTK protein degradation. In vitro studies with lymphoma cell lines have previously demonstrated that BTK degradation results in potent suppression of BCR signaling and proliferation. Importantly, BTK degraders efficiently degrade BTK C481S[60]. We can deduce that degrading kinase-deficient BTK could be beneficial in targeting B-cell lymphomas as the alternate functions we propose would no longer be an option for survival. There are two ongoing Phase 1 clinical trials using BTK degraders NX-2127 (NCT04830137) and NX-5948 (NCT05131022) in patients with advanced B-cell malignancies. Although results from patients treated on these trials have yet to be published, the potential use of BTK degraders represents a novel way to overcome BTK inhibitor resistance due to kinase deficient BTK mutations.

Conclusion

In this review, we have discussed the discovery of kinase deficient BTK mutations in resistance to BTK inhibition, their increasing recognition as having distinct mechanisms, and potential therapeutic strategies to overcome them. Resistance to ibrutinib occurs primarily from mutations that arise in the C481 drug binding site but newer covalent inhibitors like zanubrutinib may have predilection towards kinase deficient L528W mutations. Noncovalent BTK inhibitors are impervious to C481 mutations and thus far have shown less acquired resistance but appear susceptible to some kinase deficient BTK mutations. Given the novelty of these mutations and their increasing incidence with next-generation BTK inhibitors, there is an immediate and vital need to identify kinase deficient BTK mechanisms of action across B-cell malignancies in hopes of proposing alternate therapies for patients with these mutations.

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