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Protein Kinases: Emerging Therapeutic Targets in Chronic Lymphocytic Leukemia

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Abstract

Introduction—Although protein kinases are primary targets for inhibition in hematological malignancies, until recently their contribution to chronic lymphocytic leukemia (CLL) was poorly understood. Insights into B cell receptor (BCR) signaling and its role in regulating key cellular functions have shed light on candidate protein kinases that are aberrantly activated in CLL. In this regard, protein kinases are now considered as potential drug targets in CLL.

Area covered—This review has covered signaling pathways and associated protein kinases in CLL and the kinase inhibitors currently available in preclinical and clinical investigations. Individual protein kinases that are abnormally active in CLL and the functional consequences of their inhibition are discussed.

Expert opinion—A growing body of evidence suggests that protein kinases are druggable targets for patients with CLL. The emergence of novel and bio-available kinase inhibitors and their promising clinical activity in CLL underscore the oncogenic role of kinases in leukemogenesis. Further investigations directed towards their role as single agents or in combinations may provide insight into understanding the substantial role of kinase mediated signal transduction pathways and their inhibition in B- CLL.

Keywords

Chronic lymphocytic leukemia (CLL); PTK – protein tyrosine kinase; RTK – receptor tyrosine kinase; TKI – tyrosine kinase inhibitor; BCR – B cell receptor

1. Protein kinases and their classification

Kinases are a key class of enzymes that covalently attach a phosphate group to their target substrate. Among the different types of kinases that phosphorylate biological molecules (proteins, lipids, and nucleotides), protein kinases are the largest group of kinases that phosphorylate amino acid substrates. They possess a catalytic subunit that transfers the

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phosphate group from ATP to one or more amino acid residues in the protein substrate, resulting in its conformational change and/or functional alteration. The resulting process of phosphorylation in subject to post-translational modification of proteins plays a major role in various cellular processes such as metabolism, apoptosis, cell cycle, DNA replication and repair, transcription, and translation.

About 478 typical and 40 atypical protein kinase genes have been identified in humans, corresponding to 2% of all human genes. Depending on substrate specificity, they have been classified into protein-serine/threonine kinases, protein-tyrosine kinases (PTKs), and tyrosine kinase-like (TKL) proteins. PTKs are of two types: trans-membrane receptor tyrosine kinases (RTK) and cytoplasmic non-receptor tyrosine kinases. Despite the low levels of protein-tyrosine phosphate as opposed to other phosphates, they play a central role in the regulation of cellular processes (1, 2).

1.1. Protein kinases as therapeutic targets

Because protein kinases are crucial in regulating cellular functions, they are often themselves oncogenes. The activities of protein kinases are tightly regulated in normal cells, and any transforming functions that they acquire due to mutations, overexpression, or stimulation may contribute to tumorigenesis. Most importantly, the process of protein phosphorylation functions as an on/off switch in cellular functions and any aberrations in the regulation of protein kinases propagate signals through the cytoplasm and activate multiple signal transduction cascades.

Chronic myeloid leukemia is one of the few cancers caused by a single specific genetic mutation in 90-95% of cases(3). The BCR-ABL protein that results from the fused genes of BCR and ABL promotes malignancy, increases proliferation, and decreases apoptosis in CML cells. Because ABL has a catalytic domain that can add a phosphate group to tyrosine residues, the BCR-ABL fusion gene functions as a PTK. Imatinib (Gleevec) was the first tyrosine kinase inhibitor (TKI) identified to inhibit BCR-ABL kinase (4) (5), and later other TKIs such as dasatinib and nilotinib came into application.

Acute myelogenous leukemia is another myeloid leukemia that is known to have activated PTK (6). FLT3 (Fms-like tyrosine kinase 3) is mutated in about 25% of AML patients (7) with either amino acid sequence mutation or internal tandem duplication (ITD)(8). Indolinones such as SU5416 (IV) and SU1124 (oral) and others (CEP-701, MLN518 and PKC412) are the potent FLT3 inhibitors currently in clinical trials.

2. Role of protein kinases in chronic lymphocytic leukemia (CLL)

Despite the notion that protein kinases are primary targets for inhibition in hematological malignancies such as chronic myeloid leukemia and acute myeloid leukemia, until recently their involvement in CLL was not known. Insights onto B-cell receptor (BCR) and its function in regulating gene expressions have shed light on candidate protein kinases that are aberrantly activated in CLL. In this regard, protein kinases are now considered as potential drug targets, as they are relatively selective for CLL cells. This review will discuss the

various protein kinases that are particularly active in CLL and the currently available kinase inhibitors in clinical trials (Table 1 and 2).

2.1. B-Cell receptor signaling

Current research proposes a model(9) in which BCR stimulation by antigen leads to phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) located in the cytoplasmic tails of CD79a/CD79b by a Src-related PTK, LYN(10). Following ITAM phosphorylation, SYK (spleen tyrosine kinase) is subsequently recruited and activated by tyrosine phosphorylation (11, 12). PI3K (phosphatidylinositol 3-kinase) generates inositol phosphate metabolites including PIP₃, which binds to the pleckstrin homology (PH) domain of BTK (Bruton's tyrosine kinase) and localizes it to the cell membrane. Activated SYK phosphorylates the adaptor molecule BLNK (B-cell linker)(13), which enables BTK to be phosphorylated by SYK and PLC- γ 2 (phospholipase C γ 2, a phospholipase enzyme) (14) to be activated by BTK and thus coupling SYK and BTK to PLC γ 2(15). Subsequently, PI3K recruits other BCR-signaling molecules to the membrane and transmits signal to ser/thr kinase AKT. Activated AKT targets numerous substrates that are functionally pro-apoptotic. In parallel, activated PLC γ 2 cleaves the membrane phospholipid, phosphatidylinositol-4,5-bisphosphate into diacylglycerol (DAG) and inositol triphosphate, which consequently mobilize calcium and activate protein kinase C (PKC)(14). These events open up a signaling cascade promoting activation of multiple kinases and transcription factors and concurrent protein synthesis (Fig. 1). Taken together, the consequences of an imbalanced functional response of the BCR signaling axis are responsible for the deregulation of gene expression in CLL.

2.1.1. Lyn tyrosine kinase—LYN, a Src family PTK is the proximal kinase to initiate signaling cascade in BCR pathway. It effectively increases tyrosine phospho-proteins suggesting that LYN could be the only Src kinase that is mostly responsible for the abnormal cellular tyrosine phosphorylation in B-CLL (16). Compared with other Src kinases such as SRC, FYN, c-FGR, and LCK, LYN protein isoforms (p53 and p56) are expressed in higher amounts in leukemic B-cells than in normal B lymphocytes (16). LYN kinase activity is not limited to activating downstream PTKs, but it also directs the action of phosphatases such as shp-1 (src homology 2 domain-containing phosphatase 1) a known negative regulator of hematopoietic cell function (17). For instance, LYN and ZAP-70 are two key substrates of PTPROt (a protein tyrosine phosphatase receptor-type O) that is epigenetically silenced in CLL, and a critical phosphorylation site in LYN (Y397) that is essential for its activity is dephosphorylated by PTPROt(18). Thus, LYN regulates both protein kinases that positively regulate BCR pathways as well as phosphatases that negatively regulate BCR signaling(19).

In addition to molecular mechanisms, *LYN* expression is regulated by epigenetic mechanisms. Overexpression of *LYN* in B-CLL was associated with a significant downregulation of microRNA-337-5p, suggesting that the aberrant expression of this particular microRNA could be involved in post-transcriptional regulation of *LYN* mRNA (20). Additional studies reported that HSP-90 a chaperone protein is tightly bound to its client protein LYN enhancing its stability, and that the HSP-90 inhibitor geldanamycin was

capable of dissociating the LYN-HSP-90 complex and induce cell death (21). Taken together, these observations indicate the physiological importance of LYN in BCR signaling, and establish LYN as a potential target for inhibition.

2.1.1.1. Lyn inhibitors: In preclinical studies, exposure of leukemic cells to the LYN inhibitors PP2 and SU6656 was shown to block LYN kinase activity and promote apoptosis suggesting that LYN could be involved in blocking apoptosis signals in CLL cells (16). Dasatinib, which was originally synthesized as a Src kinase inhibitor and later used in CML to target ABL kinase, is now in clinical trials for CLL as a dual BCR-ABL/LYN kinase inhibitor. In vitro data suggest that subsets of CLL patients with del (17p) and unmutated immunoglobulin heavy chains (I_GV_H) might be especially sensitive to dasatinib (22-24). Dasatinib monotherapy has moderate clinical activity in CLL, however its activity is enhanced when combined with other agents. Partial responses according to NCI-WG criteria were achieved in 3 of the 15 patients (20%) (25). It induced apoptosis in peripheral blood CLL cells within 3 to 6 hours of its administration and downregulated SYK mRNA (25).

NS-187 (INNO-406), a dual BCR-ABL/LYN kinase inhibitor was developed with greater affinity for ABL and enhanced specificity for LYN compared with dasatinib (26). An orally bio-available rationally designed form of NS-187 (bafetinib) is in a phase II clinical trial for evaluation of efficacy and safety in patients with high-risk B-CLL. Preliminary results demonstrated that 11 evaluable patients demonstrated 50% elevation in their peripheral lymphocyte counts during first two months of treatment, a pattern similar to that exerted by other kinase inhibitors in B-CLL patients. Six demonstrated > 50% shrinkage in their lymph nodes and/or spleen, two patients had stable disease and three patients had progressive disease at their initial assessments(27).

Despite the fact that LYN is the proximal kinase in BCR signaling, there are not many specific inhibitors available. Because LYN is upstream of several kinases, combining Lyn inhibitors with other kinase inhibitors may prove useful. Considering the leukemic microenvironment as an essential component of CLL pathophysiology, understanding LYN function and expression in response to the microenvironment is important. Finally, the functional role of LYN in the direct or indirect regulation of expression of anti-apoptotic proteins has not been elucidated. Mechanistic studies directed towards these end points may provide further insight into functional characterization of this kinase in CLL.

2.1.2. Spleen tyrosine kinase—SYK is a non-receptor cytoplasmic member of Syk family PTK. Following antigen receptor stimulation, SYK is recruited to the BCR and phosphorylated at the Y352 site, thereby promoting an intracellular signaling cascade of several pro-survival molecules (Fig. 1). Alternatively, SYK is constitutively active in CLL, suggesting that inhibition of this kinase can disrupt both antigen-dependent and antigen-independent signaling pathways that regulate leukemic cell survival. Studies using animal models demonstrated that Syk is critical for B cell development and Syk-deficient mice displayed a severe defect of B lymphopoiesis with a block at the pro-B to pre-B transition (28, 29). Additional studies on mouse models of non-Hodgkin's lymphoma revealed that Syk is essential for the survival and maintenance of malignant B cells (30). While SYK has a pivotal role in altering functional responses in BCR signaling (31), gene expression profiling

studies identified enhanced SYK expression and activation of downstream pathways in CLL compared with healthy B cells (32).

2.1.2.1. Consequences of SYK inhibition: Pharmacologic inhibition of SYK in CLL primary cells led to down regulation of pro-survival molecules such as ERK, AKT, and MCL-1, signifying the direct regulatory role of SYK on these molecules (33-35). Alternative approaches (siRNA silencing) demonstrated that SYK regulates cell survival through protein kinase C delta (PKC δ), a downstream protein of SYK that instructs AKT to inhibit GSK-3 β function and block proteasomal degradation of MCL-1(36). As opposed to Src kinases, inhibition of SYK and PLC γ 2 quantitatively correlated with the degree of dasatinib-induced apoptotic response in CLL (37) implicating SYK as an important therapeutic target in B-CLL.

2.1.2.2. SYK in response to microenvironment: Microenvironment plays a crucial role in CLL tumorigenesis. Cross-talk between BCR and tumor microenvironment is growing evidence in CLL pathophysiology. Consistent to this notion, in vitro models representing BCR engagement modulated the surface expression of adhesion molecules, chemokine receptors, and chemotaxis associating the network between BCR signaling and the microenvironment. A preferential response observed with anti-IgM in I γ V $_H$ unmutated and ZAP-70 positive cases of CLL support the direct correlation between BCR signaling and aggressive disease. In the same setting, R406 an ATP-competitive Syk inhibitor, abrogated the BCR-driven signaling cascade as well as nurse like cell (NLC)–triggered cell homing, retention, and survival, endorsing importance of SYK with respect to lymph node microenvironment (34, 38). Additionally, Syk inhibition demonstrated a significant reduction in major inflammatory mediators such as TNF α , IL-1, IL-6, and IL-18 suggesting that SYK may also inherit pro-inflammatory properties.

2.1.2.3. Syk inhibitors in clinical trials: Syk inhibitors that are currently in clinical investigations are R788 and Bay613606. Fostamatinib (R788), an oral form of R406 blocked BCR signaling in the E μ -Tcl-1 mouse model of CLL without affecting the generation of normal B cells (39). In clinical trials, the response in small lymphocytic leukemia (SLL) and or CLL was 54% (6 PR) and responders include both ZAP-70 $^{+}$ and ZAP-70-cases (2). Before induction of remission, the response was characterized by an early transient increase in peripheral lymphocyte counts, along with decreases in lymph node sizes in a significant proportion of CLL patients (40). This observation suggests that inhibition of SYK induces mobilization of CLL cells from tissue compartments to the blood, by antagonizing signals that confine CLL cells within tissues, before or along with the induction of CLL cell death. Molecular characterization of these mobilized CLL cells and their sensitivity to chemotherapy are important in the future use of SYK inhibitors.

2.1.3. ZAP-70 tyrosine kinase—ZAP-70 another Syk family PTK, is known for key signaling in T cells, where it couples the antigen-activated T-cell receptor (TCR) to downstream signaling pathways (41). Between 1999 and 2005, several reports were published on the expression and function of ZAP-70 in CLL (42-48). Gene expression profiling studies in CLL identified ZAP-70 as the gene that was most differentially

expressed between the I_GV_H unmutated and mutated CLL and correlated with inferior clinical outcome (42). Its expression and correlation with I_GV_H mutational status was a significant finding in terms of identifying prognostic markers in CLL (43-46). Further investigations interpreted not just the associations between ZAP-70 status and I_GV_H mutation but also the associations between CD38 expression, unfavorable clinical course and chemo-resistance in B-CLL cells (47, 48).

2.1.3.1. ZAP-70 in BCR signaling: Following its detection in B-cells, the functional characterization of ZAP-70 in BCR signaling was also established. Experiments with avian lymphoma B cells demonstrated that Zap-70 can restore some BCR signaling events when Syk is not expressed, indicating a certain degree of functional homology between the two PTKs (49). ZAP-70 was shown to undergo tyrosine phosphorylation and associate with the BCR complex in antigen-stimulated CLL B-cells, suggesting ZAP-70 may be involved in BCR signal transduction even when SYK is present (50). It was later proposed that because ZAP-70 has functional homology with SYK, in addition to its role as a prognostic marker, it might also exhibit kinase function in altering BCR signaling (51). The differential expression of ZAP-70 in two subtypes of CLL displayed a differential ability to transmit BCR-derived stimuli and induced stronger signaling responses in CLL B-cells from patients with poor prognosis (48, 50, 52). Consistently, genetic modulation of ZAP-70 resulted in stronger activation of certain BCR-signaling molecules, such as SYK, BLNK, and PLC γ suggesting that ZAP-70 directly participates in the activation of BCR (53). However, when the tyrosine phosphorylations of ZAP-70 and SYK were evaluated side by side in BCR engagement, ZAP-70 phosphorylation was inefficient and negligible compared to activation of SYK (54). Similar results were generated using in vitro immune complex kinase assays, in which ZAP-70 was shown to have an approximately 100 times lower intrinsic enzymatic activity than SYK (55). In addition, ZAP-70 was expressed at lower levels than SYK in the majority of ZAP-70⁺ CLL cases (56). Regardless of activation, how ZAP-70 contributes to BCR signaling is an ongoing debate. The notion that ZAP-70 is not directly involved in activation of BCR does not necessitate the requirement of specific inhibitors for this kinase.

2.1.4. PI3K/AKT axis—PI3K (phosphatidylinositol-3-kinase) is a lipid kinase that principally phosphorylates 3' inositol on phospholipids rather than proteins. Activation of the PI3K pathway by RTK contributes to cell growth, cell cycle, cell survival, and cell motility - all important aspects of tumorigenesis (57-59). On the basis of structural similarities and substrate specificity, the PI3K family is divided into three classes (I, II, and III). Class I PI3K is further divided into two groups, class-IA and class-IB. Class-IA consists of three isoforms (p110 α , p110 β , and p110 δ), whereas the only class IB member is p110 γ . PI3K δ is expressed primarily in blood cell lineages and therefore is considered an important kinase in hematological malignancies, in particular CLL. PI3Ks are constitutively activated in CLL and over expression of *PI3K* transcript levels has been observed using the real-time quantitative polymerase chain reaction technique (60). Expression of *PI3K* correlated with unmutational status of I_GV_H, a representative aggressive clinical course of CLL (61). The cellular levels of PI3K products are controlled by a balance between PI3K activity and PTEN phosphatase activity (phosphatase and tensin homologue deleted on chromosome 10)

(62), and therefore any mutation or deficit in PTEN contributes to PI3K pathway activation (63).

PI3K or the PI3K/AKT pathway is an important component in BCR (64). It is implicated as a signaling intermediate upstream of survival gene expression that is dependent on the transcription factor NF- κ B (65, 66). In addition to BCR signaling, the PI3K/AKT pathway is also active in CD40 signaling. Mechanistic studies on purified splenic B-cells demonstrated that activation of PI3K was necessary for CD40-mediated cell proliferation, which facilitated NF- κ B/Rel nuclear translocation, AKT activation, and down regulation of cell cycle inhibitor p27^{kip} (67, 68). Taken together, these findings indicate that the PI3K/AKT axis constitutively activates multiple signaling pathways that are critically important in the maintenance of malignant clones.

2.1.4.1. The PI3K pathway and microenvironment: Several lines of evidences indicate that PI3K and its downstream targets are activated in response to the tumor microenvironment in CLL. First, the signals propagated from the microenvironment (such as cell adhesion, CXCR4 activation, and migration) activate the PI3K/AKT pathway in CLL (69-71). Second, CLL-stromal co-culture model systems that mimic representative microenvironments have demonstrated that the stromal layer provided markedly better survival and concurrent up regulation of AKT and ERK (72). Third, gene expression profiling studies on co-cultures demonstrated up regulation of PI3K/NF- κ B pro-survival pathway genes that confer chemotherapy resistance and a pro-angiogenic switch in the CLL cells (69). Fourth, inhibition of PI3K with LY294002 completely abrogated CD40-induced proliferation and impaired BCL-XL expression in both wild-type and c-Rel *knock out* B cells, demonstrating the cooperative role of PI3K toward the microenvironment and anti-apoptotic proteins (60). Fifth, treatment with the PI3K inhibitors wortmannin and LY294002 led to a dose-dependent inhibition of the CXCL12-induced migration and invasion, whereas the p38 MAP kinase inhibitor SB203580 and the MEK inhibitor PD98059 had no significant effect on CXCL12-induced migration or invasion, evidencing PI3K association with the microenvironment. Sixth, studies on a panel of novel isoform-selective PI3K inhibitors that target different isoforms of the p110 subunit showed that class I PI3K inhibitors PIK-90 and PI-103 effectively inhibited CLL cell migration and signaling responses to CXCL12, inhibited AKT phosphorylation on Ser473, and induced CLL cell apoptosis in suspension and marrow stromal cell co-cultures. Finally, PIK-90 and PI-103 displayed a more than additive cytotoxic effect in combination with fludarabine and reversed the protective effect of stromal cells (73). Together, these reports establish the essential cross-talk between the PI3K pathway and tumor microenvironment.

2.1.4.2. PI3K inhibitors: Although many PI3K inhibitors are in development, lack of sufficient activity, poor specificity, and low bio-availability limit their possible clinical use. Prototype PI3K inhibitors wortmannin and LY294002 sensitize human cancer cells to chemotherapy and radiation, but lack substrate specificity and exhibit toxicity in animal studies, precluding their clinical development. Recently, CAL-101 a selective inhibitor of PI3K δ in preclinical investigations decreased cell survival and reduced stromal interactions that retained CLL cells in protective tissue microenvironments (74-77). In clinical

investigations, plasma concentrations of chemokines CCL3, CCL4, and CXCL13 were elevated at baseline and lymphadenopathy was reduced in all 32 (100%) patients; 29/32 (91%) achieved a lymph node response. An initial increase in peripheral absolute lymphocyte counts of >50% from baseline was observed in 21/35 (60%) patients with partial responses in 11/33 (33%) of patients (78).

2.1.4.3.AKT Ser/thr kinase: Activation of PIP2 to PIP3 by PI3K recruits additional signaling molecules that phosphorylate cytosolic kinase AKT/PKB at the Thr308 and Ser473 residues (79-81), over expression of which mediates cell survival and proliferation in malignant cells (82). Upon activation, AKT potentially targets many downstream cellular substrates whose functions are predominantly proapoptotic and critically important in the regulation of signal transduction pathways. An ever-increasing number of substrates as phosphorylation targets by AKT has been identified, including members of the Forkhead family of transcription factors (83), BAD (84), caspase 8 (85), caspase 9 (86), glycogen synthase kinase-3 (GSK-3), mTOR, and many more (84, 86-88). AKT phosphorylates substrate BAD at the Ser136 residue, which then binds to the ubiquitous cytosolic protein 14-3-3 that sequesters BAD and keeps it out of function. This loss of function dissociates BAD from the BCL-2/BCL-XL complex, causing it to lose its pro-apoptotic function and ability to block the survival of malignant cells.

mTOR, a target of rapamycin, is another substrate of AKT. AKT promotes activation of mTOR through AKT-mediated cellular energy and inhibition of AMPK, which is an activator of TSC2 – a negative regulator of mTOR (89). Subsequently, mTOR promotes mRNA translation by phosphorylating S6 kinase 1 (S6K1) and inhibiting eukaryotic initiation factor 4E-binding proteins (4E-BPs) that repress mRNA translation. Importantly, a stronger activation of the AKT pathway has been related to a higher capacity for cell cycle progression in CLL cells from patients with progressive disease. Selective inhibitors of AKT are currently limited. MK-2206 (90, 91) is in early phase clinical trial for solid tumors and pediatric leukemia and perifosine is in phase II studies for patients with relapsed and refractory leukemia(92).

2.1.4.4.Glycogen synthase kinase (GSK-3 β): The serine/threonine protein kinase GSK-3 β , another substrate of AKT was first described as a component of the metabolic pathway for glycogen synthase regulation. Later, it became evident that GSK-3 modulates the function of a diverse series of proteins and is associated with a wide variety of human disorders, including cancer. GSK-3 β is regulated at multiple levels by its post-translational modifications. Phosphorylation at Ser9 promotes inhibitory actions of the kinase, and phosphorylation at Tyr216 releases the inhibition. Additional studies showed that GSK-3 β protein accumulates in the nuclei of human CLL B-cells and that pharmacological inhibition of GSK-3 β leads to depletion of its nuclear pool, suppression of NF- κ B transcriptional activity, decreased expression of anti-apoptotic proteins (XIAP, BCL-2), and enhanced apoptosis in CLL B-cells, suggesting that inhibition of GSK-3 β could be a promising approach to CLL therapy (93). Other studies have demonstrated that GSK-3 β function is important in Mcl-1 degradation because AKT-mediated phosphorylation of GSK-3 β enhances Mcl-1 stability (87). Taken together, these findings indicate that the PI3K/AKT/

GSK-3 β pathway has clinical relevance as a promising therapeutic target. GSK-3 β inhibitors tested in preclinical investigations demonstrated induction of apoptosis in CLL (94), however they have not entered clinical trials.

2.1.5. BTK tyrosine kinase—BTK is a Tec family cytoplasmic kinase that serves an essential role in B cell signaling and development. Mutations in the BTK gene have been linked to severe developmental blocks in human B-cell ontogeny, leading to X-linked agammaglobulinemia(95). BTK protein and mRNA are expressed significantly high in CLL lymphocytes as compared to normal B-cells and CLL patient samples have highly variable levels of BTK protein but modest variability in *BTK* mRNA levels (96). In contrast to normal B-cells, there was no correlation between mRNA and protein levels in CLL cells suggesting that the deregulation of BTK protein expression must be occurring at a post-transcriptional level. On the other hand, BTK expression is selectively down regulated in T lymphocytes and plasma cells (97). Three models have been proposed for activation of BTK in CLL. In the first model, BCR cross-linking promotes activation of LYN, which then recruits BTK to the plasma membrane via PIP3 generated by PI3K and phosphorylate Tyr-551 residue of BTK (98). Alternatively, in the second model, BLNK is phosphorylated by SYK and recruited to the plasma membrane via binding to Ig α . BTK is recruited to phosphorylated BLNK and then activated by SYK (99). In the third model, BTK is activated independently of PI3K that lead to calcium mobilization via activation of PLC- γ 2. In parallel, activation of PI3K leads to activation of AKT, and both BTK and PI3K are important in the activation of NF- κ B, induction of cyclin D2 and expression of BCL-XL.

BTK is the first non-receptor PTK to be identified as a dual-function regulator of apoptosis, having both anti-apoptotic and pro-apoptotic properties (100). It functions as a mediator of oxidative stress-induced apoptosis in irradiated neoplastic B-cells and B-cell precursors (101, 102); on the other hand, it inhibits Fas-activated apoptosis in B-cells suggesting that apoptosis of B-cells may be reciprocally regulated by Fas and BTK (103). Consistently, defective BTK expression in leukemic B-cell precursors from infants with t(4;11) acute lymphoblastic leukemia has been associated with radiation resistance (104). Its pro-apoptotic function in B-cells was further illustrated by down regulation of anti-apoptotic activity of STAT-3 transcription factor on exposure to reactive oxygen intermediates (104). While this set of observations demonstrates the pro-apoptotic nature of BTK, its anti-apoptotic function upstream of BCL-XL within BCR has also been studied. Investigations by Feldhahn et al. demonstrated the anti-apoptotic function of BTK in the activation of pre-BCR dependent survival signals such as activation of PLC γ 1, autonomous Ca²⁺ signaling, STAT5 phosphorylation, up regulation of BCL-XL, and inhibition of apoptosis(105, 106). BTK acts in concert with SYK to phosphorylate and activate its substrate PLC γ 2 (107, 108), which mediates production of the second messengers inositol 1,4,5-triphosphate and DAG (109). DAG subsequently stimulates the activity of PKC and increases intracellular calcium levels, resulting in the activation of downstream transcription factors (110). As a proof of concept, recent studies showed that a null mutation in BTK known to cause the xid phenotype, prevented BCR-induced activation of the transcription factor NF- κ B. This protection was reversed by reconstitution with wild-type BTK, suggesting its role in coupling NF- κ B to BCR with the involvement of PLC γ 2 and PKC (111).

2.1.5.1.BTK inhibitors: The efforts of 15 years research revealed that BTK is a new anti-apoptotic molecular target for the treatment of B-lineage leukemias and lymphomas. LFM-A13 was the first identified BTK-specific TKI (112) showing chemosensitizing and apoptosis-promoting anti-leukemic properties in mouse models (113-115). The selectivity of LFM-A13 to BTK was demonstrated in experiments with Burkitt's leukemia/lymphoma cells in which LFM-A13 caused a 70% inhibition of BTK-dependent PLC- γ 2 phosphorylation on Y753 and Y759 residues, and its effects were identical to the measure of BTK siRNA that target human *BTK* mRNA (116). Despite its promising activity in preclinical models, LFM-A13 has not advanced to clinical trials because it lacks specificity and inhibits other kinases such as Polo-like kinase and PI3K. The antibiotic terreic acid (quinone epoxide) was described as blocking the interaction between PKC and the PH domain of BTK (117) and inhibiting BTK autophosphorylation, with no effect on the activities of LYN and SYK (117). An unbiased chemical proteomics approach to detect binding proteins identified Tec kinase BTK, in addition to ABL and Src kinases, as a major binder of dasatinib (118).

The emergence of new BTK inhibitors has essentially shifted the paradigm of CLL treatment toward inhibition of kinases. In preclinical studies, PCI-32765, an irreversible, orally bio-available BTK inhibitor demonstrated its selectivity toward tumor B-cells by inhibiting BTK phosphorylation, abrogating downstream survival molecules (ERK1/2, PI3K, and NF- κ B), and inhibiting CD40 or BCR activation-induced proliferation and survival signals. Furthermore, PCI-32765 disrupted the residual support supplied by the stromal cells including soluble factors (CD40L, BAFF, IL-6, IL-4, and TNF- α), fibronectin engagement, and stromal cell contact (96). Additional in vitro and in vivo studies on PCI-32765 and simultaneous correlative studies on specimens from patients receiving therapy revealed that this agent significantly inhibit cell survival, DNA synthesis, migration in response to tissue homing chemokines (CXCL12, CXCL13) and secretion of BCR-dependent chemokines (CCL3, CCL4)(119).

A phase I dose-escalation study of PCI-32765 in recurrent B-cell lymphoma demonstrated safety and tolerability under conditions of BTK-drug occupancy with potent bioactivity in targeted cell populations. A phase IB/II multi-cohort study of single-agent PCI-32765 in CLL/SLL patients with relapsed/refractory disease demonstrated tolerability and promising clinical activity in CLL with grade 3 toxicities in 10 of 30 patients(120). Of the 14 patients evaluated using the predefined criteria, the overall response rate was 64% (1 CR, 8 PR, and 4 SD). A characteristic pattern of rapid reduction of lymph node disease and a corresponding initial phase of lymphocytosis was observed (120). A phase Ib, multicenter, open-label, parallel-group safety study of PCI-32765 in combination with chemotherapies such as fludarabine, cyclophosphamide, and rituximab (FCR), or in combination with bendamustine and rituximab (BR) in subjects with CLL/SLL is under way.

Another oral agent CNX-774 (AVL-292-001) that selectively and covalently binds to BTK irreversibly inhibited and silenced BTK in preclinical studies. A double-blind, placebo-controlled, single ascending dose study of AVL-292-001 in healthy volunteers demonstrated favorable safety, tolerability, and pharmacokinetics (121). Further investigations on these inhibitors in preclinical and clinical studies may shed light on the functional role of this

kinase in CLL and mechanism based combinations with chemotherapeutic agents may eradicate peripheral blood lymphocytes.

2.1.6. Protein kinase C—PKC, a serine/threonine protein kinase, plays a key role in transmembrane signal transduction pathways. On the basis of interaction with calcium and DAG, PKC is subclassified into conventional (α , β , γ), novel (δ , ϵ , μ), and atypical isoforms. Over expression of the PKC β 2 isoform is a characteristic feature of CLL, and the activity of this enzyme strongly correlates with B-CLL response to BCR engagement. Functional characterization of PKC variants demonstrated that isoforms β , γ , δ , and ζ are consistently expressed in CLL (122), and similar to the PI3K/AKT pathway, the PI3K/PKC δ pathway plays an integral role in defective apoptosis (60). AKT that is downstream from PI3K is also phosphorylated by PKC β , suggesting that AKT is activated by both PI3K-dependent and PI3K-independent pathways (123).

PKC inhibitors such as bisindolylmaleimide I (BisI), 7-hydroxystaurosporine (UCN-01), and bisindolylmaleimide IX (BisIX) are proven potent inducers of apoptosis in CLL cells, supporting the notion that PKC mediates survival signals within CLL cells (124-126). Inhibition of PKC by the specific inhibitor Rottlerin enhanced apoptosis in CLL but not in normal B cells (60). Mechanistically, treatment with the PKC inhibitor BisIX precipitated apoptosis via an intrinsic mitochondrial pathway that involves subcellular redistribution of BAX from the cytosol to the mitochondria, mitochondrial release of cytochrome c, activation of effector caspases, and downregulation of MCL-1 and XIAP levels (125, 126). Consistently, the PKC agonist bryostatin induced upregulation of MCL-1, PKC-dependent phosphorylation of BCL-2 at Ser70, and disruption of its association with BAX (127). Activating PKC and PI3K pathways using bryostatin and PMA, induced survival and differentiation in CLL cells underscoring the cytoprotective effect of these pathways in B-CLL cells. Other agents in preclinical investigations are the PKC β -specific inhibitor LY379196, (128), safinol, and calphostin (122), which downregulated certain target genes in response to PKC inhibition.

A novel compound, serratamolide (AT514), has been shown to induce caspase-dependent apoptosis of CLL cells via the intrinsic pathway, including cells from patients with fludarabine-resistant disease, while having no effect on normal peripheral blood lymphocytes. Importantly, the mechanism of AT514 occurred through PI3K- and PKC-mediated signaling pathways as it demonstrated synergism with LY294002 and BisI inhibitors (129). PKC inhibitors in phase II clinical trials—such as *N*-benzoyl-staurosporine (PKC412), a staurosporine derivative that possesses selectivity towards classical PKC isoforms—induced a reduction in PKC activity and apoptosis in CLL cells (130). PKC412 was effective at treating CLL patients who had already displayed a resistance to chlorambucil and fludarabine (131). In a phase I trial of UCN-01 (7-hydroxystaurosporine) in combination with fludarabine phosphate, UCN-01 without significantly increasing the toxicity of FAMP, induced apoptosis of the CLL cells (132). Enzastaurin, an inhibitor targeting both PKC β and AKT, induced apoptosis of CLL cells but not T cells in vitro (133). A phase I study to characterize the inhibitory effects of enzastaurin on the pGSK3 beta level in B-CLL cells has been completed (NCT00452257). These studies showed that PKC

isoforms are potential regulators of cell survival and that inhibition of PKC activity induces apoptosis of primary CLL cells insisting its combination with classical CLL therapies.

2.1.7. Mitogen-activated protein kinases (MAPKs)—In addition to PI3K activation, mitogen-activated protein kinase (MAPK) pathways are functional in B-CLL cells. Because the MAPK pathways have been implicated in cell growth, differentiation, survival, and apoptosis, they are key regulators of cell fate in many systems, including B-cells (134). The family of MAP kinases includes extracellular signal-regulated kinase (ERK) 1/2, ERK3/4, ERK5, p38, and c-Jun NH2-terminal kinase (JNK). BCR cross-linking resulted in ERK activation, suggesting that some signaling pathways such as activation of ERK through BCR are functional in B-CLL cells despite the extensive impairment of signaling pathways (135, 136). Studies on freshly isolated primary leukemia cells from patients with B-CLL demonstrated that rituximab activates a CD20-mediated signaling pathway that results in apoptosis dependent on p38 MAPK activation (137). Furthermore, constitutive activation of the MAPK p38 is critical for MMP-9 production and survival of B-CLL cells on bone marrow stromal cells (138). Sorafenib, a multi-kinase inhibitor induced cytotoxic effects on CLL cells that are subjected to microenvironment and reduced phosphorylated B-RAF, C-RAF, ERK, STAT3, and Mcl-1 associating sorafenib cytotoxicity to its impact on RAF and Mcl-1 (139). Sorafenib is also shown to block SDF-1 induced phosphorylation of ERK and MEK in CLL (140).

3. BCR pathway-independent kinases

In addition to kinases that are downstream of BCR signaling, there are other functional kinases in CLL that are independent of BCR pathways. Gene profiling studies had revealed increased expression of *ROR1* mRNA, a cell surface RTK, in all patients with CLL whereas enriched blood B and T cells as well as granulocytes from healthy donors were negative (141, 142). Proviral integration of murine leukemia viral (PIM) kinase, an anti-apoptotic serine/threonine kinase, is present in high levels in heme malignancies and its inhibition in CLL results in cell death (143). Epigenetic silencing and/or germline mutations in *death-associated protein kinase 1* (*DAPK1*), a positive mediator of apoptosis contribute to familial CLL and this gene is silenced in virtually all cases of sporadic CLL, suggesting that aberrant silencing of *DAPK1* is an early and required event in leukemogenesis. (144). *DAPK* is preferentially hypermethylated in APL (145).

Cyclin-dependent kinases (CDKs) generally regulate cell cycle events, whereas members CDK7 and CDK9 phosphorylate C-terminal domain of RNA polymerase II and hence associate with transcription processes such as initiation and elongation steps in transcription (146). Importantly, inhibition of CDK7 and CDK9 primarily affects transcripts and proteins with relatively short half-lives (147), such as MCL-1 and XIAP that are crucial for CLL cell survival. Consistent to this observation, CDK inhibitors flavopiridol, roscovitine, and SNS-032 showed impressive preclinical and clinical activity in CLL (148, 149).

Expert opinion

Despite the evidence that protein kinases are potential targets for inhibition in hematological malignancies, until recently their substantial role in CLL was poorly understood. CLL pathophysiology that is associated with defective BCR signaling and resistance to chemotherapy evidence the existence of constitutively activated signaling pathways in this disease. Mechanistic studies have revealed the expression and functional characterizations of several families of protein kinases (Src, Syk, and Tec family kinases) that are primarily active in CLL. Accordingly, selective and specific inhibition of target protein kinases opens up a new avenue of treatment options in this leukemia.

Nonetheless, identification of specific and selective kinase inhibitors has always been a challenge. For instance, dasatinib, which was formerly synthesized as Src kinase inhibitor, later was shown to bind to several other kinases (ABL, LYN, SYK, BTK, polo-like kinase, and PI3K). Erlotinib was originally synthesized as an EGFR kinase inhibitor but is now known that SYK is a candidate erlotinib target in AML (150). Although there are advantages in dual or multi-kinase inhibitors, lack of specificity or selectivity could strengthen off-target effects and may lead to variable outcomes.

In the current therapeutic options for CLL, conventional chemotherapies and DNA-targeted agents demonstrate clinical activity in selective patients; however, refractory or recurrent disease is a major downside with the current agents having an “all or none” effect. Additionally these DNA-damaging agents are highly associated with emergence of secondary malignancies (151). Although transcriptional inhibitors are often promising in preclinical investigations, their activity is limited to transient inhibition of oncogene transcripts, while the existing cellular messages may potentially trigger survival signals, shifting the paradigm towards chemoresistance. Furthermore, their clinical tolerance has been rate-limiting for their use. The targeted therapies called BH3 mimetics have indeed demonstrated significant preclinical and activity in several clinical trials. However, the best known inhibitor (ABT-737) does not target Mcl-1 and novel combinations are needed (152). Monoclonal antibodies are efficient but their targets are essentially surface molecules, which are far beyond the molecular pathways and generation of protein oncogenes. Finally, the use of protein kinase inhibitors seems to be a novel strategy in terms of directly decreasing CLL cell survival while reducing interactions that retain CLL cells in protective tissue microenvironments. The emergence of bio-available and irreversible inhibitors of PI3K δ (CAL-101), SYK (R788), and BTK (PCI-32765 and CNX-774) has drawn interest towards kinase inhibition in CLL treatment. Importantly, these agents demonstrated tolerability and promising clinical activity in patients. Preclinical studies demonstrated their ability to inhibit kinase activities as well as down-regulate the anti-apoptotic molecules MCL-1 and XIAP. This effect primarily gives the combined benefit of targeting both defective BCR as well as deficit apoptosis in CLL. The functional consequences of disrupting intrinsic and extrinsic survival signals could be a critical mechanism for the clinical activity of these agents. In vitro studies, animal model studies and clinical trials could determine which inhibitors of which targets are likely to be most effective and least toxic, either in single or in combination.

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Figure 1.

BCR stimulation by antigen leads to phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) located in the cytoplasmic tails of CD79a/CD79b by a Src-related PTK, Lyn. Following ITAM phosphorylation, Syk is subsequently recruited and activated by tyrosine phosphorylation, which in turn propagates signal via BLNK, a linker protein to activate Bruton's tyrosine kinase (BTK). Syk and BTK subsequently phosphorylate phosphatidylinositol 3-kinase (PI3K) and phospholipaseC γ 2 (PLC γ 2), respectively. PI3K, a lipid kinase, phosphorylates phosphatidylinositol-3,4,5-triphosphate, which then recruits other BCR-signaling molecules to the membrane and transmits signal to ser/thr kinase AKT. Activated AKT targets numerous substrates that are functionally pro-apoptotic. In parallel, activated PLC γ 2, a phospholipase enzyme, cleaves the membrane phospholipid, phosphatidylinositol-4,5-bisphosphate into diacylglycerol (DAG) and inositol triphosphate, which consequently mobilize calcium and activate protein kinase C (PKC). These events

open up a signaling cascade promoting activation of multiple kinases and transcription factors and concurrent protein synthesis. In addition, stromal cells also contribute to the high levels of anti-apoptotic proteins.

Table 1

Kinase targets in CLL

Kinase	Family	Type	RTK	Inhibitor	In clinic
LYN	Src	Tyr	NO	Dasatinib, Bafetinib	YES
SYK	Syk/zap-70	Tyr	NO	Fostamatinib	YES
PI3K	Lipid kinase	N/A	NO	CAL-101	YES
AKT	AKT	Ser/thr	NO	MK-2206 perifosine	YES YES
GSK-3 β	GSK-3	Ser/thr	NO	NP031112	YES
BTK	Tec	Tyr	NO	PCI-32765 AVL-292	YES YES
PKC	PKC	Ser/thr	NO	PKC412	YES
ERK	MAPK	Ser/thr	NO	AEZS-131 GW5074	NO

Table 2

Kinase inhibitors in clinical trials for CLL

Inhibitor	kinase	Phase	Reference
Dasatinib	BCR-ABL/LYN kinase	II	Amrein et al(25)
Bafetinib	BCR-ABL/LYN kinase	II	Kadia et al(27)
Fostamatinib	SYK	II	Friedberg et al(40)
CAL-101	PI3K5	II	Furman et al(78)
PCI-32765	BTK	II	Burger et al(120)
AVL-292-001	BTK	I	Evans et al (121)
PKC412	PKC	II	Virchis et al(131)
Flavopiridol	CDK	II	Lin et al(149)
SNS-032	CDK	I	Tong et al(148)