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Review Article

BH3 mimetics and TKI combined therapy for Chronic Myeloid Leukemia

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Chronic myeloid leukemia (CML) was considered for a long time one of the most hostile leukemia that was incurable for most of the patients, predominantly due to the extreme resistance to chemotherapy. Part of the resistance to cell death (apoptosis) is the result of increased levels of anti-apoptotic and decreased levels of pro-apoptotic member of the BCL-2 family induced by the BCR-ABL1 oncoprotein. BCR-ABL1 is a constitutively active tyrosine kinase responsible for initiating multiple and oncogenic signaling pathways. With the development of specific BCR-ABL1 tyrosine kinase inhibitors (TKIs) CML became a much more tractable disease. Nevertheless, TKIs do not cure CML patients and a substantial number of them develop intolerance or become resistant to the treatment. Therefore, novel anti-cancer strategies must be developed to treat CML patients independently or in combination with TKIs. Here, we will discuss the mechanisms of BCR-ABL1-dependent and -independent resistance to TKIs and the use of BH3-mimetics as a potential tool to fight CML.

Introduction

Chronic Myeloid Leukemia (CML) is a Philadelphia chromosome (Ph1)-derived myeloproliferative disease [1] characterized by the expansion of leukemic cells extremely resistant to chemotherapy [2–8]. The reciprocal translocation between chromosomes 9 and 22 — t(9;22)(q34;q11) that originates the Ph1 chromosome also generates the chimeric BCR-ABL1 tyrosine-kinase. BCR-ABL1 constitutive enzymatic activity is responsible for a complex signaling transduction cascade [9] that accounts for the malignant phenotype observed in CML, including the strong resistance to chemotherapy-induced cell death (largely apoptosis).

BCR-ABL1 inhibits apoptosis induced by a variety of stimuli, including chemotherapeutic drugs, oxidative stress, UV or γ-irradiation, growth factor withdrawal and death receptor ligands [5,6,8]. To a great extent, BCR-ABL1-mediated resistance to apoptosis involves up-regulation of anti-apoptotic and down-regulation of pro-apoptotic BCL-2 (B-cell lymphoma/leukemia-2 gene) family members [5,6,10] thereby preventing the translocation of cytochrome c from the mitochondria to the cytosol and the consequent activation of effector caspases [5,6].

BCL-2 regulated apoptotic pathway

Over the past three decades, the BCL-2 family of intracellular proteins have emerged as central regulators of apoptosis signaling, dictating cellular fate by inhibiting or promoting cell death. Apoptosis can occur via two distinct cellular pathways — the *intrinsic* (aka mitochondrial, stress-induced, BCL-2-regulated) and the *extrinsic* (aka death receptor-induced) pathways [11,12]. The intrinsic or BCL-2-regulated apoptotic pathway can be initiated in conceivably all cell types in response to a plethora of intrinsic stress stimuli. These stimuli include DNA damage, oxidative stress, endoplasmic

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reticulum (ER) stress, aberrant calcium flux and nutrient deprivation. Once activated, the intrinsic apoptotic pathway leads to mitochondrial outer membrane permeabilization (MOMP). This is mediated by the pro-apoptotic multi BH (BCL-2 homology) domain BCL-2 family members BAX and BAK [13]. MOMP causes release of apoptogenic proteins from the mitochondrial intermembrane space into the cytoplasm. This includes cytochrome c as well as or SMAC/DIABLO, which both drive activation of the cascade of aspartate-specific cysteine proteases (caspases) that bring about the ordered demolition of the dying cells.

The members of the BCL-2 family proteins contain up to four (relatively loosely) conserved BCL-2 homology (BH) domains, BH1–BH4. Based on structure and function, the BCL-2 proteins can be classified into three groups: (**a**) the pro-survival proteins (BCL-2, BCL-X_L, BCL-W, MCL-1, BFL-1/A1); (**b**) the pro-apoptotic multi-BH domain containing effectors of apoptosis (BAK, BAX, BOK), and (**c**) the pro-apoptotic BH3-only proteins (BIM, PUMA, BID, NOXA, BMF, BIK, BAD, HRK) [13]. Apoptosis signaling is controlled by complex interactions between the pro-survival BCL-2 family members, the pro-apoptotic BH3-only proteins and the effectors of apoptosis, BAX and BAK. The pro-survival BCL-2 proteins can either directly inhibit the effector proteins BAX and BAK by binding to them or by binding to the BH3-only proteins, thereby preventing them from activating the effector proteins.

The BH3-only proteins are essential for the initiation of apoptosis signaling. Their levels are increased in response to diverse stresses through a broad range of transcriptional, post-transcriptional and post-translational processes [13–15]. The BH3-only proteins such as BIM, PUMA and t-BID (the proteolytically activated form of BID), can bind to and inhibit the pro-survival BCL-2 proteins [16,17], thereby releasing BAX and BAK from their restraint, unleashing MOMP and the downstream events in apoptosis. BAD selectively binds to BCL-2, BCL-X_L and BCL-W, whereas NOXA selectively binds to MCL-1 and A1 [16]. Importantly, certain BH3-only proteins, including PUMA and BIM, can also directly activate BAX and BAK. BAX and BAK also differ in their ability to bind to the pro-survival BCL-2 proteins. BAX associates with all the pro-survival proteins whereas BAK associates specifically with MCL-1 and BCL-X_L but surprisingly not with BCL-2, BCL-W, or A1 [18]. Interestingly, the effector protein BOK is not regulated by any of the pro-survival or the BH3-only proteins.

Therefore, the balance between the different pro-survival and pro-apoptotic BCL-2 family members and the interactions between them dictate cellular outcome. Once activated, the effector proteins BAX and BAK oligomerize to form pores in the outer mitochondrial membrane leading to MOMP [19], which is considered the point of no return in apoptosis signaling [20,21]. While BAK is already located at the mitochondrial outer membrane in unstressed cells, BAX translocates from the cytosol to the mitochondrial membrane in response to stresses that induce apoptosis [22,23]. Following MOMP, the formation of the apoptosome, comprising cytochrome c, APAF-1 and dATP, leads to the recruitment and activation of the initiator caspase, caspase-9, which then unleashes the downstream executioner caspases, caspase-3, -6, -7, that dismantle the cell in an orderly manner.

BCR-ABL1 modulation of BCL-2 family members

As mentioned above, the extreme resistance to apoptosis conferred by the expression of BCR-ABL1 is largely due to its multi-layered modulation of BCL-2 family members [2,4–8]. BCR-ABL1 was shown to up-regulate the expression of the anti-apoptotic members BCL-2, BCL-X_L, MCL-1 and A1 [6,10,24,25]. BCL-X_L and MCL-1 up-regulation occurs downstream of the activation of STAT5 [10,26,27], whereas BCL-2 appears to rely on Ras pathway [28] and A1 is associated with NFκB activation [29]. Although overexpression of BCL-2 or BCL-X_L was shown to confer resistance to imatinib [30], not a single anti-apoptotic member of the BCL-2 family seems to account for the extraordinary resistant phenotype observed in CML cells [8].

BCR-ABL1 also modulates pro-apoptotic BH3-only members of the Bcl-2 family, particularly BIM, BAD and BMF [31–34]. BCR-ABL1 was shown to interfere with the expression of BIM and BMF and post-translationally regulate BIM and BAD [32,33]. Whereas BMF seems to play a minor role on the BCR-ABL1-mediated resistance to apoptosis, BIM and BAD appears to cooperate and fully account for the anti-apoptotic phenotype, at least against TKIs, since imatinib does not induced apoptosis in BCR-ABL1-transformed $bim^{-/-}bad^{-/-}$ cell lines [33]. Whether these double-knockout cells are also resistant to the full spectrum of chemotherapeutic drugs remains elusive. Interestingly enough, it was recently shown that BCR-ABL1 mediates the formation of a HSP70/BIM complex in a kinase-independent way [35,36]. This complex apparently contributes to BCR-ABL1-driven leukemogenesis via the stabilization and consequence up-regulation of AKT and eIF4E protein levels and inactivation of BAK [36]. Finally, BIM was shown to be

epigenetically downmodulated in a set of patients that showed decreased cytogenetic and molecular responses to imatinib [37]. This observation indicates that combinations of TKIs and demethylating agents could improve the response of certain BIM-deficient patients.

BCR-ABL1 tyrosine kinase inhibitors

The constitutive activity of the BCR-ABL1 oncoprotein is the trigger to the signaling cascades responsible for the extreme resistance to apoptosis found in CML cells. This understanding guided scientists to the design of specific TKIs that could stop this primary oncogenic signal and be used in clinic to treat CML patients. In short, TKIs are small molecules capable of interacting with BCR-ABL1 tyrosine kinase at the adenosine triphosphate (ATP) pocket, thereby obstructing its enzymatic activity [38,39].

Imatinib mesylate (IM) was the first TKI approved by the Food and Drug Administration (FDA) to treat CML patients in the chronic phase (CML-CP) [38]. In IRIS study (International Randomized Study of Interferon and STI571), IM induced complete cytogenetic remission in ∼74% of patients with CML-CP and complete hematologic response in 11% of patients with CML in blastic phase (CML-BP) [40,41]. Patients from the IRIS trial who achieved more than 3 log reduction in BCR-ABL1 transcripts levels had low risk for disease progression [42]. Despite the high rates of cytogenetic and molecular responses to IM, the emerging resistance to this drug has been recognized as a major problem in the treatment of Ph-positive leukemia, particularly for CML patients in accelerated and blastic phases [43,44]. Almost 17% of CML patients from the IRIS study developed IM resistance after five years of follow-up.

The second and third generation TKI (dasatinib, nilotinib, bosutinib, and ponatinib) have been developed for CML therapy as an attempt to overcome IM resistance and intolerance [45–49]. Although these TKIs provide a better rate of molecular remission, none of them can cure CML patients, prevent disease progression or overcome the patients' resistance to therapy.

BCR-ABL1-dependent and -independent mechanisms of TKI resistance in chronic myeloid leukemia

Before introduction of TKIs, CML patients diagnosed at chronic phase progressed to accelerated or blastic phases after three to five years [39]. This scenario changed significantly after introduction of TKIs as first-line therapy. However, still several patients develop resistance to TKIs leading to relapse of the disease and death of these patients. Therefore, a better knowledge about the mechanisms underlying CML resistance to TKIs is essential to design novel BCR-ABL1-dependent or -independent therapeutic strategies that can serve as more broad and effective therapy approaches.

The resistance to TKI treatment is defined as *primary resistance* — the absence of response to therapy at the very beginning of treatment; or *acquired or secondary resistance* — when patients' leukemic cells develop molecular alterations that enable them to escape from ongoing therapy [50,51]. Importantly, the mechanisms involved in CML resistance to TKI therapy is divided into BCR-ABL1-dependent and -independent categories, as discussed below (Figure 1) [52,53].

BCR-ABL1-dependent mechanisms of resistance to TKIs

It is well-known that some BCR-ABL1-positive cells persist even in CML patients who respond to TKI therapy, indicating that these drugs do not eliminate all leukemic cells, particularly a BCR-ABL1-positive quiescent leukemic stem cells (LSC) population. Consequently, CML patients need to undergo continuous treatment with TKIs to keep the disease in check and to avoid progression to blastic phase. Unfortunately, it has been proved that the prolonged treatment with TKIs frequently leads to acquired resistance [54].

CML patients treated with IM may develop point mutations in the ABL1 kinase domain (KD) of BCR-ABL1 protein that reduces IM binding. Mutation in KD domain alters the protein structure and impairs the TKI and BCR-ABL1 interaction or stabilizes the kinase structure and reactivates the tyrosine kinase enzymatic activity [53,55]. These KD mutations are the most frequent mechanism of TKI resistance in CML patients (Figure 1), accounting for 9 to 48% of primary resistance and 10 to 68% of secondary resistance to IM [56–58]. Notably, CML patients at accelerated and blastic phases acquire KD mutations more frequently than CML-CP patients [59]. The KD mutations can be categorized into three types: (1) A-loop: mutation located at activation loop region that regulates the interaction with catalytic site; (2) P-loop: the ATP-binding loop, and (3) ATP-binding site [53,55].

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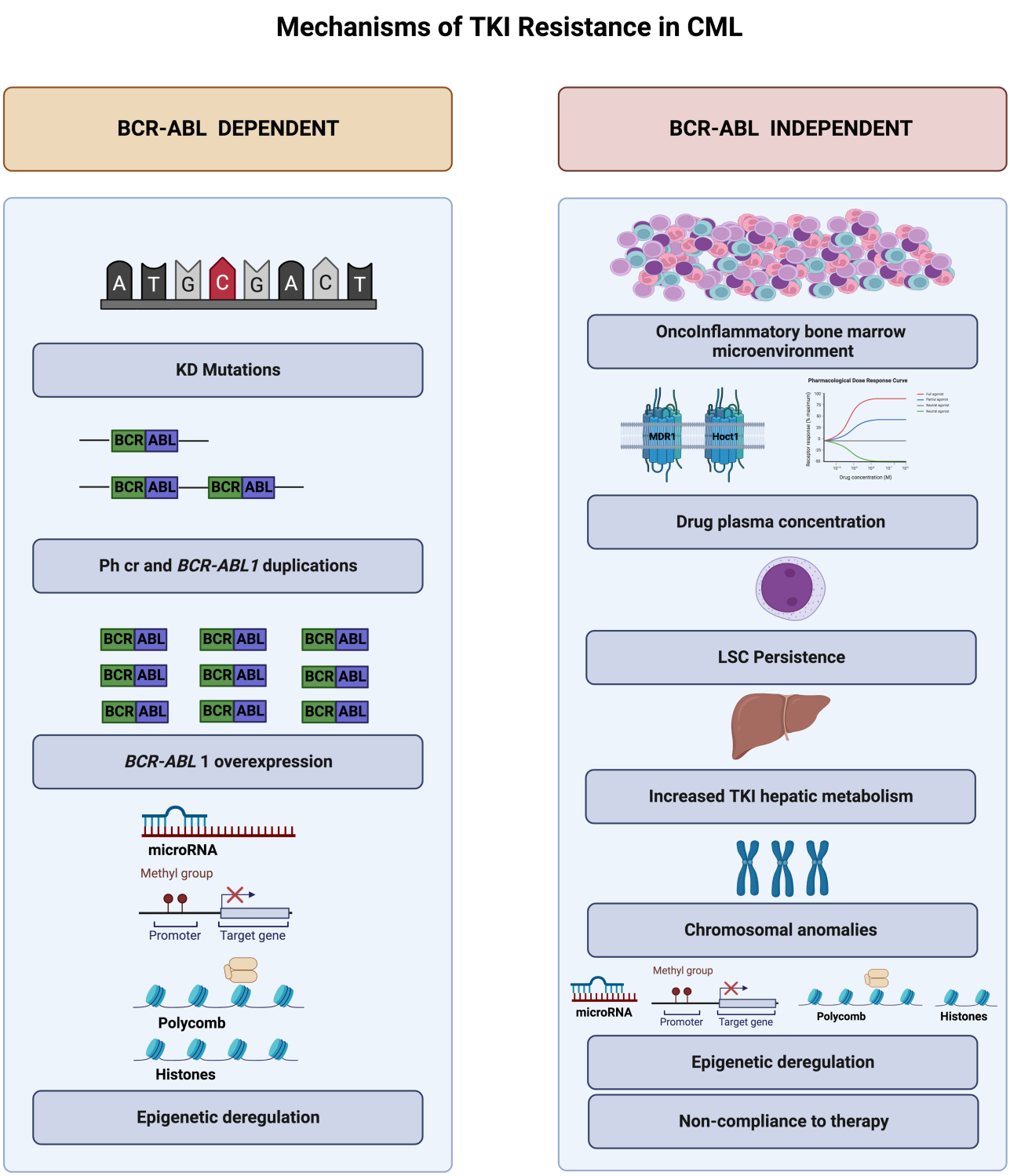

Figure 1. BCR-ABL1-independent and -dependent mechanisms of resistance in chronic myeloid leukemia.

BCR-ABL1-dependent mechanisms are BCR-ABL1 duplication and overexpression, microRNAs deregulated expression, and Kinase-domain mutations. The BCR-ABL1-independent mechanisms are the chronic myeloid leukemic stem cell (CML-LSC) persistence and high stemness, the overexpression of ATP binding cassette transporters such as P-glycoprotein and ABCG2 efflux pumps, epigenetic deregulation (microRNAs, histonemarking systems and the polycomb repressive complex 1 and 2), oncoinflammatory bone marrow microenvironment, increased drug metabolism, and non-compliance to therapy (low drug adherence).

Although Dasatinib, nilotinib, bosutinib, and ponatinib therapy have been successful for CML patients with the major KD mutations, these TKIs lack efficacy in a minimal number of different leukemic clones and all of them but ponatinib lack efficacy against the T315I mutation. The most frequent KD mutations associated with CML resistance to imatinib, nilotinib, dasatinib, bosutinib, and ponatinib according to their potency and region of BCR-ABL1 that each of these mutations target are shown at Figure 2 [52].

Additional BCR-ABL1-dependent mechanisms of resistance have been described, including BCR-ABL1 overexpression due to BCR-ABL1 gene amplification, Ph chromosome duplication, and differential regulation of oncogene transcription (Figure 1). BCR-ABL1 gene overexpression enables constitutive activation of tyrosine kinase despite the presence of TKI, which leads to leukemic cell expansion until a KD mutation is acquired and confers overt resistance [60]. As mentioned above, to overcome KD mutation, physicians should detect the type of mutation linked to a particular CML patient and select an adequate and effective new generation TKIs.

BCR-ABL1-independent mechanisms of resistance to TKIs

The BCR-ABL1-independent mechanisms of TKI resistance (Figures 1 and 3) in CML are particularly associated with persistence of LSC (Figure 3) [52,61,62], which may be related to their interactions with bone marrow niche microenvironment [61] as well as deregulated drug influx/efflux pumps [63,64], other chromosomal abnormalities, altered transcriptional regulatory and signaling networks or mitochondrial/metabolic changes [65], epigenetic alterations, and patient nonadherence to medication (Figures 1 and 3). In this regard,

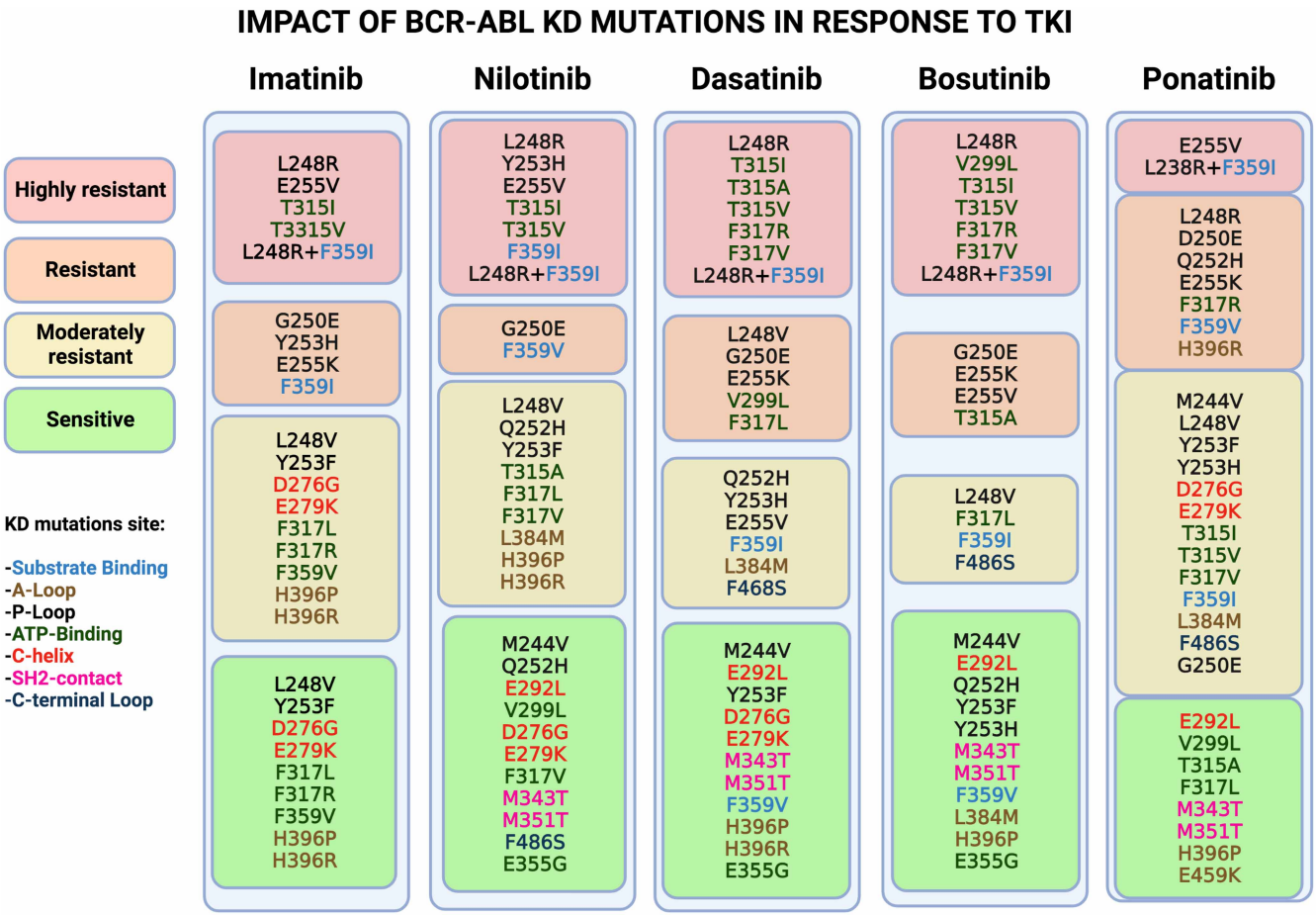

Figure 2. The most frequent KD mutations associated with CML resistance to imatinib, nilotinib, dasatinib, bosutinib, and ponatinib according to their potency and region of *BCR-ABL1* that each of these mutations target.

Green: BCR-ABL1 KD mutation sensitive to TKI therapy; Yellow: BCR-ABL1 KD mutation resistant to TKI therapy; Orange: BCR-ABL1 KD mutation moderately resistant to TKI therapy; Red: BCR-ABL1 KD mutation highly resistant to TKI therapy (adapted from [52]).

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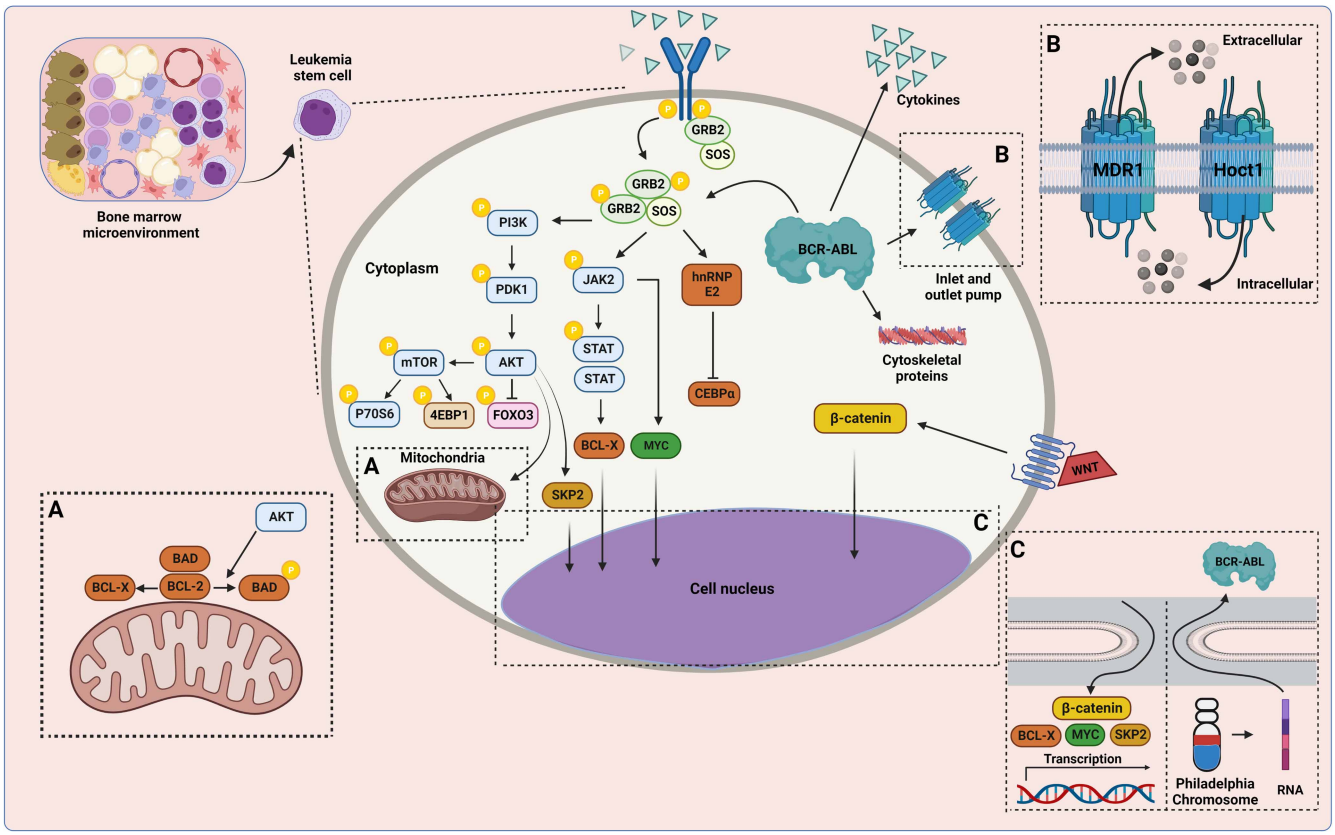

Figure 3. Tyrosine kinase inhibitor-resistant leukemia stem cell.

The chronic myeloid leukemia stem cell (CML-LSC) is resistant to TKI due to oncoinflammatory bone marrow niche, PI3-kinase and JAK/STAT signaling pathways; overexpression of anti-apoptotic-related genes (**A**); the low activity of the organic-cation transporter-1 (OCT-1) and overexpression of ATP binding cassette transporters such as P-glycoprotein and ABCG2 efflux pumps (**B**); BCR-ABL1 overexpression and aberrant activation Beta-catenin (**C**). Taken together all these alterations lead to CML-LSC exacerbated stemness, inhibition of apoptosis, genetic instability and TKI resistance.

persistence of CML-LSC in the bone marrow have been described as the cause of the minimal residual disease during TKI treatment, which can also lead to TKI resistance over time and relapse after therapy discontinuation [66]. CML-LSC have high stemness potential, are resistant to TKI-induced-apoptosis, interact with bone marrow stromal cells, and express high levels of BCR-ABL1 and drug influx/efflux proteins.

Deregulated drug influx/efflux pumps and drug sequestration are also related to TKI resistance because they interfere with intracellular drug availability. The low activity of the organic-cation transporter-1 (OCT-1), a cellular influx pump for IM, impairs the drug influx into leukemic cells. In contrast, elevated OCT-1 activity is associated with molecular response and high overall survival of IM-treated CML patients [63,64]. The high expression of the ATP-binding cassette (ABC) efflux transporters P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) reduces intracellular TKI accumulation and results in CML resistance to therapy [67,68]. The high expression of the influx transporter SLC22A1 is associated with a successful IM therapy in CML patients [67]. In addition, genetic polymorphisms of membrane transporters also contribute to CML resistance to IM. The SLCO1B3 699GG and 344TT genotypes are associated with lack of response to IM, while the ABCA3 4548-91 CC/CA genotypes are linked to poor prognosis in CML patients treated with IM standard dose [69]. Importantly, increase in TKI doses overcomes the low intracellular drug availability caused by alterations in drug influx/efflux pumps and sequestration processes.

The signals from the bone marrow (BM) microenvironment (BMM), especially from oncoinflammatory signals (pro-inflammatory cytokines and chemokines) and mesenchymal stromal cells also support LSC persistence. The alterations in CML-BMM, including the presence of high levels of inflammatory cytokines and chemokines (IL-1α, IL-1β, IL-6, G-CSF, TNF-α, CCL3, and CCL4), contribute to CML oncoinflammation,

CML-LSC genetic instability, and to TKI resistance [70]. In a mice model, the reduction in Cxcl12, a chemokine secreted by mesenchymal stromal cells (MSC) leads to normal HSC numbers but promotes LSC expansion by increasing self-renewing cell divisions. On the other hand, the absence of endothelial cell-specific Cxcl12 reduces LSC proliferation, suggesting niche-specific effects in CML [71]. Activation of alternative pathways such as PI3K/AKT, WNT/β catenin and STAT provides several survival signals to CML-LSC in the bone marrow niche [53,72]. The up-regulation of PI3K/AKT signaling pathway is important for CML LSCs maintenance in bone marrow niche and seems to correlates with P210BCRABL1 transcripts levels [73]. Airiau et al. [74] also reported that PI3K/mTOR inhibitors is capable of sensitize CML LSC to nilotinib, a potent TKI. The STAT3 pathway seems to favor leukemic cells survival via intrinsic signaling and via bone marrow stromal cells modulation [53,75,76]. In CML LSC many survival genes (*MCL-1*, *BCL-2* and *BCL-X_L*) are up-regulated because of STAT5 increased activation [77] leading to apoptosis resistance.The WNT/β-catenin is relevant for CML LSC stemness and quiescence. CML animal model deficient in β-catenin present an impairment of CML LSC self-renewal potential [78]. In addition, the β-catenin overexpression is related to CML progression to advanced phase and TKI resistance [79] (Figure 3).

In another avenue, there is limited knowledge about the relationship between resistance to TKI and the metabolome of CML patients. Recently, the analysis of lipid metabolism (sphingolipids and eicosanoids) in CML showed that patients who are resistant to TKI exhibited higher ceramide and lower sphingomyelin levels compared with CML patients responsive to TKI therapy. The enzyme sirtuin 1 (SIRT1) deacetylase is overexpressed in CML $CD3^{4+}$ cell regulating their metabolism and leukemogenic potential. SIRT1 regulates many cellular pathways, including energy metabolism and stress response to tumorigenesis. Recently, this enzyme overexpression was associated with CML-LSC survival as well as for acquisition of genetic mutations that promote TKI resistance [80,81]. In addition, SIRT1 overexpression contributes to genetic mutation occurrence in CML leading to TKI resistance [81]. It was also reported that the SIRT1 substrate, the PGC-1α, contributed to oxidative phosphorylation and TKI resistance in CML by interfering in LSC mitochondrial respiration [81].

Furthermore, the deregulation of epigenetic intrinsic cell-regulatory mechanisms has been notably associated with TKI resistance and CML-LSC persistence after TKI treatment. The alteration in microRNAs, histonemarking systems and the polycomb repressive complex 1 and 2 is frequent in hematological malignancies.

Altered expression of microRNAs (miRs) is implicated in the acquisition of TKI resistance in CML patients. The miRs can not only regulate BCR-ABL1 expression but also modulate the expression of genes involved in apoptosis regulation (apoptomiRs), drug transport or activation of essential signaling pathways. Several authors have described the association between TKI resistance and miRs. For instance, the apoptomiRs miR-26a, miR-29c, miR-130b and miR-146a expression are down-regulated in IM-resistant CML patients while their predicted target genes *C-IAP-1* and *MCL-1* are overexpressed. Deregulation of these miRs and their predicted targets can be a mechanism of IM resistance, since these genes are anti-apoptotic and inhibit the TKI-induced BCR-ABL1-positive cell death [82].

The deregulation of epigenetic intrinsic cell-regulatory mechanisms has been also notably associated with the CML-LSC persistence after TKI treatment. For instance, the histone methyltransferase *enhancer of zeste homolog 2* (EZH2) is overexpressed in CML-LSC and is associated with LSC protection from apoptosis and TKI-resistance [83]. Importantly, treatment with an EZH2-specific inhibitor resulted in increased sensitivity to dasatinib that is associated with up-regulated levels of p53 and the p53 targets BAX, BIM, PUMA and NOXA [83]. These results suggest that increased levels of EZH2, observed in CML-LSC, prevent p53 mediated intrinsic apoptosis. In addition, our group has previously shown that EZH2 also contributes to the resistance to TRAIL-mediated extrinsic apoptosis in CML cell lines [84,85]. Down-regulation of EZH2 by shRNA up-regulates TRAIL and sensitizes CML cell lines to imatinib (IM) [84]. Importantly, blockage of TRAIL signaling by treatment with TRAILR2-Fc prevented the combined effect of knocking-down of EZH2 and imatinib. Inhibition of TRAIL by EZH2 requires the expression of the tumor antigen *preferentially expressed antigen of melanoma* (PRAME) [84]. EZH2 forms a complex with PRAME to mediate epigenetic silencing of retinoic acid (RA)-target genes [86]. Indeed, we found that EZH2 is enriched at the *retinoic acid responsive element* (RARE) located at the TRAIL promoter in K562 cells and the down-regulation of PRAME resulted in significant loss of EZH2 from the TRAIL promoter [84]. Moreover, increased expression of PRAME, but not EZH2, was associated with the progression of disease in CML patients and knocking down of PRAME also sensitizes CML cell lines to chemotherapy, including IM [84,85].

Additionally, the overexpression of BMI1 a polycomb repressive complex 1 (PRC1) member is associated with CML progression, poor patient outcome and low response to TKI. In contrast, the increased expression of

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polycomb repressive complex 2 (PRC2) members, the PRC1-like and chromobox homolog 6 and 7 is linked to a better CML response to TKI [87–89].

Finally, drug adherence is one of most important steps for CML patients' response to TKI therapy. Oral TKI therapy is a prolonged and a non-curative treatment for CML. Recent studies have reported that 20 to 30% of CML patients fail to adhere to TKI treatment due to lack of medication, forgetfulness, disbelief in the drug potential, drug side effects, and size and number of pills to take. The patients' low compliance leads to low TKI plasma levels and suboptimal or no response to therapy. Pharmaceutical intervention showing the effectiveness of medication for disease control could improve the patients' compliance. Therefore, description of the processes involved in BCR-ABL1-independent mechanisms of TKI resistance in CML may contribute to design new therapeutic strategies capable of circumventing TKI resistance and eliminating CML-LSC [39].

BH3-mimetics in cancer

The discovery that anti-apoptotic BCL-2 family of proteins are implicated in the pathogenesis of several human cancers, including hematological malignancies such as CML [6,24,90], multiple myeloma [91,92]; and AML [93–95], let to the development of synthetic drugs that could inhibit prosurvival BCL-2 proteins. These drugs, so called 'BH3-mimetics', mimic BH3-only proteins by antagonizing the pro-survival function of anti-apoptotic proteins, unleashing the mitochondrial apoptosis machinery to eliminate cancer cells [96]. After 30 years of numerous scientific efforts several BH3-mimetic drugs have reached clinical trials for hematopoietic malignancies. Of those, five are BCL-2 targeting drugs, including the Pan-BCL-2 inhibitors GX15-070 (obatoclax) [97] and AT-101 (Gossypol) [98], the BCL-2 specific drugs BCL201 [99] and ABT-199 (venetoclax) [100] and the BCL-2/BCL-X_L/BCL-w inhibitor ABT-263 (navitoclax) [101].

The pharmaceutical companies AbbVie and Idun developed the first BH3-mimetic ABT-737 [102]. With high affinity for BCL-2, BCL-X_L and BCL-W, this inhibitor was shown to kill primary cells from patients with B-cell malignancies [103] and, in preclinical studies, presented efficacy on eliminating small cell lung cancer cells (SCLC) and other blood cancers [104]. Unfortunately, ABT-737 was not bioavailable after oral administration, leading to the development of an orally available drug ABT-263 or Navitoclax [101]. In preclinical studies ABT-263 showed efficacy as both a single and combined treatment for Chronic Lymphocytic Leukemia (CLL) [105], SCLC [106,107], triple negative breast cancer [108], and several other cancers. However, *in vivo* preclinical studies revealed that inhibition of BCL-X_L by ABT-737 and ABT-263 promoted cell death of platelets and immature B and T cells [109,110]. This cytotoxicity was confirmed in Phase I clinical trials where CLL and SCLC patients treated with ABT-263 developed thrombocytopenia [105]. These results limited the progression of navitoclax for the treatment of cancer. Importantly, the subsequent development of a highly specific BCL-2 inhibitor ABT-199 or Venetoclax, allows the elimination of cancer cells with less induced thrombocytopenia [100]. In clinical trials, Venetoclax was well-tolerated in chemotherapy refractory CLL patients [111]. These results led to the FDA approval of Venetoclax/ABT-199 in 2016, for the treatment of CLL and Small Lymphocytic Lymphoma (SLL).

More recently, in combination with other anti-cancer drugs or non-chemotherapies, ABT-199 has been approved for untreated CLL patients and newly diagnosed AML patients who are unable to receive intensive induction chemotherapy [112]. Venetoclax combined therapy in other cancers, including CML, has been explored and will be further discussed in this review. Despite the clinical success of ABT-199, recent studies show an intrinsic resistance to this drug in leukemia patients. Although Venetoclax resistance has been strongly linked to molecular deregulation of BCL-2 family of proteins including, increased expression of prosurvival proteins BCL-xL and MCL-1, acquired mutations and amplifications in BCL-2 protein and decrease in one or more pro-apoptotic proteins [113–115], recent studies have also shown that loss of TP53 and overactivation of signaling pathways such as FLT3 or Ras may also be important markers for venetoclax treatment response [116].

The evidence that MCL-1 is involved in disease relapse and that it has an important role in the growth and survival of malignant cells from several types of cancer [10,117–121], led to the generation of potent MCL-1 inhibitors. The MCL1 inhibitor S63845, from the pharmaceutical company Servier, was one of the first anti-MCL-1 drugs to provide insights into the efficacy of MCL-1 targeting for the elimination of cancer cells [122,123]. Anti-tumor activity of S63845 was reported in leukemia, multiple myeloma, lymphoma, SCLC, prostate and breast cancer [122,124–126]. To date, six MCL-1 inhibitors have entered phase 1 clinical trials S64315/MIK665 [127] (NCT02992483, NCT02979366, NCT03672695), AZD5991 [128] (NCT03218683, NCT03013998), ABBV-467 (NCT04178902), PRT1419 [129] (NCT05107856, NCT04837677, NCT04543305) and AMGEN MCL-1 inhibitors AMG-176 [130] (NCT 03797261, NCT05209152, NCT02675452) and

AMG-397 [131] (NCT03465540). Despite the AMGEN compounds, all the other MCL-1 inhibitors are currently being evaluated for their safety, tolerability in Phase I clinical trials in recurrent hematologic malignancies including AML, multiple myeloma and lymphoma (NCT02992483; NCT02979366; NCT04178902 and NCT04543305). The MCL-1 inhibitors AMG-176 and AMG-397 were also in clinical trials for relapse or refractory MM or AML. However due to cardiotoxicity effects the trials have been suspended. Finally, preclinical and clinical studies combining two BH3-mimetics such as Venetoclax and MCL-1 inhibitors (NCT03672695; NCT03218683; NCT02675452) [132,133] may also provide further evidence on the most effective treatment for recurrent malignancies.

BH3 mimetics combined therapy in CML

The discovery that CML cell survival and resistance to TKIs is associated with deregulation of pro and anti-apoptotic BCL-2 family of proteins such as BIM, BAD BCL-2, BCL-X_L and MCL-1 [5,6,26,37,134,135], led to the investigation of BH3-mimetics for the treatment of CML.

The observation that overexpression of BCR-ABL in leukemia cell lines results in increased expression of BCL-X_L and resistance to mitochondrial-mediated apoptosis [5,6], whereas inhibition of BCR-ABL kinase leads to down-regulation of BCL-X_L and sensitization of CML chronic phase cells [26], suggest a strong involvement of BCL-X_L in disease progression and a potential target for the treatment of CML. The BH3-mimetic ABT-737, was one of the first of its kind to be tested in BCR-ABL positive leukemias. The combination of ABT-737 with INNO-46, an BCR-ABL tyrosine kinase inhibitor (TKI), increased the expression of pro-apoptotic BH3-only proteins (Bim, Bad, Bmf and Bik) and sensitized Philadelphia-positive (Ph(+)) leukemias to intrinsic apoptosis [34]. Studies using ABT-263, another BCL-X_L antagonist, have also shown that inhibition of BCL-X_L combined with activation of BAD increased apoptosis in CML blast crisis cell lines [136].

Table 1 Clinical trials involving BH3 mimetics for BCR-ABL1-positive leukemia

Clinical trial	Study	Start date	Treatment	Status
Phase 1 NCT00438178	Obatoclax mesylate, safety and efficacy in hematological Malignancies (AML, MDS, CML in myeloid blast phase, myelofibrosis, CLL and Aplastic Anemia)	October 2005	Obatoclax	Completed
Phase 1 NCT00933985	Study of side effects and best dose of obatoclax in combination with chemotherapies for young patients with relapse/refractory solid tumors and leukemias, including blastic Phase and childhood CML	June 2009	Obatoclax + Vincristine, Doxorubicin, Dexrazoxane	Terminated
Phase 2 NCT02115295	Cladribine plus Idarubicin plus cytarabine plus venetoclax in patients with blastic phase chronic myeloid leukemia (BCR-ABL1 positive) or AML or MDS.	May 2014	Cladribine, Cytarabine, Gilteritinib, Idarubicin, Midostaurin, Venetoclax	Recruiting
Phase 2 NCT02689440	Dasatinib and Venetoclax in Treating Patients with Philadelphia Chromosome Positive or BCR-ABL1 Positive Early CML	February 2016	Dasatinib, Venetoclax	Recruiting
Phase 1/2 NCT03576547	Venetoclax, Ponatinib, and Dexamethasone in Participants with Philadelphia Chromosome or BCR-ABL Positive Relapsed or Refractory ALL or CML	July 2018	Dexamethasone, Ponatinib Hydrochloride, Venetoclax	Active, not recruiting
Phase 2 NCT04188405	Decitabine, Venetoclax, and Ponatinib for the Treatment of Philadelphia Chromosome-Positive AML or Myeloid Blast Phase or CML Accelerated Phase	December 2019	Decitabine, Venetoclax, Ponatinib	Recruiting
Phase 2 NCT05433532	Azacitidine, Venetoclax and Flumatinib in newly diagnosed Ph-positive Acute Leukemia and CML accelerated phase or blast phase	June 2022	Azacitidine, Venetoclax, Flumatinib	Recruiting

CML, chronic myeloid leukemia; CLL, chronic lymphoid leukemia; AML, acute myeloid leukemia; MDS, myelodysplastic syndromes; ALL, Acute Lymphoblastic leukemia.

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Although BCL-X_L appears to be the anti-apoptotic BCL-2 protein mostly affected by BCR-ABL, deregulation of anti-apoptotic protein BCL-2 have also been associated with CML TKI resistance [134,137,138]. BCR-ABL TKIs such as Imatinib and Dasatinib were shown to be efficient in inducing cell death of newly CML cells, they are inactive against quiescent CML stem cells, leading to disease relapse is a common feature once treatment has stopped [139,140]. When compared with normal hematopoietic cells, CML-LSC and blast crisis CML cells show increased expression of anti-apoptotic BCL-2 [134]. Inhibition of BCL-2 by sabutoclax, a pan-BCL-2 antagonist, sensitized CML-LSC to low doses of TKI, suggesting that the combination treatment of TKI plus BCL-2 inhibitors may eliminate CML-LSC, reduce disease relapse and prolong patients survival [134]. Furthermore, *in vivo* studies have shown that co-treatment of TKIs and the clinical BCL-2 inhibitor Venetoclax, reduced the engraftment capacity of CML-LSC and prolonged survival in a mice CML model [138]. Importantly, recent studies have also shown that CD26-target, immunoliposome loaded with Venetoclax, selective induced apoptosis of CML-LSCs sparing normal HSCs [141].

Whilst BCL-2/BCL-X_L inhibitors provided promising results in inducing apoptosis in BCR-ABL(+) CML cell lines and primary cells and prolonged the survival of CML mouse models, their effectivity appear to be enhanced limited in leukemic cells harboring ABL kinase mutations such as T3151 [142]. This resistance could be overcome by addition of an MCL-1 inhibitor, suggesting that multi-targeting of BCL-2 proteins may be required for eradication of the disease. Indeed, a recent study shows that dual-inhibition of BCL-2 anti-apoptotic proteins dramatically increases the killing of CML cell lines and blast-phase CML primary cells [143]. The investigation of pan-BH3-mimetic in CML has also been extended to combined therapy with alternative chemotherapeutic agents such as Paclitaxel, used to overcome TKI primary and secondary resistance in CML [144]. While TKI resistance has been strongly linked to overexpression of BCL-2 and BCL-X_L, studies using CML-derived cell line K562 show that Paclitaxel resistance is associated with overexpression of anti-

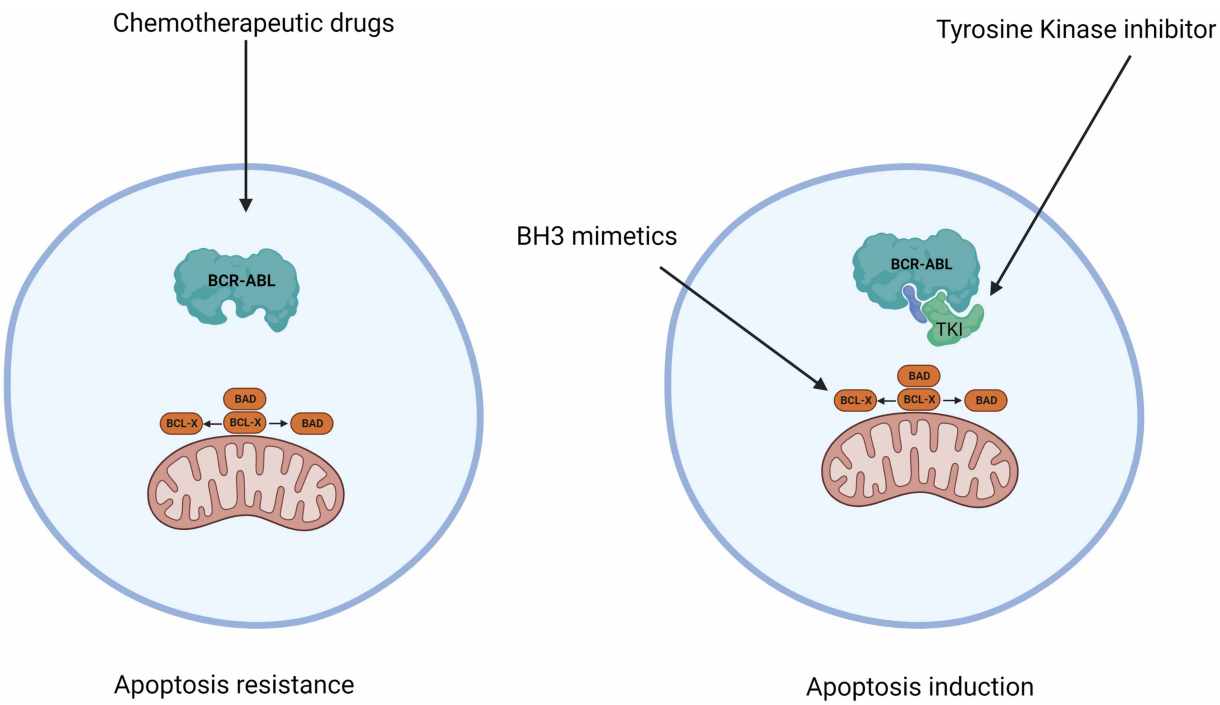

Figure 4. Graphic overview of BCR-ABL1-induced resistance to cell death and the proposed effect of BH3 mimetics and TKI combined therapy for Chronic Myeloid Leukemia.

Expression of BCR-ABL1 induces a complex signaling cascade that confers resistance to apoptosis, including down-regulation of pro-apoptotic and up-regulation of anti-apoptotic Bcl-2 family members, which control the mitochondrial/intrinsic pathway of apoptosis. TKIs may stop signals emanating from BCR-ABL1 kinase but do not interfere with BCR-ABL1-independent resistance to cell death that emerges during the advanced phases of the disease. Combination of TKI and BH3 mimetics provides synergistic death-inducing signal that can be used to eliminate leukemic cells in CML patients.

apoptotic protein MCL-1. Treatment of TKI/Paclitaxel resistant CML cell lines with the pan-BH3-mimetic S1, a BCL-2/BCL-X_L/MCL-1 inhibitor, could induce apoptosis in leukemic cells. These results once again suggest that the combination of TKI and inhibitors of BCL-2/BCL-X_L/MCL-1 may provide a more effective therapy strategy for CML patients.

The strong evidence that BCL-2 family of proteins are associated with cell death resistance and poor response to TKI treatment, led to clinical trials combining BH3-mimetics and TKI inhibitors clinical trials. Four phase II trials combining Venetoclax with different TKI are in progress (Table 1). The comparison between Dasatinib single therapy and combination treatment Dasatinib/Venetoclax (NCT02689440) is being investigated for the treatment of BCR-ABL1-positive early chronic phase CML patients. The efficacy of venetoclax triple therapy Decitabine/ Venetoclax/ Ponatininb (NCT04188405) Azacitidine/Venetoclax/Flumatinib (NCT05433532) and Venetoclax/Ponatinib/Dexamethasone (NCT03576547) are being investigated for the treatment of Ph^+ AML, accelerated phase or blast CML patients, and BCR-ABL1-positive relapse or refractory ALL and CML, respectively.

Indubitably, the results obtained from these clinical studies will provide further and critical information about the efficacy of BH3-mimetics in CML. Notwithstanding, if the promising preclinical results are correct, is predicted that BH3-mimetic combination therapies will overcome TKI resistance, improve elimination of CML stem cells and prolong survival of CML patients (Figure 4).

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Gustavo P. Amarante-Mendes: Conceptualization, Resources, Funding acquisition, Writing — original draft, Writing — review and editing. **Gabriela Brumatti**: Conceptualization, Resources, Funding acquisition, Writing — original draft, Writing — review and editing. **Deeksha Kaloni:** Writing — original draft, Writing — review and editing. **Fabíola Attié Castro:** Conceptualization, Resources, Funding acquisition, Writing — original draft, Writing — review and editing.

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Abbreviations

BH, BCL-2 homology; BM, bone marrow; BMM, bone marrow microenvironment; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; EZH2, enhancer of zeste homolog 2; FDA, Food and Drug Administration; IM, Imatinib mesylate; KD, kinase domain; LSC, leukemic stem cells; MOMP, mitochondrial outer membrane permeabilization; PRAME, preferentially expressed antigen of melanoma; PRC, polycomb repressive complex; SCLC, small cell lung cancer cells; SIRT1, sirtuin 1; TKIs, tyrosine kinase inhibitors.

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