Mechanisms of ibrutinib resistance in chronic lymphocytic leukaemia and non-Hodgkin lymphoma

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Summary

Bruton tyrosine kinase (BTK), a mediator of B-cell receptor (BCR) signalling, has been implicated in the pathogenesis of chronic lymphocytic leukaemia (CLL) and other B-cell malignancies. Ibrutinib is an orally bioavailable and highly specific BTK inhibitor that was recently approved for treatment of patients with recurrent CLL and mantle cell lymphoma (MCL). In addition, ibrutinib has shown efficacy in subsets of patients with diffuse large B cell lymphoma (DLBCL) and Waldenstrom macroglobulinaemia (WM). However, despite ibrutinib’s activity in multiple B-cell malignancies, cases of primary and secondary resistance have emerged. The overall reported frequency of resistance is low, but follow-up in many trials was short, and we predict that the incidence of observed resistance will increase as clinical use outside clinical trials expands over time. Mutations within BTK have been described and clearly interfere with drug binding; however, there are also emerging alternative mechanisms that bypass BTK entirely and offer new opportunities for other targeted agents. Improved understanding of mechanisms of primary and secondary resistance is essential to developing appropriate therapeutic strategies to both prevent and address resistance. This review provides a comprehensive analysis of ibrutinib resistance in CLL, MCL, DLBCL and WM and considers potential strategies for further study.

Keywords: B-cell receptor, cell signalling, DNA mutation, drug resistance, lymphoid malignancy.

Bruton tyrosine kinase (BTK) is a cytoplasmic tyrosine kinase that plays a critical role in the development, activation and differentiation of B cells (Mohamed et al, 2009). B cells express the B cell receptor (BCR) on the surface, which consists of primarily four subunits: antigen binding immunoglobulin heavy chain and light chain subunits, coupled non-covalently with disulfide-linked CD79A and CD79B heterodimer (Fig 1). CD79A and CD79B each contain a conserved signalling module called immune-receptor tyrosine-based activation motifs (ITAMs). Upon antigen stimulation of BCR, ITAM tyrosine residues on CD79A and CD79B are phosphorylated by the SRC-family kinases, including LYN. This subsequently creates a binding site for the SH2 domains of cytosolic tyrosine kinase SYK and recruits SYK from cytoplasm to cell membrane. At this location, SYK is phosphorylated and activated by LYN. Phosphorylated SYK then phosphorylates and activates BTK. BTK subsequently phosphorylates and activates phospholipase C gamma 2 (PLCγ2), which catalyses the cleavage of membrane phosphatidylinositol-4,5 bisphosphate (PIP2) into inositol trisphosphate (IP3) and diacylglycerol (DAG). This mobilizes calcium and activates protein kinase C beta (PRKCB) and downstream proteins. In addition, the BCR co-receptor transmembrane protein CD19 is phosphorylated by LYN during BCR signalling. This recruits phosphoinositide 3-kinase (PIK3) to BCR with subsequent phosphorylation of PIP2 to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3). Collectively, these signalling pathways induce activation of the nuclear factor (NF)-κB, AKT, RAS, mitogen-activated protein kinase and nuclear factor of activated T cells pathways (Dal Porto et al, 2004; Kurosaki et al, 2010; Buggy & Elias, 2012; Macias-Perez & Flinn, 2012), resulting in B-cell cellular changes including cell survival, proliferation, adhesion, migration and homing.

Bruton tyrosine kinase, an early component of BCR signalling, has been implicated in the development of B-cell malignancies including chronic lymphocytic leukaemia (CLL), mantle cell lymphoma (MCL), follicular lymphoma (FL), diffuse large B cell lymphoma (DLBCL) and acute lymphoblastic leukaemia (ALL). Pre-clinical and clinical studies have shown that targeting BTK in several B-cell malignancies have been highly effective. Ibrutinib (PCI-32765) is an orally administered drug that has produced remarkable clinical response in patients with CLL and non-Hodgkin lymphoma (NHL) (Novero et al, 2014). The drug binds covalently to the active site of BTK at cysteine 481 (C481) and irreversibly...
inhibits phosphorylation of BTK and its downstream signalling activities (Pan et al., 2007; Honigberg et al., 2010). Due to the restricted expression of BTK to B cells, toxicities have been minimal and generally do not include myelosuppression. The US Food and Drug Administration approved ibrutinib in November 2013 for treatment of patients with recurrent MCL and in July 2014 for patients with CLL. In addition, ibrutinib has been found to have efficacy in patients with DLBCL [activated B cell (ABC)-like subtype] (Wilson et al., 2012) as well as in patients with Waldenström macroglobulinaemia (WM) (Yang et al., 2013).

Despite ibrutinib’s promising activity across multiple lymphoid malignancies, cases of primary and secondary resistance have emerged. In primary resistance, patients demonstrate lack of response at initial therapy, whereas secondary resistance is characterized by an initial disease response that is subsequently lost. Although the reported frequency of overall resistance is relatively low, most patients now receive ibrutinib therapy outside of clinical trials and the exact incidence remains to be determined. In this article, we systematically review the literature that addresses primary or secondary resistance of ibrutinib therapy in CLL and other lymphoid malignancies.
lymphoid malignancies, including MCL, DLBCL and WM. Understanding the molecular mechanisms underlying primary and secondary ibrutinib resistance will be critical in preparing patient care teams to identify patients with emerging resistance and prompt initiation of therapeutic measures to address and overcome such resistance.

Understanding these mechanisms will also prompt the development of next generation therapeutic strategies to prevent or reduce the incidence of resistance.

**Ibrutinib resistance in CLL**

**Role of BCR/BTK in the pathogenesis of CLL**

Both BCR signalling and BTK have direct pathogenic roles in CLL through several mechanisms (Burger & Chiorazzi, 2013). The BCR is constitutively active through both ligand-independent and ligand-dependent mechanisms. Ligand-dependent BCR signalling may be triggered by antigens present in the tissue microenvironment (Burger & Chiorazzi, 2013) while ligand-independent BCR signalling may be a result of autonomous stimulation of BCR by the heavy-chain complementarity-determining region and an internal epitope of the BCR (Duhren-von Minden et al, 2012). BTK itself also contributes to CLL pathogenesis. We have previously shown that levels of BTK phosphorylation are significantly higher in CLL than in normal B cells, consistent with aberrant BCR pathway activation (Cheng et al, 2014a). Furthermore, BTK directly promotes CLL cell proliferation, as demonstrated in BTK transfection and knock-down experiments (Cheng et al, 2014b). In addition to its role in BCR signalling, BTK also plays a fundamental role in B-cell trafficking and tissue homing by mediating chemokine receptors (CXCR4 and CXCR5) and adhesion molecules (integrins)-triggered pathways. Inhibition of BTK reduces CLL-derived chemokines and chemokine receptor-related intracellular signalling. Thus, a BTK inhibitor will not only antagonize BCR-related activation signals, but also impact CLL cell migration and tissue homing via inhibition of chemokine receptor function (Ponader et al, 2012).

**Clinical trial results in CLL**

Ibrutinib has significant clinical activity in patients with relapsed/refractory (R/R) CLL (Burger & Chiorazzi, 2013) (Table II). A pivotal phase I dose escalation study enrolled 56 patients with relapsed, refractory B-cell malignancies, including CLL (Advani et al, 2013). Pharmacokinetic studies showed rapid elimination, yet BTK occupancy was maintained for a minimum of 24 h, indicative of the irreversible covalent bond between ibrutinib and BTK. The overall response rate (ORR) in 50 evaluable patients was 60%, including a complete response (CR) rate of 16%. Median progression-free survival (PFS) in all patients was 13-6 months (Advani et al, 2013). Importantly, ibrutinib was well tolerated and a maximum tolerated dose was not identified. A phase Ib-II multicentre study enrolled 85 patients with R/R CLL/small lymphocytic lymphoma and showed an ORR of 71% (Byrd et al, 2013). An additional 15–20% of patients had a partial response (PR) with persistent lymphocytosis. The lymphocytosis is a unique, and probably mechanism-related phenomenon and should not be confused with clinical progression. At 26 months, the estimated PFS and overall survival (OS) rate among patients treated with ibrutinib was 75% and 83%, respectively (Byrd et al, 2013). Furthermore, a pooled analysis of 246 CLL patients treated with ibrutinib found 8% (20/246) disease progression after a median 14 months of therapy, including 8 patients with Richter transformation (Chang et al, 2013). Based on published data, the overall incidence of primary resistance is variable between patient populations and ranges between 13% in the general R/R CLL patients to 43% in high-risk patients (Byrd et al, 2013). Secondary resistance seems less frequent in CLL, but median follow-up on most of these trials is quite short, and the true incidence will probably become more evident over time.

**Secondary ibrutinib resistance in CLL: BTKC481S and PLCG2 mutations**

Two groups have published their findings on the mechanisms of secondary ibrutinib resistance. We provided a longitudinal mutational analysis of a CLL patient who progressed following 21 months of ibrutinib therapy (Furman et al, 2014a). RNA sequencing revealed a thymidine to adenine mutation at nucleotide 1634 (NM_000061.2), resulting in a cysteine to serine missense mutation at residue 481 (C481S) localized at the active site of the BTK enzyme (Fig 1C and Table I). The mutation was detected only during progressive disease and was not present prior to therapy or during disease response. No other genetic changes were identified that correlated with the patient’s clinical course in the same manner as the BTK mutation (Furman et al, 2014a). Similarly, Woyach et al (2014) performed whole-exome sequencing of 6 patients with progressive CLL. Prior to ibrutinib therapy, the C481S mutation was absent in these patients. Of these six patients, five were found to have the BTKC481S mutation (Woyach et al, 2014). In an update presented at the August 2014 American Society of Hematology (ASH) Lymphoma Biology conference, Byrd (2014) reported that two of six CLL patients (33%) with Richter transformation after ibrutinib therapy had the BTKC481S mutation, while 10 of 10 CLL patients (100%) with progression but no Richter transformation had either one or both BTKC481 and PLCG2 mutations (see below) (Byrd, 2014). Thus, molecular mechanisms of ibrutinib resistance may differ in patients with Richter transformation versus simple progression.

Efforts have been made to understand the functional consequences of the BTK mutation. On a structural level, the C481S mutation disrupts covalent binding, allowing for
reversible, instead of strong irreversible, binding of BTK by ibrutinib. The critical biochemical role of covalent-bond formation was revealed when fluorescently tagged-ibrutinib labelled the wild-type (WT) BTK, but not the BTKC481S mutant (Furman et al., 2014a). Woyach et al. (2014) showed that ibrutinib produced inhibition of recombinant BTKC481S 25-fold less potently than WT; thus, the intrinsic affinity of ibrutinib for BTK is significantly weakened by the mutation. The impaired binding leads to ineffective BTK inhibition and ultimately results in ibrutinib resistance.

On a functional level, consequence of the BTKC481S mutation is characterized from multiple aspects, including cell signalling, gene expression and cellular behaviour. Our laboratory demonstrated decreased BTK and ERK activity during clinical response to ibrutinib therapy, and the activity was increased to that of the pretreatment level upon disease progression (Cheng et al., 2014b). Moreover, a 27-gene BCR signature showed parallel changes throughout the patient’s clinical course of response and relapse. These data suggest that the BTKC481S mutation restores BCR signalling with subsequent clinical relapse. On a cellular level, increase of the Ki67 proliferative CLL population is associated with disease activity and clinical relapse (Cheng et al., 2014b). A clear cause-and-effect relationship was established with the introduction of WT or mutant BTK into a lymphoma cell line. While cells bearing WT BTK suffered growth inhibition with ibrutinib exposure, cells bearing mutant BTK continued their growth. Taken together, these investigations functionally demonstrate that a genetic change in BTK directly affects molecular signalling, gene expression and cellular behaviour. The accelerated cellular proliferation ultimately manifests in the patient as increasing lymphocytosis, worsening lymphadenopathy and clinical disease progression.

In addition to the BTKC481S mutation, PLCG2 mutations have also been implicated in secondary ibrutinib resistance. In the six ibrutinib-resistant patients reported by Woyach et al. (2014), three distinct PLCG2 mutations were found in two CLL patients with secondary resistance to ibrutinib therapy: arginine-to-tryptophan mutation at position 665 (R665W), leucine-to-phenylalanine at position 845 (L845F), and a serine-to-tyrosine mutation at position 707 (S707Y) (Fig 1C). Specifically, the S707Y mutation has a gain-of-function effect due to disruption of an auto-inhibitory SH2 domain (Zhou et al., 2012). These mutations were detected

#### Table II. Clinical trials of ibrutinib in CLL, MCL, DLBCL, and WM.

<table>
<thead>
<tr>
<th>Study</th>
<th>Year</th>
<th>Phase</th>
<th>Regimen</th>
<th>N</th>
<th>Response (ORR %)</th>
<th>PFS (months)</th>
<th>OS (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL</td>
<td>2013</td>
<td>I</td>
<td>DE</td>
<td>16</td>
<td>9 PR, 2 CR (69)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Byrd et al</td>
<td>2013</td>
<td>I/II</td>
<td>Ibrutinib 420 mg/d PO</td>
<td>51</td>
<td>34 PR, 2 CR (71)</td>
<td>75% (26)</td>
<td>83% (26)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ibrutinib 840 mg/d PO</td>
<td>34</td>
<td>24 PR (71)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MCL</td>
<td>2013</td>
<td>I</td>
<td>DE</td>
<td>9</td>
<td>4 PR, 3 CR (78)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Wang et al</td>
<td>2013</td>
<td>II</td>
<td>Ibrutinib 560 mg/d PO</td>
<td>111</td>
<td>52 PR, 23 CR (68)</td>
<td>–</td>
<td>58% (18)</td>
</tr>
<tr>
<td>Advani et al</td>
<td>2013</td>
<td>I</td>
<td>DE</td>
<td>7</td>
<td>2 (29)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Wilson et al</td>
<td>NCT 01325701</td>
<td>II</td>
<td>Ibrutinib 560 mg/d PO</td>
<td>39 (ABC)</td>
<td>15 (38)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Wilson et al</td>
<td>NCT 01325701</td>
<td>II</td>
<td>Ibrutinib 560 mg/d PO</td>
<td>20 (GCB)</td>
<td>1 (5)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>WM</td>
<td>2013</td>
<td>I</td>
<td>DE</td>
<td>4</td>
<td>3 (75)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Treon et al</td>
<td>NCT 0164821</td>
<td>II</td>
<td>Ibrutinib 420 mg/d PO</td>
<td>63</td>
<td>4VGPR, 32 PR, 15 MR (81)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

ABC, activated B cell-like subtype; CLL, chronic lymphocytic leukaemia; CR, complete response; DE, dose escalation (ranging 1-25 mg/kg-125 mg/kg, 28 d on/7 d off schedule or continuous daily dosing of 8-3 mg/kg); DLBCL, diffuse large B-cell lymphoma; GCB, germinal centre B cell-like subtype; m, months; MCL, mantle cell lymphoma; MR, minimal response; N, number of patients enrolled; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; PR, partial response; PO, orally; VPGR, very good partial response; WM, Waldenström macroglobulinaemia; “–” data not available.
only after ibrutinib therapy; none were detected before drug exposure. No other recurrent mutations were noted at time of relapse in these patients.

In contrast to the BTK<sup>C481S</sup> mutation, which causes eventual loss of BTK inhibition by ibrutinib, PLCG2 mutations are all potentially gain-of-function mutations. Situated downstream from BTK, PLCG2 mutations allow for continued signalling regardless of BTK activity. After stimulation with anti-IgM antibody, cells with either the PLCG2<sup>R665W</sup> or PLCG2<sup>2A855F</sup> mutations were found to have sustained BCR signalling that was not inhibited by ibrutinib, as measured by calcium-flux assays and phosphorylation of ERK and AKT (Woyach et al., 2014). These findings suggest that PLCG2 mutations activate BCR signalling distal to BTK and confer secondary resistance to ibrutinib therapy in patients with CLL. Overall, acquired resistance to ibrutinib is due, at least in part, to recurrent mutations in BTK and PLCG2; understanding the precise mechanism of secondary resistance in individual patients may prompt development of new strategies and is discussed further below.

*Sensitivity of ibrutinib-resistant CLL cells to other kinase inhibitors*

Given that BTK mutations simply cause loss of inhibition, it is reasonable to assume that BTK-mutated CLL cells may remain sensitive to other agents that target the BCR signalling pathway. Several other kinase inhibitors were tested using an *in vitro* CLL proliferation model (Cheng et al., 2014b). It was found that CLL cells are sensitive to inhibition by dasatinib, idelalisib, SYK inhibitors [Cerdulatinib (PRT062070) and PRT060318], while insensitive to tofacitinib, a JAK inhibitor. Structural modelling suggests that dasatinib inhibits BTK by acting as a reversible antagonist, occupying the ATP pocket of both the WT and mutant BTK enzymes (Cheng et al., 2014b). Idelalisib, known to be effective against R/R CLL (Brown et al., 2014; Furman et al., 2014b), was also assessed for its potential to overcome ibrutinib resistance. After testing a range of idelalisib concentrations, it was found that at a clinically achievable concentration of 5 μmol/l, the population of proliferative CLL cells was reduced from 22-7% in untreated cells to 1.9% in treated cells in an *in vitro* model. These results suggest that idelalisib and other kinase inhibitors may serve as alternative therapy in the setting of ibrutinib secondary resistance (Cheng et al., 2014b). Notably, these experiments were performed in an *in vitro* model of CLL proliferation; clinical applicability and relevance remain to be determined.

*Primary ibrutinib sensitivity/resistance in CLL*

The vast majority of CLL patients respond to ibrutinib, as reflected by the aggregate observation of PR and CR as well as prolonged stable disease with nodal response despite peripheral lymphocytosis. However, there are currently no upfront clinical biomarkers to predict sensitivity or resistance to ibrutinib therapy in CLL. Interestingly, Byrd et al. (2013) reported that 4 of 12 patients with mutated *IGHV* (33%) achieved PR or CR, while 53 of 69 patients with unmutated *IGHV* (77%) had a PR or CR response to ibrutinib therapy. This difference in ORR was highly statistically significant (P = 0.005) (Byrd et al., 2013). Other clinical trials of ibrutinib-containing therapies published to date have similar observations (Burger et al., 2014; Foa, 2014). Given that unmutated CLL responds poorly to chemoimmunotherapeutic regimens, it is both biologically intriguing and clinically relevant to determine why CLL patients with unmutated *IGHV* are more sensitive to ibrutinib inhibition. Unmutated CLL was reported to have a higher proliferative response, compared to mutated CLL, upon antigenic stimulation of the BCR receptor (Guarini et al., 2008), suggesting that unmutated CLL is more dependent on the BCR pathway. Will mutation status or any surrogate markers guide future therapy selection? Understanding the molecular mechanisms underlying this difference will impact future clinical practice.

*Ibrutinib resistance in mantle cell lymphoma*

**Role of BCR/BTK in the pathogenesis of MCL**

Mantle cell lymphoma, a NHL of pre-germinal centre mature B cells, is an incurable malignancy for which there are no universally accepted therapeutic standards. BCR signalling is essential for the proliferation of MCL cells, based on several lines of evidence. For instance, SYK RNA and SYK protein were found to be overexpressed in a subset of primary MCL cells (Rinaldi et al., 2006). Furthermore, BTK, LYN and SYK were noted to be the most abundant tyrosine-phosphorylated proteins in MCL cell lines (Rinaldi et al., 2006; Boyd et al., 2009). We compared BTK activity of MCL primary tumour cells with B cells from healthy donors: although they express similar levels of total BTK, the level of phosphorylated BTK (p-BTK) was significantly higher in primary MCL cells than normal resting B cells, indicating that BTK is constitutively active in MCL. (Ma et al., 2014). In addition, Cinar et al. (2013) demonstrated higher BTK expression in MCL cases (n = 19) compared to benign lymphoid tissues (n = 10) with immunohistochemistry.

**Clinical trial results in MCL**

Current frontline therapies for MCL include combination chemotherapy and stem cell transplantation, which have improved patient outcomes, though most patients eventually suffer disease relapse. Ibrutinib shows significant single-agent activity in R/R MCL (Table II). In a phase I study in multiple lymphoma subtypes, Advani et al. (2013) observed response to ibrutinib in seven of nine patients with R/R MCL, prompting a subsequent international multicentre phase 2 trial. In total, 111 patients with R/R MCL were trea-
ated with ibrutinib and demonstrated an unprecedented 68% ORR (21% CR, 47% PR). At median follow-up of 15.3 months, estimated median PFS was 13.9 months and median OS was not reached (Wang et al., 2013). The overall rate of adverse events was low and only eight patients discontinued due to adverse effects (Wang et al., 2013).

Secondary ibrutinib resistance in MCL: BTK\textsuperscript{C481S}

The mechanisms of resistance in MCL are under active investigation. Chiron et al. (2014) evaluated two patients who progressed on ibrutinib after a PR of 14 and 30 months, respectively. The BTK\textsuperscript{C481S} mutation identified in CLL was also identified in both of these MCL patients at relapse (Fig 1C and Table I). The authors obtained five serial tissue biopsies from the MCL patient who progressed following 14 months of therapy. Longitudinal whole transcriptome sequencing revealed high abundance of BTK\textsuperscript{C481S} exclusively at relapse in MCL cells from both the bone marrow and spleen. The mutation was not detected in ibrutinib-naive primary MCL cells.

Primary ibrutinib resistance in MCL: sustained PIK3-AKT activity

In MCL, studies suggest presence of alternative mechanisms for primary resistance unrelated to BTK mutations. For instance, BTK\textsuperscript{C481S} was not identified in biopsies from six patients with primary or early acquired resistance to ibrutinib within 5 months of therapy (Chiron et al., 2014). Further investigation reveals that downstream PIK3-AKT activity, rather than BTK activity, correlates with clinical response in MCL (Fig 1B and Table I). AKT was inactivated in a responding MCL patient on ibrutinib therapy; in contrast, AKT activity was maintained in primary resistant patients (n = 3) (Chiron et al., 2014). While this data was based on a small number of patients, our studies with MCL cell lines and primary MCL tumour cells supported these findings (Ma et al., 2014). Treatment of three MCL cell lines with ibrutinib resulted in a highly variable apoptosis and cell growth response (Ma et al., 2014). Only one MCL cell line of the three was susceptible to BTK inhibition. Interestingly, activity of ERK and AKT, but not BTK, correlated with cellular responses of primary MCL tumour cells to ibrutinib. Although levels of phosphorylated-BTK were reduced by ibrutinib in all samples, only the degree of ERK or AKT inhibition predicted cellular sensitivities. In short, effectiveness of ERK and AKT inhibition correlated with extent of cell death (Ma et al., 2014). Taken together, primary ibrutinib resistance or transient response is not caused by ineffective ibrutinib inhibition of BTK, but rather involves sustained distal BCR signalling, specifically via PIK3-AKT activation. These observations provide a strong rationale for combining PIK3-AKT inhibitors with ibrutinib therapy to address primary ibrutinib resistance.

Primary resistance in MCL: classical NFkB pathway versus alternative NFkB pathway

Laboratory investigation also indicates another mechanism of primary ibrutinib resistance in MCL (Rahal et al., 2014). After pharmacological evaluation of 10 MCL cell lines, two pathogenic mechanisms of MCL were revealed: the classical NFkB pathway and the alternative NFkB pathway. MCL cell lines exhibiting ibrutinib sensitivity were found to have chronic activation of the BCR leading to the activation of the classical NFkB pathway via BTK. In contrast, ibrutinib-resistant MCL cell lines were dependent on the alternative NFkB pathway not mediated by BTK (Fig 1A,C and Table I). Genomic studies using RNA-Seq and SNP array revealed somatic mutations in these cell lines including non-sense mutations in NTRK3 and deletions in TRAF3, TRAF2 and TRAF3 which altogether represent ~17% of all MCL tumour tissues. Collectively, the authors concluded that MCL pathogenesis depends on either the BCR-BTK-NFKB (classical) or MAP3K14-NFKB (alternative) pathways. It is predicted that the former patient group is sensitive to ibrutinib while the latter group is unlikely to benefit from anti-BTK therapy. It remains to be determined whether mutations in the alternative NFkB pathway are associated with primary resistance in patients receiving ibrutinib. Based on Rahal et al. (2014), these patients may respond to MAP3K14 inhibition, a promising option for the future development of targeted therapy.

Ibrutinib resistance in DLBCL

Diffuse large B cell lymphoma comprises a heterogeneous group of lymphomas, defined by proliferation of intermediate and large-sized B-cells that obliterate the nodal architecture. While DLBCL constitutes the single most common NHL entity, it is a heterogeneous disease with a wide range of variability in clinical presentation and final outcome. Landmark gene expression profiles of DLBCL were conducted to elucidate the molecular heterogeneity that underlies the clinical heterogeneity. Based on relatedness of gene expression profiles to normal B-cell subsets, DLBCL were classified into three cell-of-origin subtypes: germinal centre B-cell (GC B) subtype, ABC subtype and primary mediastinal B-cell lymphoma (PMBL) (Alizadeh et al., 2000; Rosenwald et al., 2002, 2003; Wright et al., 2003). These molecular subtypes are clinically relevant as patient outcomes and responses to chemoimmunotherapeutic regimens are different; GC B DLBCL has a much higher response rate than ABC subtype to R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone).
Ibrutinib resistance in Waldenstrom macroglobulinaemia

A preliminary analysis by Treon and colleagues reported data from a prospective multicentre phase II study of ibrutinib in 63 patients with WM, of which 17 had refractory disease (Table II). The aim of the study was to evaluate both the efficacy of single-agent ibrutinib and to assess the impact of MYD88<sup>WT</sup> and CXCR4<sup>WHIM-like</sup> mutations on ibrutinib response. MYD88<sup>265P</sup> is present in 90–95% of WM patients (Treon et al, 2012; Treon & Hunter, 2013; Hunter et al, 2014). Based on pre-clinical findings that MYD88<sup>265P</sup> triggers activation of BTK (Yang et al, 2013), it was hypothesized that WM patients with the MYD88<sup>265P</sup> mutation would be particularly responsive to BTK inhibition. Ibrutinib was well tolerated and effective in patients with R/R WM, as indicated by rapid reduction of serum immunoglobulin M and improved haematocrit levels; the best ORR was 81%. Notably, patients with CXCR4<sup>WT</sup> and CXCR4<sup>WHIM-like</sup> mutations experienced 77% and 30% response rate, respectively (Treon et al, 2013). Details regarding response in patients with MYD88<sup>WT</sup> and MYD88<sup>265P</sup> were not reported, perhaps due to the fact that the majority of the WM patients carry MYD88<sup>265P</sup>. Taken together, MYD88<sup>265P</sup> renders WM cells sensitive to BTK inhibition while CXCR4 mutations confer primary resistance to ibrutinib treatment.

CXCR4 is a transmembrane chemokine receptor that is internalized upon binding to its ligand CXCL12 and subsequently signals through G-proteins to activate the Akt and ERK pathways. The CXCR4 pathway plays an important role in lymphocyte migration and homing. CXCR4<sup>WHIM-like</sup> are prevalent somatic mutations, present in 30% of patients with WM (Cao et al, 2015). WHIM denotes that the mutations, including non-sense and frameshift mutations affecting the C-terminal regions of CXCR4, are present in the germline DNA of patients with Warts, Hypogammaglobulinaemia, Infections and Myelokathexis syndrome (WHIM). The WHIM-like mutations impair receptor internalization and prolong CXCR4-mediated G-protein signalling. It was recently demonstrated that CXCR4<sup>S338X</sup>, the most common WHIM-like mutation, reduces CXCR4 receptor internalization and allows for sustained enzymatic activity of Akt and ERK and subsequent increased cell survival (Cao et al, 2015). When cells are exposed to ibrutinib, CXCR4<sup>S338X</sup>-carrying WM cells, compared to CXCR4<sup>WT</sup> cells, exhibit reduced apoptosis. These findings indicate that WHIM-like mutations confer resistance to ibrutinib-triggered apoptosis in WM cells. Because of the prevalence of the MYD88<sup>265P</sup> mutation in 90–95% of WM patients, many patients with CXCR4 mutations also carry MYD88 mutations. Thus, it appears that the presence of resistant CXCR4 mutations trumps the sensitive MYD88 mutation, rendering patients with reduced sensitivity to ibrutinib (77% vs. 30%). Interestingly, CXCR4<sup>S338X</sup>-related survival advantages were blocked by AMD3100, a CXCR4 inhibitor, suggesting that CXCR4 antagonists may be used in a combination regimen to restore sensitivity of CXCR4<sup>S338X</sup> and possibly other CXCR4<sup>WHIM</sup>-expressing WM cells to ibrutinib (Cao et al, 2015).

MYD88 mutations: DLBCL versus WM

As discussed above, the MYD88<sup>TIR</sup> mutations, including L265P, are associated with ibrutinib resistance in ABC DLBCL, but MYD88<sup>265P</sup> is associated with ibrutinib sensitivity in WM. How to explain this conundrum? There are no clear answers at present, but based on the available data, one can speculate the following (Fig 1D,E): In ABC DLBCL, at least two pathways lead to oncogenic activation of NFKB (Davis et al, 2010; Staudt, 2010; Young & Staudt, 2013). One is through the BCR-BTK-PKC-CBM complex and the other through TLR-MYD88-IRAK1/4-TRAF6. Mutations in MYD88 result in oncogenic activation of NF-kB that is independent of BCR-BTK activity, and establishes a pathway for ibrutinib...
resistance (Fig 1D). However, this theory does not explain why ABC DLBCL carrying both MYD88 and CD79A/B mutations become sensitive to ibrutinib. In the case of WM, Yang et al (2013) demonstrated that MYD88<sup>C481S</sup> is a regulator of BTK activation. The mutant, as opposed to MYD88<sup>WT</sup>, preferentially binds to p-BTK and subsequently activates NFKB (Fig 1E). Ibrutinib treatment reduces such binding, therefore blocking downstream NFKB activation. Thus, the oncogenic activity of MYD88<sup>C481S</sup> is mediated through BTK in WM and renders cells sensitive to ibrutinib’s inhibition. The fact that MYD88 mutations function differently in different cells highlight the notion that impact of a particular genetic mutation has to be determined and understood within the particular cellular context.

**Potential strategies to address ibrutinib resistance**

Based on improved understanding of ibrutinib resistance, there are a number of considerations worthy of exploration to either mitigate or attenuate clinical resistance. In patients with primary ibrutinib resistance, therapeutic options include traditional therapies or drugs targeting alternative pathways independent of BTK signalling. As discussed above, studies so far implicate (i) PI3K and alternative NFKB pathway activity in MCL; (ii) MYD88-IRAK pathway in DLBCL and (iii) CXCR4 pathway in WM. These signalling pathways can be potentially targeted with PI3K inhibitors, mTOR inhibitors, MAP3K14 (NIK) inhibitors and CXCR4 antagonists, as discussed below.

When dealing with secondary ibrutinib resistance, there are two distinct but related issues: (i) management of ibrutinib-treated patients developing secondary resistance after an initial response and (ii) prevention/reduction of emergence of drug resistance. Regarding the first issue, the routine approach would be to stop ibrutinib and change to an alternate therapy. As discussed earlier, our group investigated alternative kinase inhibitors in the ibrutinib-resistant CLL patient carrying BTK<sup>C481S</sup>. Based on our in vitro CLL proliferation model, we demonstrated that the proliferative activity of CLL cells was sensitive to inhibition of LYN (dasatinib) and SYK [Cerdulatinib (PRT062070) and PRT060318] and PIK3CD (PI3Kδ) (idelalisib) (Cheng et al, 2014b). In this reported case, the patient was treated with dasatinib at a dose used for CML patients and showed some improvement in lymphadenopathy before she succumbed to sepsis. Anecdotes such as this, as well as accumulating preclinical data, support rational investigation into several classes of drugs that may be useful in overcoming or circumventing secondary resistance: (i) other BTK inhibitors; (ii) inhibitors targeting other BCR components; (iii) inhibitors targeting other oncogenic pathways and (iv) inhibitors targeting other cellular processes.

In light of the clinical success of ibrutinib, a number of small molecules with BTK-inhibitory properties have been developed and are under intense preclinical and clinical development. Clinical trials have already been initiated using these agents, including GDC-0834, HM-71224, CC-292 and ONO-4059. Most of these drugs are orally available and have highly selective reversible BTK inhibitory properties with variable potencies (no covalent bond is formed between the drug and the BTK enzyme; therefore, C481 residue is not required) (Akinleye et al, 2013). In addition, LFM-A13 (Mahajan et al, 1999), a first in-class dual BTK/ Polo-like kinases inhibitor, has demonstrated anti-proliferative effect in both lymphoma and breast cancer cells in preclinical studies (Akinleye et al, 2013).

An alternative approach is to develop inhibitors targeting other BCR pathway components. For example, LYN and SYK are both situated upstream from BTK in the BCR pathway (Fig 1C). In the case of the BTK<sup>C481S</sup> mutation, the loss of covalent bonding between ibrutinib and BTK leads to persistent BCR signalling which can be overcome by LYN and SYK inhibitors (Cheng et al, 2014b). Both dasatinib and fostamatinib (inhibits SYK) have shown clinical efficacy in patients with CLL and NHL (Friedberg et al, 2010; Amrein et al, 2011; Herman et al, 2013). Cells from CLL patients treated with fostamatinib exhibited reduced BTK phosphorylation (Herman et al, 2013). A phase I clinical trial of cerdulatinib, an agent with activity against both SYK and JAK, is ongoing in CLL and other NHL (Flinn et al, 2014). However, we predict that inhibition of LYN, SYK or BTK will be ineffective with PLCG2 mutations, because these activating mutations act further downstream to reactivate the BCR pathway without involving the upstream kinases. In this case, other approaches would have to be developed.

A third approach would be to target other oncogenic pathways that are related to BCR signalling, including inhibitors of PI3K-mTOR, NFKB and alternative NFKB. Targeting these pathways using a PIK3CD inhibitor, such as idelalisib, a PIK3CG/PIK3CD dual inhibitor like IPI-145, an mTOR inhibitor (e.g. everolimus) or a MAP3K14 inhibitor in the future would be rational. In addition, it may be effective to target cell survival with BCL2 inhibition or to target cell cycle with CDK inhibitors. BCL2 family proteins are anti-apoptotic proteins that play a critical role in the pathogenesis of CLL and NHL. (Buggins & Pepper, 2010). At present, much focus has been placed on ABT-199, an orally bioavailable, second-generation BH3-mimetic that selectively inhibits BCL2 and achieves potent anti-tumour activity (Davids & Letai, 2013). Preliminary results of a phase I ABT-199 study in patients with relapsed/refractory NHL show impressive efficacy in MCL, where six of six MCL patients experienced >50% reduction in target lesions (Davids et al, 2012). Naturally occurring compounds may also ameliorate ibrutinib resistance. Pre-clinical models showed that nimbiolide, a potent Neem leaf tetraterpenoid extract, is highly active in WM cells by inducing cytotoxicity through a BCL2-dependent apoptotic mechanism (Chitta et al, 2014). Interestingly, ibrutinib-resistant WM cells were shown to be particularly
sensitive to nimboline in the pre-clinical setting (Chitta et al., 2014). In the future, the compound may have potential to serve as an adjunct therapy.

Finally, inhibitors targeting other cellular processes associated with BCR signalling may also have utility. Multi-targeting inhibitors, such as Heat Shock Protein 90 (HSP90AA1, also termed HSP90) inhibitors or selinexor, an inhibitor of nuclear export (XPO1) may have the ability to overcome or circumvent ibrutinib-resistance. HSP90AA1 is a molecular chaperone protein that aids its client proteins to fold, thus allowing them to function properly. Many of clients of HSP90AA1 are bona-fide oncoproteins including mutated TP53, BCR-ABL1, BRAF, ERBB2 (HER2), EGFR and AKT1. It contributes to multiple oncogenic pathways by stabilizing these oncoproteins. Inhibition of HSP90AA1 induces destabilization of these client proteins, promotes their ubiquitination and proteasome degradation (Trepel et al., 2010). Many phase I and II clinical trials are ongoing for several second-generation HSP90AA1 inhibitors with improved bioavailability and toxicity profiles (Jhaveri et al., 2012). To date, pre-clinical data suggest activity in melanoma, acute myeloid leukaemia, castrate-resistant prostate cancer, HER-positive metastatic breast cancer and non-small cell lung cancer with different genetic lesions (Trepel et al., 2010; Jhaveri et al., 2012). A phase I clinical trial in lymphoid malignancies is ongoing (NCT00964873). Selinexor (also termed KPT-330) is an inhibitor of nuclear export (NCT00964873). Selinexor (also termed KPT-330) is another multi-targeting inhibitor. It inhibits nuclear export of many tumour suppressor genes (TSG). Cancer cells inactivate their TSG via nuclear export (Gerecitano, 2014). Inhibition of this process would maintain TSG nuclear levels, achieving an antitumour effect. In ongoing clinical trials of patients with AML, R/R multiple myeloma, WM, CLL and NHL, selinexor has been well tolerated with a favourable pharmacokinetic profile, and in some cases, durable clinical response (Chen et al., 2013; Kuruvilla et al., 2013, 2014; Savona et al., 2013). In summary, as we better understand the molecular basis of resistance, targeted therapies in these areas may have clinical utility in addressing either emerged secondary resistance or to pre-emptively prevent its development.

Conclusion

There is no doubt that BCR inhibition via the BTK-inhibitor ibrutinib offers an important and clinically relevant option for patients with relapsed and refractory CLL and MCL. However, as more patients are treated with ibrutinib over longer intervals, resistance is and will become an even more important clinical challenge. It is therefore increasingly crucial to understand the mechanisms of primary and secondary resistance. Such understanding would eventually lead to early detection, pre-emptive treatment and rational combinations of ibrutinib with other novel agents to overcome or prevent resistance development.

Acknowledgements

The authors thank the Leukaemia and Lymphoma Society and Portola Pharmaceuticals for their generous support of ibrutinib resistance-related work conducted in YLW’s laboratory.

Author contributions


Disclosure of potential conflict of interest

YLW receives research funding from Portola Pharmaceuticals. SMS has participated as a consultant to Pharmacyclics and to Janssen. Other authors declare no competing financial interests.

References


Review


