Targeting Bruton’s Tyrosine Kinase in Chronic Lymphocytic Leukemia at the Crossroad between Intrinsic and Extrinsic Pro-survival Signals

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Chemo immunotherapies for chronic lymphocytic leukemia (CLL) showed a positive impact on clinical outcome, but many patients relapsed or become refractory to the available treatments. The main goal of the researchers in CLL is the identification of specific targets in order to develop new therapeutic strategies to cure the disease. The B-cell receptor-signalling pathway is necessary for survival of malignant B cells and its related molecules recently become new targets for therapy. Moreover, leukemic microenvironment delivers survival signals to neoplastic cells also overcoming the apoptotic effect induced by traditional drugs. In this context, the investigation of Bruton’s tyrosine kinase (Btk) is useful in: i) dissecting CLL pathogenesis; ii) finding new therapeutic approaches striking simultaneously intrinsic as well as extrinsic pro-survival signals in CLL. This paper will review these main topics.

Abstract

Chemo immunotherapies for chronic lymphocytic leukemia (CLL) showed a positive impact on clinical outcome, but many patients relapsed or become refractory to the available treatments. The main goal of the researchers in CLL is the identification of specific targets in order to develop new therapeutic strategies to cure the disease. The B-cell receptor-signalling pathway is necessary for survival of malignant B cells and its related molecules recently become new targets for therapy. Moreover, leukemic microenvironment delivers survival signals to neoplastic cells also overcoming the apoptotic effect induced by traditional drugs. In this context, the investigation of Bruton’s tyrosine kinase (Btk) is useful in: i) dissecting CLL pathogenesis; ii) finding new therapeutic approaches striking simultaneously intrinsic as well as extrinsic pro-survival signals in CLL. This paper will review these main topics.

Keywords: Chronic lymphocytic leukemia; Bruton’s tyrosine kinase; Inhibitors; Therapy

Introduction to Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is a lymphoproliferative disease characterized by a progressive accumulation of CD19+/CD5+/CD23+ B cells in the blood, bone marrow and lymphatic tissues. The levels of surface immunoglobulins (Ig) and the expression of CD20 and CD79b are characteristically low when compared with normal B cells. Leukemic cells are restricted to the expression of either k or λ immunoglobulin light chains. CLL is the most common leukemia in western country, with an estimated incidence of 3-5 cases/100,000/year. The median age at diagnosis is 72 years; however, almost 10% of subjects have less than 55 years at disease onset [1,2]. The diagnosis of CLL is established by the following the IWCLL-2008 criteria [3]: i) the presence in the peripheral blood of ≥5,000 monoclonal B lymphocytes/μl for at least 3 months with less than 55% of prolymphocytes; ii) the clonality of circulating B lymphocytes as assessed by flow cytometry; iii) the typical immunophenotype and iv) the features of leukemia cells found in the blood smear which are small, mature lymphocytes with a narrow border of cytoplasm and a dense nucleus lacking nucleoli and with partially aggregated chromatin. The clinical heterogeneity characterizing CLL, with survival time ranging from months to decades reflects the biological diversity of the disease [4]. Researches on the molecular pathogenesis of CLL allowed the identification of differences in morphology, immunophenotype, specific chromosomal abnormalities, aberrations in the B-cell receptor (BCR) signalling and mutations of cancer related genes [5]. This biological heterogeneity reflects the wide spectrum of clinical behaviours of the disease, ranging from patients with a slow accumulation of leukemic cells to subjects with rapidly increasing lymph nodes. Clinical markers include clinical stage systems (Rai and Binet), lymphocyte doubling time (LDT) and high levels of serum markers as LDH, beta-2 microglobulin and thymidine kinase have been used to predicted tumor burden and progression [6-10]. Anyway, the limit of these markers is the inability to provide survival and treatment responses.

Cytogenetic alterations are the most important prognostic markers, and among them deletions of 11q23, 13q14, 17p13 and trisomy 12 are the most common. Deletions of 17p13 and/or TP53 mutations are related to a very aggressive phenotype with low response to conventional chemo-immunotherapies and, as consequence, are recommended to direct treatment decision [3]. A well as recognized a most relevant prognostic marker is represented by somatic hypermutation of the IGHV genes. Presence of mutations, defined as gene homology < 98% from the germline sequence, in the IGHV genes is one of the most stable and reliable indicators of favorable clinical outcome [11]. Several other prognostic markers have been identified and, among them the most important are CD38, ZAP70 and CD49d; however their use in clinical practice is still matter of controversy [3]. Some groups have tried to combine prognostic markers in order to identify a reliable prognostic index; for example our group has recently combined data of cytogenetic by FISH analysis, IGHV mutational status and CD38 expression into the “Integrated CLL Scoring System” [4]. In addition, recent reports on whole genome sequencing in CLL have revealed a number of recurrent somatic gene mutations that occur in parallel to the above-mentioned structural genomic aberrations. These include the genes NOTCH1, MYD88, TP53, ATM, SF3B1, FBXW7, POT1, CHD2, RPS15 and others [12-14]. The accumulation of leukemic B cells results from a complex balance between cell proliferation and apoptosis. In fact, CLL is not a static disease simply resulting from the accumulation of long-lived lymphocytes arrested in G0/G1 phases of cell cycle, but rather a disease in which proliferative and accumulative pools coexist [15]. Important advances in the molecular pathogenesis of CLL were obtained through the study of structural and functional features of the BCR such as IGHV mutations and deregulation of the BCR-dependent signaling pathways. Several lines of evidence indicate that key molecules of the
signaling pathway downstream BCR triggering are constitutively active in CLL B cells, resulting in a ligand-independent BCR signaling, defined as tonic signalling [16]; among them Lyn [17], Syk [18], PI3K (phosphoinositide 3-kinase) [19,20] and mitogen-activated protein kinase p38 [21] play the most important role. Aberrant miRNA expression and DNA methylation have been also implicated in the initiation and progression of CLL. Down-regulation of miR-15a/miR-16-1, as consequence of 13q14 deletion, is implicated in the up-regulation of BCL2 and thus blocking apoptosis [22]. The decrease of miR-29c and miR-223 levels is reported to take place during the progression of disease [23], whereas overexpression of miR-21 and down-modulation of miR-181b are unfavorable prognostic markers [24]. Interestingly, miR-18a and miR-34a levels are correlated with a shortened time to treatment and with TP53 status [25]. Finally, aberrant methylation has been described for genes that are specifically deregulated in CLL (i.e. TWIST-2, CD38, BTG4, HOXA4, DAPK1, p16INK4a and p15INK4B). Interestingly, epigenetic regulation is likely to have a role in altered miRNA expression in CLL [26]. Despite new genetic prognostic markers and increasing discoveries on CLL pathogenesis and progression, this disease is still incurable, although readily controllable, with combination therapies, including purine analogues, monoclonal antibodies, and newer targeted agents, such as Btk inhibitors [27].

Brief Overview of Current Treatment Options and Patient Outcomes

CLL is a slow-growing disease and most of patients do not require treatment at diagnosis. The 2008 workshop on CLL provided specific indications on which patients need treatment according to tumor burden (LDT < 6 months and/or rapidly increasing lymphoid organs), cytopenias and B symptoms. For these reasons, many subjects are only “watched” until disease progression. The choice of specific treatment is oriented on the basis of the number and kind of previous therapies (first vs further lines, fludarabine refractory vs sensitive), fitness (fit vs unfit vs frail patients) and on presence of TP53 abnormalities (17p deletion and/or TP53 mutations) [28]. The gold standard treatment for young (<65-70 years) and fit patients without TP53 abnormalities is the chemotherapy with rituximab-fludarabine-cyclosphamide (RFC). This regimen showed, for the first time, an improvement of both progression free survival (PFS) and overall survival (OS) [29]. However, RFC is too toxic for elderly unfit patients in particular for the high-rate of infections; for this reason, Bendamustine-Rituximab (BR) is considered a good alternative. Preliminary result from CLL10 trial showed the superior efficacy of RFC vs RB in previously untreated and physically fit patients with CLL but not in the unfit elderly group, likely related to the higher number of infective events [30]. Another international trial from the German group, the CLL11 trial, showed a high response rate, an improved PFS and secures toxicities profile of the combination chlorambucil (CLB)-obinutuzumab vs. CLB-R vs CLB alone [31]. Thanks to this study, the combination of CLB with anti-CD20 antibody (obinutuzumab or R) is considered the standard of untreated frail patients without TP53 abnormalities. Considering patients with TP53 abnormalities, both at presentation and at relapse, the conventional regimens with chemotherapies showed limited, if any, effects and short remissions. For these reasons, these subjects should be treated within a clinical trial, with BCR inhibitors or alentuzumab-containing regimens. For young and fit patients with TP53 genetic lesions, allogenic stem cell transplantation should also be considered balancing type of transplantation (matched related or unrelated donor), condition regimens (i.e. whether or not to use reduced-intensity regimens), transplant-related mortality, graft versus host disease and immunosuppressive therapy [28]. Patients, who relapsed after 24 months from the last therapy, can be retreated with the previous regimens (i.e. RFC or RB). For those subjects who experienced progression within 2 years since last treatment they should be recruited in a clinical trial, or treated with BCR inhibitors, Ofatumumab or CLB with anti-CD20. In particular, clinical trials investigating new agents should be considered for refractory patients. Refractoriness defined by a disease progression within 6 months from the last treatment. Some current ongoing studies are also investigating the role of a maintenance therapy after induction chemotherapy in CLL [28]. For example, the CLL1M and the CLL002 continuum trials, from the CLL German study group, are evaluating the efficacy/safety of Lenalidomide during first and second remission, respectively. No data are available from both studies and maintenance therapy is not recommended from international guidelines. Nowadays, an increased number of new compounds targeting impaired signaling pathways are in clinical development and, of particular interest, are BCR signaling molecules. Despite the pivotal role of Lyn and Syk in BCR signaling initiation, the activity of their inhibitors (Dasatinib and Fostamatin, respectively) in clinical trial was only modest without long-lasting responses [28]. On the other hand, the development of PI3K inhibitors (i.e. Idelalisib, Duvelisib or Copanlisib), anti-BLC2 (i.e. Venetoclax) and Bruton’s tyrosine kinase inhibitors (i.e. Ibrutinib and Acalabrutinib) are an exciting field for biological researches and clinical applications [28].

Introduction to Btk-Known Physiological Functions

Bruton’s tyrosine kinase (Btk) is a non-receptor protein tyrosine kinase belonging to the Tec family [32] and firstly identified in 1993 [33]. The protein consists of 659 amino acids for a molecular weight of 77 kDa and the BTK gene is located in the human X chromosome (Xq21.3-22) [34]. BTK gene mutations result in a hereditary immunodeficiency named X-linked agammaglobulinemia (XLA), discovered by O.C. Bruton in 1952 [35,36] and characterized by the lack of B lymphocytes development. Most of the Tec family kinases (TFKs) (Figure 1), including Btk, are predominantly expressed in hematopoietic cells with Btk absent in plasmacells and T lymphocytes [32,37]. Like other TFKs, Btk contains different domains as illustrated in Figure 2: a pleckstrin homology (PH), a Tec homology (TH), two Src homology (SH) -3 and -2, which are all essential for the interaction with other intracellular signaling proteins, and the catalytic domain. TH domain is, in turn, composed of a region of homology between GTPase activating protein (GAP), the Btk homology (BH) domain and a proline-rich (PR) motif [37]. Following antigen ligation to BCR, the Src family kinase (SKF) Lyn phosphorylates the immunoreceptor tyrosine activation motifs (ITAMs) of CD79a and CD79b leading to the activation of Syk, which in turn phosphorylates different substrates. Among them, and and following BCR engagement, Btk translocates to the plasma membrane, by the binding of the PH domain to phosphatidylinositol 4,5-bisphosphate (PIP2), [38,39] where it is phosphorylated at Tyr551 by Lyn. This event is followed by an auto-phosphorylation at Tyr223 necessary for Btk full activation [40,41]. As a consequence, and in tight cooperation with the adapter protein B-cell linker (BLNK), Btk phosphorylates PLC-γ2 starting the downstream signaling events. Following additional phosphorylation by Syk, the adaptor protein SLP-65 functions as a scaffold for Btk and PLCγ-2 that form a complex that initiates Calcium (Ca++) signaling. Activated PLCγ-2 hydrolyzes membrane PIP2 into diacylglycerol (DAG) and IP3, which binds to and activates IP3 receptors, through

which Ca\(^{++}\) leaves the endoplasmic reticulum. In this context, the fraction of Btk localized at the cell membrane positively regulates the magnitude of Ca\(^{++}\) entry through calcium-release activated (CRAC) channels following store depletion and this effect is blocked by protein kinase C\(\beta\) (PKC\(\beta\)) dependent phosphorylation of Btk. In fact, Btk is also negatively regulated by PKC\(\beta\) through phosphorylation at serine (Ser) 180, resulting in an elegant mechanism of negative feedback [42]. Other proteins known to inhibit Btk activity are IBtk (inhibitor of Btk), physically associated with it [43], and Sab (SH3BP5, SH3 domain-binding protein 5) [44]. It has been demonstrated that also cavelin-1, in B lymphocytes, functionally interacts with the kinase domain of Btk leading to a drastic downregulation of Btk kinase activity [45]. Btk plays a crucial role in B lymphocyte development, differentiation, proliferation, signaling, and survival [46]. Accordingly, B lymphocytes lacking Btk do not reach the mature state and are probably condemned to premature death. Individuals who have mutations in the gene encoding Btk lack circulating mature B lymphocytes and serum immunoglobulins of all isotypes thus being unable to mount a proper immune response. This immunodeficiency is the previously mentioned XLA [47]. When Btk is absent, B cell development is stopped between the pro- and the pre-B-cell stages [48]. In addition to its role in BCR signalling, Btk participates in the signal mediated by different others surface receptors that regulate B lymphocytes-microenvironment interactions (Figure 3). Btk takes part of o4β1 integrin signalling that culminates in cytoskeletal reorganization [49], B cell migration and tissue homing mediated by CXCL12-CXCR4 axis [50]. Moreover, Btk is involved in the signalling mediated by different Toll-like receptors (TLRs) and other crucial proteins belonging to these pathways thus playing a prominent role in both the innate and adaptive immunity. In particular, Btk, by targeting PLC-γ2 and IP-3, is required for IL-6 production in response to TLR9 and BCR activation and is necessary for TLR9-BCR co-localization within an auto-phagosome-like compartment [51]. In addition to B cells, defective functions related to Btk have been detected in other cell types such as platelets and macrophages [52,53] with impairments in macrophage polarization [54]. The evidence that Btk is also present in the nucleus suggests its involvement in transcriptional regulation. Btk, in fact, interacts with different regulators of transcription by phosphorylating some of these (i.e. NF-κB) and thus inducing their activity [55,56]. Btk has also been shown to phosphorylate signal transducer and activator of transcription 5A (STAT5A) in a B cell line [57].

Role of Btk in Chronic Lymphocytic Leukemia

In CLL, Btk protein expression is quite variable among patients and apparently not correlated with patients' clinical features. On the other hand, Btk mRNA is significantly higher in CLL with respect to normal B cells showing no correlation with protein expression, thus suggesting that the deregulation of Btk can take place at a post-transcriptional level. Moreover, Western blotting analysis found a constitutive phosphorylation of Btk, at basal conditions, in CLL cells varying among patients [58]. Literature on Btk in CLL is mainly associated to its inhibition by Ibrutinib (PCI-32765). These studies argue that Btk inhibition sensitizes leukemic B cells to apoptosis and alters their migratory activity. In fact, Ibrutinib exhibited a significant dose-dependent induction of cytotoxicity in CLL cells in a caspase-dependent manner as demonstrated by induced PARP cleavage and caspase-3 enzymatic activity [58]. Ibrutinib-induced apoptosis of leukemic B cell uses a mechanism involving the inhibition of Lyn and ERK kinases, two relevant proteins in CLL pathogenesis [59,60]. When studying Btk in CLL, it must be taken into account that it is a Lyn substrate after BCR engagement. In CLL, Lyn is overexpressed and constitutively active [17], thus this molecule could modulate Btk phosphorylation, and therefore its activity, with significant impact on CLL B cell fate. NF-κB signaling is also down-modulated in CLL cells from both the peripheral blood and tissue compartments during Ibrutinib treatment [61]. These data demonstrate that Ibrutinib effectively inhibits pathways that promote tumor cell activation and proliferation. Moreover, secretion of CCL3 and CCL4 chemokines is significantly inhibited by Ibrutinib in CLL-nurse like cells (NLCS) co-cultures, thus indirectly demonstrating the implication of Btk in the connection of leukemic B cells with CLL microenvironment [62]. In this context, Ibrutinib does not affect CLL B cell migration toward conditioned medium of mesenchymal stromal cells (MSCs) isolated from bone marrow (BM) of CLL patients, rich in cytokines and chemokines, suggesting that neoplastic B cells do not lose their ability to move toward a protective niche. In addition, during a co-culture of CLL B cells with MSC, Ibrutinib is able to interfere with the expression levels of chemokine receptors involved in cell migration and over-expressed on CLL cells surface; in particular, Ibrutinib increase CXCR4 and decrease CXCR5 levels, while it does not affect CCR7 and CXCR3 expression [59,63]. Taking into account the importance of cell-cell contact, it was demonstrated that Ibrutinib inhibits pseudopemperipilosiss in CLL B cells co-cultured with MSCs, a process that involves the down-modulation of the integrin CD49d expression [59]. This evidence suggests that Ibrutinib not only is able to reduce CLL B cell viability by blocking key molecules for cell survival, but it interferes with leukemic B clone-microenvironment interaction; this aspect finds confirmation by the typical transient lymphocytosis during Ibrutinib treatment due to the egress from the lymph nodes. In this context, Baldari's group and we demonstrated that Ibrutinib was able to restore the expression of the cytokine receptor S1P1, which plays a pivotal role in lymph node egress [63]. This finding, together with a decrease in surface CXCR4 levels through the inhibition of CXCR4 phosphorylation at Ser339 by Ibrutinib, results in the rapid leukemic cell redistribution from secondary lymphoid organs to blood [64].

Preclinical studies have shown that Ibrutinib effectively inhibits the proliferation of malignant B lymphocytes and their survival in vivo, as well as cell migration and adhesion to the substrate in vitro [65]. Results obtained in our lab (unpublished data), demonstrated that...
Ibrutinib is able to down-regulate both the expression and the phosphorylation of Cortactin, a protein involved in CLL pathogenesis, aggressiveness and migration [66].

Kil et al. [67] tempted to investigate Btk signaling in CLL development, by modulating Btk expression in the EμTCL1 mouse model. They demonstrated that CLL development is fully dependent on Btk since in the Btk-deficient model they did not observe CLL formation [67]. The importance of Btk in CLL has been confirmed by Woyach et al. that come to the same conclusions by genetic inactivation of Btk thus validating this molecule as a target for future drug development [68].

Critical Analysis of the Clinical Potential for BTK Inhibition in Chronic Lymphocytic Leukemia

Although therapies for CLL have shown a positive impact on prognosis and clinical outcome, some patients relapse or become unresponsive to treatment. Since several abnormalities of molecules involved in different signal transduction pathways are connected to CLL pathogenesis, the main goals of researchers in this field is the identification and characterization of specific targets in order to develop new therapeutic strategies to cure this malignancy. Taking into account that the study of abnormalities in the signals mediated by BCR is mandatory to deep into CLL pathogenesis, attention has increasingly focused on BCR signaling molecules to be targeted. In CLL, the other event contributing to the acquisition of the transformed phenotype and the diffusion of the disease is the cross-talk between tumor cells and the surrounding microenvironment. In fact, despite their in vivo prolonged lifespan CLL leukemic cells rapidly undergo spontaneous apoptosis when cultured in vitro, highlighting the need of signals delivered by accessory cells, collectively referred as "the CLL microenvironment", which modulate the apoptotic status of CLL cells [69]. Leukemic cells co-cultured with different marrow adherent cell types show greater survival, migration and drug resistance. The targeting of these two events (intrinsic/BCR signaling and extrinsic/microenvironment) has become increasingly urgent over the past year. In this context, Btk is an ideal candidate because closely participates in both BCR signaling and microenvironment-mediated events, thus representing a great option to simultaneously target both intrinsic and extrinsic pathogenic events leading to CLL. The recent attention, focused on Btk inhibition, have provided a better knowledge of the molecular and cellular mechanisms that lead to leukemic transformation, designating Btk as a critical kinase at the cross-talk between intrinsic and extrinsic survival mechanisms. At present, different clinical trial with Btk inhibitors (PCI-32765/Ibrutinib alone or in combinations, AYL-292, ACP-196 and ONO-4059) are under investigation as detailed in Table 1.

O’Brien et al. [70] published the results of a phase Ib/II trial (NCT01105247) which used Ibrutinib as initial therapy for elderly previously untreated patients with CLL. Patients received 28-day cycles of once-daily Ibrutinib 420 or 840 mg. The reported toxicity profile was acceptable and 71% of the enrolled patients achieved an objective response [70].

The international phase 3 RESONATE™ trial compared Ibrutinib 420 mg once-day vs. Ofatumumab in relapsed refractory patients [71], the primary end-point was PFS, while the duration of response rate (RR) and OS were secondary end-points. At a median follow-up of 9.4 months, Ibrutinib significantly improved RR, PFS and OS as compared with Ofatumumab. Similar effects were observed regardless of whether patients had unmutated IGHV, 17p13 deletion or resistance to purine analogs.
### Table 1: Ongoing clinical trials using BTK inhibitors.

<table>
<thead>
<tr>
<th>MOLECULE(s)</th>
<th>CT.gov IDENTIFIER</th>
<th>PHASE</th>
<th>SUBJECTS</th>
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<td>NCT01886872</td>
<td>III</td>
<td>Older patients with previously untreated CLL</td>
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<td>PCI-32765 + R vs. RFC</td>
<td>NCT02048813</td>
<td>III</td>
<td>Untreated CLL</td>
</tr>
<tr>
<td>PCI-32765 + OFA(1,2,3,4)</td>
<td>NCT01217749</td>
<td>I</td>
<td>Relapsed/Refractory CLL</td>
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<td>PCI-32765 vs. R</td>
<td>NCT01973387</td>
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<tr>
<td>PCI-32765 vs. OFA(2)</td>
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<td>CLL</td>
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<td>III</td>
<td>Previously treated with High Risk CLL</td>
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</table>

CT.gov: ClinicalTrials.gov; R: Rituximab; B: Bendamustine; F: Fludarabine; C: Cyclophosphamide; LEN: Lenalidomide; OFA: Ofatumumab; SEL: Selinexor; OBI: Obinutuzumab; PEM: Pembrolizumab (anti-PD1); ACP-196: Acalabrutinib; GDC-0199: Venetoclax; DUV: Duvelisib (anti-PI3K); BMT: bone marrow transplant. http://www.clinicaltrials.gov
Almost 20% of ibrutinib-treated patients showed a rapid decrease of lymph nodes size together an increase of absolute lymphocyte count, a condition, which has been called partial response with lymphocytosis. 51% of patients treated with Ibrutinib developed grade 3 or 4 adverse events, as compared with 39% rate in the Ofatumumab group. The most common were neutropenia (16% vs. 14%), pneumonia (7% vs. 5%) and diarrhea (4% vs. 2%). In addition, bleeding complications, most commonly petechiae and ecchymoses, and atrial fibrillation were more common in the Ibrutinib group (44% vs. 12% and 5% vs. 0.5%, respectively). The durable responses and the low toxicity profile observed with Ibrutinib, allows the majority of patients to receive continuous therapy for an extended period. Byrd et al. [71] reported long-term observation (median follow-up was 3 years) from 132 patients with symptomatic treatment-naive and relapsed/refractory CLL or small lymphocytic lymphoma. Longer treatment with Ibrutinib was associated with improvement in the quality of response over time and durable remissions. Toxicity with longer follow-up diminished with respect to occurrence of hypertension, cytopenias, fatigue and infections. Progression remains uncommon; however, they occurred primarily in some patients with 17p13 or 11q23 deletion [72]. However, a work from MD Anderson Cancer Institute demonstrated that a complex karyotype was a stronger predictor of outcomes from Ibrutinib-treated patients than TP53 abnormalities; in fact, patients with complex karyotypes had shorter PFS and OS [73]. Very recently, Burger et al. reported results from the RESONATE™-2 indicating that Ibrutinib was superior to ClB in previously untreated patients with CLL/SLL, as assessed by RR (86% vs. 35%), PFS (rate at 18 months, 90% vs. 52%), OS (rate at 24 months, 98% vs. 85%). This study established the role of oral-administered single-agent Ibrutinib as initial treatment of elderly unfit patients with CLL [74]. Thanks to these pivotal trials and strikingly results, Ibrutinib have recently become world widely available. However, some patients developed resistance to Ibrutinib. The mechanisms leading to resistance involved mutations of BTK binding-site or of the downstream molecule PLCγ2. The mechanisms leading to resistance involved mutations of BTK binding-site or of the downstream molecule PLCγ2. A substitution of serine for cysteine at residue 481 (C481S) of BTK was shown leading to the disruption of the Ibrutinib-BTK covalent binding, changing irreversible to reversible binding. In this way, the impaired binding leads to a loss of inhibition of BTK enzymatic activity ultimately resulting in Ibrutinib resistance in the patient [75]. The R665W and L845F mutations in PLCγ2 are both potentially gain-of-function mutations that lead to autonomous BCR activity regardless upstream molecules inhibition [75]. The group of Rossi D. and Gaidano G. provide evidence for the absence of these mutations in Ibrutinib-naive patients and their acquisition during the selective pressure induced by Ibrutinib itself.

Several ongoing clinical trial are combining Ibrutinib with traditional chemo-immunotherapy (R, FCR, BR, Obinutuzumab, etc.) or investigating other more specific Btk inhibitors (AVL-292, ACP-196 and ONO-405; Table 1). The HELIOS trial was an international, double-blind placebo-controlled, phase 3 study for CLL patients. Patients were randomly assigned to receive RB with either Ibrutinib or placebo. The primary end-point was PFS and crossover to Ibrutinib was permitted for patients in the placebo group who experienced progression. After a median follow-up of 17 months, PFS was significantly improved in the Ibrutinib group compared with the placebo group (not reached vs. 13.3 months, respectively). Grade 3 or higher adverse events were similar in both arms. The conclusions of authors were that in patients eligible for RB, the addition of Ibrutinib results in significant improvements in outcome with a manageable safety profile [76]. Hing et al. [77] have proposed to combine the XPO1 inhibitor Selinexor with Ibrutinib to overcome Ibrutinib resistance.

The finding of new Btk specific inhibitors is mandatory considering that the Ibrutinib concentrations, used in CLL, inhibit many other protein tyrosine kinases, thus its anti-leukemic activity cannot be completely ascribed to Btk inhibition alone [78]. In this context, Acalabrutinib (ACP-196) is a more selective, irreversible BTK inhibitor, specifically designed to improve the safety/efficacy of the first-generation BTK inhibitors. Byrd et al. reported the results from 1/II phase multicenter study NCT02029443. Administration of twice-daily Acalabrutinib, achieved a complete and continuous level of drug binding to BTK, without inhibiting other kinases. Acalabrutinib therapy showed a high RR, in particular in patients with 17p deletion it was 100%, and durable remissions with a favorable safety [79]. Among other Btk inhibitors, AVL-292 (also named CC-292) is a covalent highly selective orally active Btk inhibitor with IC50 of <0.5 nM, displaying at least 1400-fold selectivity over the other kinases assayed, thus seem not appreciably inhibiting other kinases involved in BCR signaling [80]. A Phase I escalating-dose completed trial (NCT01351935) in subjects with relapsed or refractory B cell Non-Hodgkin Lymphoma including CLL studied the use of AVL-292/CC-292 monotherapy. Brown et al. [81] reported the first results at the 2013 ASH annual meeting showing that AVL-292/CC-292 was well tolerated as an oral daily therapy being sufficient to achieve high nodal and partial response rates in relapsed or refractory CLL patients, including high-risk [81]. As regard ONO-4059, it is administered as monotherapy in a Phase I clinical study in patients with relapsed or refractory NHL and CLL. As reported by Salles et al. [82] responses have occurred across all dose levels with patients experiencing reductions in lymph nodes during the first cycle (28 days) of therapy. An improvement in the hematological parameters occurred in all the enrolled patients after at least 3 months of therapy with responses observed in relapsed, refractory and 17p- patients [82]. At present, Ibrutinib is also being tested in combination with more traditional drugs in relapsed CLL patients. Burger et al. updated the results obtained in a trial utilizing Ibrutinib combined with rituximab (14 months follow up) in high-risk CLL patients both treated and untreated. 87% of the enrolled patients achieved partial remission and 8% complete remission. Despite some adverse events, treatment was generally well tolerated, with infectious complications being the most common [83]. Similar results have been reported for a trial including Ibrutinib combined with R and B with a demonstrated safety profile such of those obtained with BR containing regimen and Ibrutinib as monotherapy. Also in this case, responses appeared to be independent of high-risk features.

Concluding Remarks

Data so far collected from clinical studies involving the use of Ibrutinib or other molecules inhibiting Btk, alone or in combination with traditional therapies (ie: R, FLU, etc…), reported a good safety profile and a high activity of the Btk inhibitors in CLL. Despite in the last years chemo immunotherapy had considerably improved the outcome of CLL patients, those having 17p deletion and/or TP53 mutations fail to respond to standard therapies and still represent an unmet clinical need in the management of CLL. Btk inhibitors equally affect patients with a good or poor prognosis, including those with 17p deletion related to unfavorable prognosis. More generally, responses to Ibrutinib were independent of high-risk clinical or genetic features. For this reason, results from clinical trials demonstrated the efficacy and
feasibility of Btk inhibitors for all patients with CLL, irrespective of prognostic factors. In some cases, questionnaires proposed during the clinical studies, revealed a significantly improved overall health and quality of life after 6 months of treatment (R + Ibrutinib). Since CLL is mainly a disease of the elderly, this type of therapy would help in reducing disability to cancer. In this context, several new molecules, which target key elements in leukemic cell survival, are entering clinical practice, alone or in association to standard therapies, with the ultimate goal to tailor therapy in each patient. This will optimize the efficiency and effectiveness of health care. Overall data obtained in these studies, despite some of them are still preliminary, seem to encourage and promote the enrollment of refractory/relapsed CLL patients in those protocols involving the use of Btk inhibitors. Further, these data support the hypothesis of a first-line treatment with Btk inhibitors for those patients with high genomic risk.

Disclosure of Potential Conflict of Interest

The authors declare no competing financial interests.

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