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Hematopoietic Stem Cells in Chronic Myeloid Leukemia

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1. Introduction

Chronic Myeloid Leukemia (CML) is a clonal disease, originated at the level of Hematopoietic Stem Cells (HSC) and characterized by the presence of the *Philadelphia* (Ph) chromosome and its oncogenic product p210Bcr-Abl. Such a protein has been shown to be essential for malignant transformation, since it is capable of altering cell adhesion, proliferation and apoptosis.

Current treatment options in CML include tyrosine kinase inhibitors (Imatinib, Nilotinib and Dasatinib), compounds that inhibit the activity of the BCR-ABL protein. However some patients will develop resistance or intolerance to these drugs and resistance has been associated with different mechanism including the quiescence of leukemic stem cells and Pgp or Src kinase overexpression.

In this chapter we focus on the basic biology of hematopoietic stem and progenitor cells from CML and analyze the most relevant and current concepts in this area.

2. Chronic myeloid leukemia

Chronic myeloid leukemia (CML) is a lethal hematological malignancy characterized by the abnormal amplification of the myeloid (mainly granulocityc) compartment of the hemato-poietic system. It originates from the transformation of a primitive hematopoietic cell that suffers a t(9;22) (q34; q11) balanced reciprocal translocation that results in the generation of the Philadelphia chromosome (Ph). Ph produces BCR-ABL, a constitutively active tyrosine kinase that drives a wide variety of physiological alterations [1].

CML was initially described in 1845 by John Hughes Bennett, who reported the case of a patient with "milky" blood and suggested that it was an infectious disease that caused hypertrophy of the liver and spleen, leading to the patient's death. A few weeks later, Rudolf Virchow reported a similar case, but, in contrast to Bennett, he suggested that the disease was not infectious and implied an increase in the number of blood cells. He coined the term leukemia (from the Greek *leukos*, white, and "Aemia", blood). In 1870, Neumann described that leukemia cells originate in the bone marrow; almost one hundred years later, in 1960, Nowel and Hungerford reported that in all cases of this malignancy there was a small, abnormal chromosome 22. However, was until 1973 that Janet Rowley described that the abnormal chromosome was caused by a reciprocal translocation between the long arms of chromosomes 9 and 22, designating the name of Philadelphia (Ph) chromosome[2, 3].

## 2.1. Epidemiology and clinical characteristics

Chronic myelogenous leukemia has a worldwide incidence of 1-2 cases per 100,000 individuals [4]. The average age at diagnosis is 60 years; it occurs less frequently in young people and a tendency to increase exponentially with age has been observed. There is no geographic or genetic predisposition to acquire this condition, although some authors have associated it with exposure to high doses of ionizing radiation. The current CML prevalence of 24,000 affected patients in the United Sates is relatively low; it is expected to increase significantly over the next 20 years as a result of widespread use of BCR-ABL tyrosine kinase inhibitor therapy [5]. In Mexico, there are no official data on the incidence of such a disease, however, it has been estimated that there are about 80,000 cases of leukemia and 10% corresponds to CML [6].

The clinical presentation often includes granulocytosis, spenomegaly and marrow hypercellularity; however about 40% of patients are asymptomatic and their diagnosis is based on abnormal blood cell counts [1]. The natural course of the disease involves three sequential phases, namely chronic, accelerated and blast crises. Ninety percent of patients are diagnosed in chronic phase and they remain in it for 3 to 8 years. In this phase, the blood cells retain their ability to differentiate until the illness progresses to the accelerated phase, which is characterized by the egress of immature cells into the bloodstream. Finally, the disease progresses to the blast crisis, defined by the presence of 30 percent or more leukemic cells in peripheral blood or marrow or extramedullary infiltrates of blast. During this phase the survival of patients is reduced to months and even weeks [7].

2.2. Molecular events (Bcr-Abl oncogene)

As mentioned before, the Philadelphia chromosome, which defines CML, is a shortened chromosome 22 originated from the reciprocal translocation between the long arms of chromosomes 9 and 22 [t (9; 22)] and involves addition of 3' segments of the *abl* gene (9q34) to 5' segments of the *bcr* gene (22q11) given rise to a *bcr-abl* fusion gene that transcribes a chimeric mRNA of 8.5 kb that, in turn, gives rise to a BCR-ABL fusion protein [7]. t(9;22) is evident in more than 95% of CML patients; between 5% and 10% of CML patients also present complex rearrangements that may involve one or more chromosomes in addition to 9 and 22 [8].

The normal human ABL gene encodes for a non-receptor tyrosine kinase that is ubiquitously expressed. Such a 145 kDa protein is involved in the regulation of the cell cycle, the response to genotoxic stress, and intracellular signaling mediated by the integrin family [9]. There are three isoforms of the BCR-ABL fusion protein all of which encode the same portion of the ABL tyrosine kinase, but differ in the length of the BCR sequence at the N-terminus. p185/p190 BCR-ABL is expressed in Acute Lypmphoblastic Leukaemia (ALL), p210 BCR-ABL is characteristic of Chronic Myeloid Leukemia, and p230 BCR-ABL has been associated with a subgroup of CML patients with a more indolent disease (Figure 1) [4].
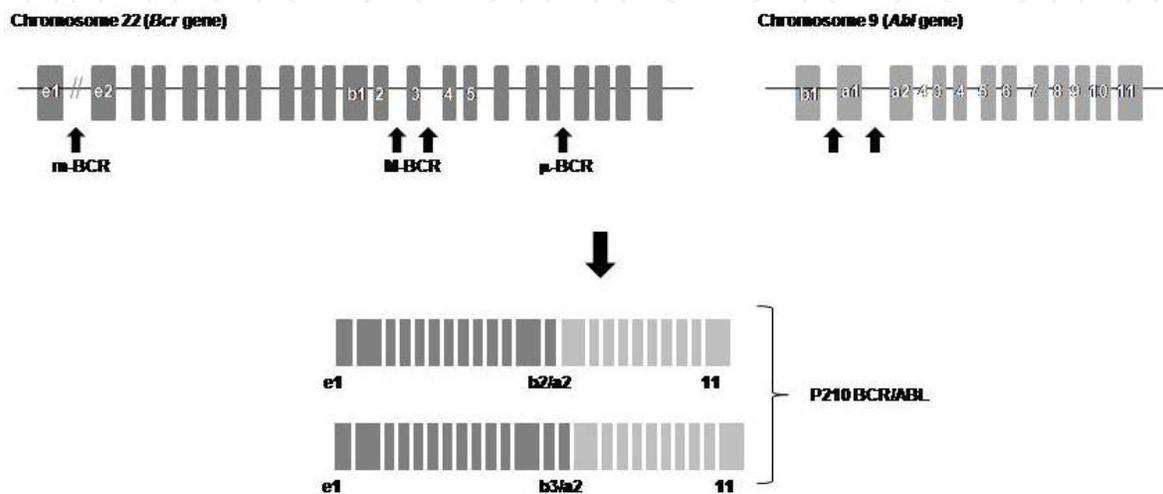
Figure 1. Structure of the Bcr-Abl gene. It is formed by a reciprocal translocation between chromosomes 22 (Bcr gene) and 9 (Abl gene). Ther M-BCR breakpoint resulting in a P210 BCR/ABL fusion transcripts b2a2 or b3a2 and they encode a protein of 210 kDa (BCR-ABLp210) present in almost all patients with Chronic Mieloid Leukemia (modified to [9]).

BCR-ABL fusion protein localizes in the cytoplasm and shows an increased and constitutive tyrosine kinase activity as a result of oligomerization of its coiled region and deletion of the SH domain of ABL. It activates a number of cytoplasmic and nuclear signal-transduction pathways involved in cell adherence, migration, inhibition of apoptosis, and induction of cell proliferation through activation of signaling proteins such as p21RAS, c-Myc, lipid kinasse PI3k, MAPk (mitogen-activated protein kinase family), tyrosine phosphatases, and signal transducer and activator of transcription (STATs) factors [9, 10].

2.3. Leukemic Stem Cells in chronic myeloid leukemia

There is an increasing body of evidence indicating that, similar to normal hematopoiesis, a quiescent stem cell population -within the CD34+ cell compartment- exists in the bone marrow of CML patients. Such Leukemic Stem Cells (LSC) seem to be the ones driving CML progression, following a similar pattern to the one observed in normal hematopoiesis. That is to say, LSC give rise to CML progenitor cells, which, in turn, give rise to more mature cells.

Just like normal hematopoietic stem cells (HSC), CML stem cells express high levels of CD34, and lack the cell surface markers CD38, CD45RA, or CD71, as well as lineage-specific markers.

However, LSC are Ph+/BCR-ABL+, which is not present in their normal counterparts. Interestingly, it has recently been shown that a novel population of lineage-negative, CD34-negative hematopoietic stem cells from CML patients also correspond to BCR-ABL+ leukemic stem cells capable to engraft immunodeficient mice [13]. Thus, it seems that most LSC are CD34+ but a subpopulation may be CD34-. Importantly, despite the predominance of LSC in CML, a residual population of normal hematopoietic stem cells (BCR-ABL- CD34+) persists in the marrow's patient, which seems to be responsible for hematopoietic recovery after a successful treatment using Tyrosine Kinase Inhibitors (TKIs).

As mentioned before, LSC are in a quiescent state, however, they can spontaneously exit G_0 to enter a proliferating state and are capable of engrafting inmunodeficient mice [11]. In this regard, several studies have shown that TKIs, like Imatinib, Nilotinib, Dasatinib, Bosutinib, and Lonafarnib, have antiproliferative or apoptotic effects in almost all dividing CML cells; however, the population of stem cells remains viable in a quiescent state [16-21].

In vitro studies indicate that LSCs are capable of surviving for several weeks in the absence of added growth factors due to autocrine mechanisms involving production of granulocyte colony-stimulating factor (G-CSF) and Interleukin 3 (IL-3) [12]. This, in fact, is an important difference between normal and CML HSC, since the former depends on the presence of exogenous cytokines for their growth, whereas the latter, as just mentioned, can utilize autocrine mechanisms. Although there is strong evidence that Bcr-Abl is sufficient to induce CML-like disease in transduction and transgenic murine models [14], it is still unclear whether Bcr-Abl is always the first hit in CML, since in some patients with a complete cytogenetic response after treatment, BCR-ABL transcripts are still detectable by RT-PCR, which indicates that leukemic cells persist even when the disease is reduced below detectable limits [15].

3. Functional characteristic of leukemic stem cells in CML

3.1. Proliferation

Proliferation of leukemic stem and progenitor cells is regulated by Bcr-Abl. Such a tyrosine kinase activates the Ras/Raf/MEK/ERK and JAK/STAT signal transduction pathways, and this results in an amplified proliferative state [22]. Bcr-Abl causes hyperactivity of Ras, Raf and JAK/STAT, which can occur by multiple mechanisms; i.e., by Bcr-Abl activating these pathways directly, or by the induction of autocrine cytokines, which in turn activate these pathways [23]. Bcr-Abl autophosphorylation of tyrosine 177 provides a docking site for the adapter molecule Grb-2. Grb-2, after binding to the Sos protein, stabilizes Ras in its active GTP-bound form. Two other adapter molecules, Shc and Crkl, can also activate Ras [9, 24]. Ras activates Raf, and finally, Raf initiates a signaling cascade through the serine–threonine kinases Mek1/Mek2 and Erk, which ultimately leads to the transcription of genes involved in cell proliferation and survival (Figure 1), such as c-Myc, Cyclin D, Cyclin A, Bcl-2, cytokines, etc [22].

The JAK/STAT pathway has been demonstrated to be constitutively activated In CML. Among all the molecules participating in these pathways, STAT1 and STAT5 have been found to be

the two major STATs phosphorilated by Bcr-Abl. STAT5 has pleiotropic physiologic functions, and its main effect in Bcr-Abl-transformed cells appears to be primarily anti-apoptotic, involving transcriptional activation of Bcl-xL [25]. Also, in some experimental systems there is evidence that Bcr-Abl induces an IL-3 and G-CSF autocrine loop in early progenitor cells [12].

3.2. Inhibition of apoptosis

Leukemic Stem Cells acquire the ability for long-term survival primarily by deregulation of apoptosis. In CML, blocking of apoptosis is mediated by Bcr-Abl. Bcr-Abl may block the release of cytochrome C from mitochondria and thus activation of caspases. This effect upstream of caspase activation might be mediated by the Bcl-2 family of proteins [26]. Bcr-Abl has been shown to up-regulate anti-apoptotic protein Bcl-xL in a STAT5-depend manner, as mention above [27]. Another link between Bcr-Abl and the inhibition of apoptosis might be the phosphorylation of the pro-apoptotic protein Bad through PI3k pathway. Bcr-Abl forms multimeric complexes with PI3 kinase, Cbl, and the adapter molecules Crk and Crkl, in which PI3 kinase is activated. The next substrate in this cascade appears to be the serine-threonine kinase Akt. This kinase had previously been implicated in antiapoptotic signaling and protein Bad as a key substrate of Akt (Figure 1). Phosphorylated Bad is inactive because it is no longer able to bind anti-apoptotic proteins such as Bcl-xL and it is trapped by cytoplasmic 14-3-3 proteins [28].

3.3. Altered adhesion properties

In CML, progenitor cells exhibit decreased adhesion to bone marrow stroma cells and extracellular matrix. From this point of view, adhesion to stroma negatively regulates cell proliferation, and CML cells escape this regulation by virtue of their perturbed adhesion properties. Bcr-Abl directly phosphorylates Crkl, a protein involved in the regulation of cell motility and in integrin-mediated cell adhesion by association with other focal adhesion proteins such as paxillin, the focal adhesion kinase Fak, p130 Cas and Hef1 [29, 30] (Figure 1). In addition to this, it has been demonstrated that the activity of Bcr-Abl promotes expression of integrin β1, a variant not found in the normal counterpart that inhibits adhesion to stroma and cell matrix, together with the effect of expansion and premature exit of myeloid progenitors and precursors to bloodstream [31].

3.4. Self-renewal

Deregulation of self-renewal has been recognized as an important event in disease progression. In normal hematopoietic stem cells, self-renewal capacity involves several signaling pathways: Notch, Wnt, Sonic Hedgehog (Shh), FoxO and Alox5 [32-34].

Notch pathway

Notch receptors are an evolutionarily conserved family of trans-membrane receptors that are known to be expressed and activated in normal HSC. Binding to their physiological ligands, which are part of the Delta and Serrata families, leads to separation of an intracellular portion of Notch. This fragment is capable of entering the nucleus where it binds transcriptional

repressor CBF-1. Interconnection of Notch, CBF-1 and the co-factor MAML-1 (mastermind-like-1) leads to transcriptional activation of target genes [35]. Constitutively active Notch is able to mediate multilineage potential *in vivo*. Differentiation of cells leads conversely to downregulation of Notch [36].

Notch signaling may also be important in advanced stages of CML. Hes1, a key Notch target gene, was found to be highly expressed in 8 out of 20 patients with CML in blast crisis, but was not seen in the chronic phase. In mice, the combination of Hes1 and BCR-ABL expression in myeloid lineage progenitor cells resulted in an acute leukemia resembling blast crisis CML [37]. This suggests that Notch inhibitors may be useful in strategies aimed at eradicating CML LSC.

Wnt pathway

In normal hematopoiesis, Wnt pathway activity is required in the bone marrow niche to regulate HSC proliferation and to preserve self-renewal capacity [38]. Activation of the canonical Wnt/β-catenin pathway consists of binding of Wnt proteins to members of the Frizzled and low-density lipoprotein receptor related (LPR) families on the cell surface. In the absence of Wnt signals, β-catenin is associated with a large multiprotein complex that includes Axin, APC, and glycogen synthase kinase 3β (GSK3β), among others. Through a mechanism not entirely understood, when Wnt proteins bind to their target, Axin facilitates phosphorylation of β-catenin by GSK3β. Phosphorylation, in turn, results in ubiquitination, targeting β-catenin for degradation. Thus, axin serves as an inhibitor of β-catenin activity. Binding of Wnt proteins to their receptors leads to activation of Dis-shevled (Dsh), which inhibits phosphorylation of β-catenin by GSKβ, so it accumulates in the cytoplasm and translocates to the nucleus, where it activates transcription factors, such as LEF/TEF and allows expression of target genes [39].

This pathway has been implicated in CML. Indeed, in blast crisis CML, the LSC, which resemble granulocyte-macrophage progenitor cells (GMP), have aberrant activation of β-catenin via the canonical Wnt signaling pathway. In a proportion of these cases, the pathway is activated through abnormal missplicing of GSK3β [40].

Sonic Hedgehog (Shh) pathway

The Hedgehog (Hh) pathway is a highly conserved developmental pathway, which regulates the proliferation, migration and differentiation of cells during development [41]. It is typically active during development, but silenced in adult tissues, except during tissue regeneration and injury repair [42]. Three distinct ligands, i.e., Sonic (Shh), Indian (Ihh) and Desert (Dhh) Hedgehog exist in humans. Upon ligand binding to the receptor patched (Ptch), inhibition of smoothened (Smo) receptor is relieved. Smo then activates members of the Gli family of zinc-finger transcription factors, which translocate to the nucleus to regulate the transcription of Hh target genes, including Gli1, Gli2, Ptch and regulators of cell proliferation and survival [43].

Based on murine embryonic stem cell studies, it has been found that Hh signaling plays major roles during primitive hematopoiesis. Ihh is a primitive endoderm-secreted signal and is sufficient to activate embryonic hematopoiesis and vasculogenesis [44]. Further-

more, a study of zebrafish showed that the mutations of the Hh pathway members or in-hibition of the Hh pathway with the Hh inhibitor cyclopamine can cause a developmental defect in adult HSC [45]. In addition, activation of Hh pathway has been observed in different human cancers. In CML patients, more than four-fold induction of the transcript levels of Gli1 and Ptch was observed in CD34+ cells in both chronic phase and blast crisis. In two studies using a CML mouse model, recipients of the Bcr-Abl transduced bone marrow cells from Smo-/- donor mice developed CML significantly slow-er than recipients of Bcr-Abl transduced bone marrow cells from wild-type donor mice. When the frequency and function of the LSCs were examined, Smo deletion caused a significant reduction of the percentage or LSCs [46]. By contrast, over expression of Smo led to an increased percentage of LSC and accelerated the progression of CML [47].

FoxO pathway

The FoxO (Forkhead-O) subfamily of transcription factors regulate cell cycle, stress resistance, differentiation, and long-term regenerative potential of HSC [48], and protect integrity of the stem cell pool. There are four members (FoxO1, FoxO3, FoxO4 and FoxO6) and are known to be effectors of the PI3k/AKT pathway, which is frequently mutated or hyperactivated in hematologic malignancies, and are abundantly expressed in the hematopoietic system. Akt directly phosporylates the FoxO members from the nucleus and promotes its degradation in the cytoplasm. FoxO members localize to the nucleus and regulate apoptosis, cell cycle progression and oxidative stress responses [49]. In a model of deficient FoxO mice it was shown a defect in the long-term expansion capacity of the HSC pool. Such a defect has been correlated with increased cell division and apoptosis of HSCs.

FoxO transcription factors have also been shown to have essential roles in the maintenance of CML LSCs [50]. FoxO3 localizes to the cell nucleus and it causes a decrease in Akt phosphor-ylation in the LSC population. In addition, serial CML transplantation showed that FoxO3 deficiency severely impairs the ability of LSCs to induce CML. Furthermore, transforming growth factor-β (TGF-β) is a crucial regulator of Akt activation and controls FoxO3 localization in LSCs of CML. A combination strategy of TGF-β inhibition, FoxO3 deficiency and Bcr-Abl kinase inhibition results in efficient LSCs depletion and suppression of CML development [51].

Alox5 pathway

The Alox5 pathway is the only one signaling pathway not shared by LSC with normal HSC. The *Alox5* gene encoding arachidonate 5-lipoxygenase (5-LO) is involved in numerous physiological and pathological processes, including oxidative stress response, inflammation and cancer [52]. 5-LO is responsible for producing leukotrienes, a group of inflammatory substances that cause human asthma [53]. Altered arachidonate metabolism by leukocytes and platelets was reported in association with myeloproliferative disorders [54]. Several selective 5-LO inhibitors were found to reduce proliferation and induce apoptosis of CML cells in vitro [55]. Recently, human CML microarray studies have shown that Alox5 is differentially expressed in CD34+ CML cells suggesting a role for Alox5 in human CML stem cells. However, the function of Alox5 in LSCs needs to be tested. Other microarray analysis of gene expression in LSCs in CML mice showed that the ALox5 gene was up-regulated by Bcr-Abl and that this

up-regulation was not inhibited by Imatinib treatment, providing a possible explanation of

why LSCs are not sensitive to inhibition by Bcr-Abl kinase inhibitors [56].
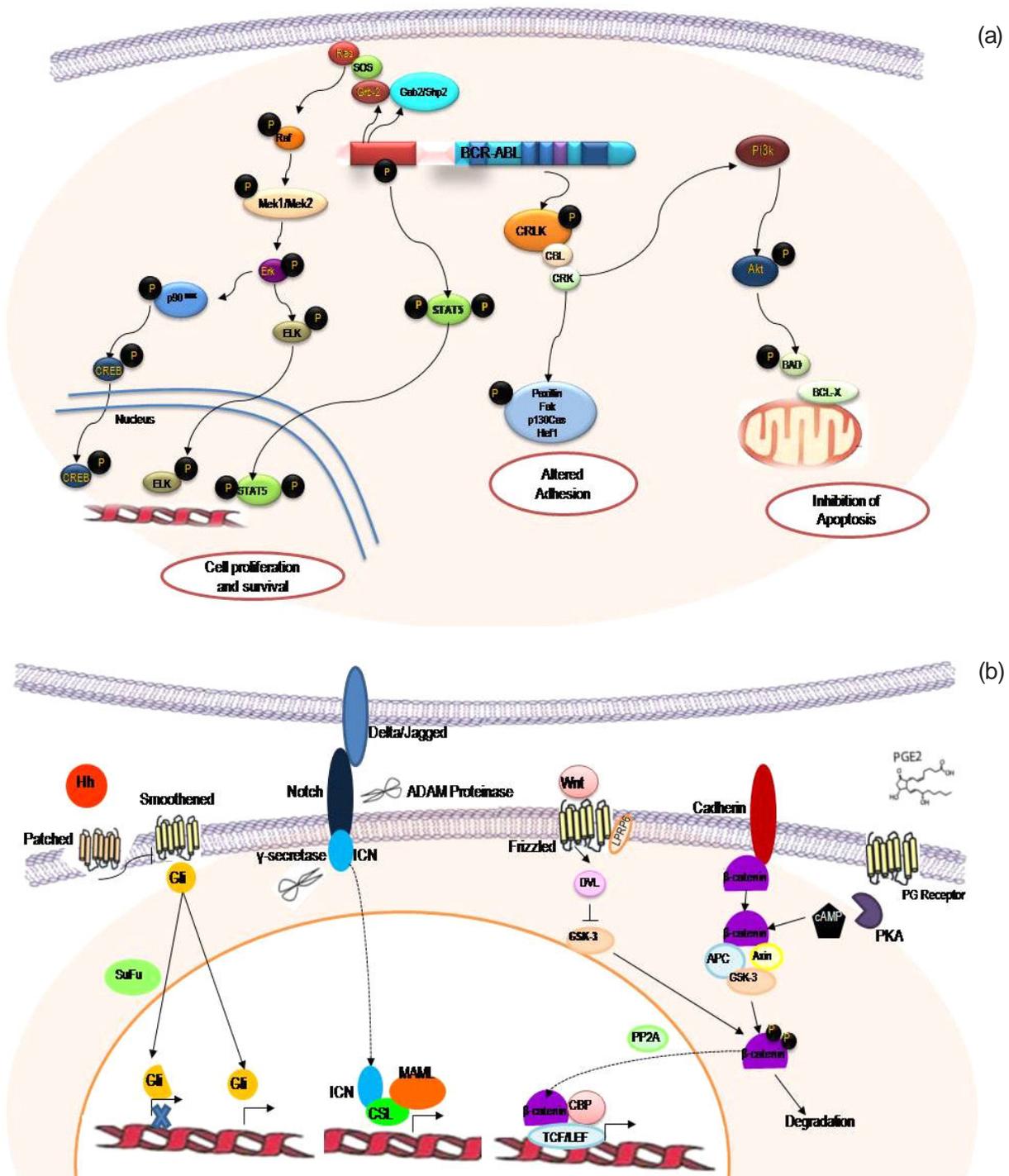
Figure 2. Signaling pathways involved in the signaling of BCR-ABL. A) Schematic representation of principal molecules that participate in proliferation, adhesion and apoptosis. B) Pathways involved in self-renewal.

4. Current therapies

The first effective treatment for CML was the solution of Fowler's, which contained arsenic as active component and was used in the early 20th century. Later between 1920 y1930 irradiation to the spleen was the main therapeutic option, since it offered patients the decrease of symptoms, although it did not prolong their lives. In 1953, busulfan was included in CML treatment. This compound provided benefit in terms of survival, although it was shown to be extremely toxic for hematopoietic progenitor cells. The next drugs effective in the treatment of CML were hydroxyurea and cytosine arabinoside, both less toxic than busulfan and able to block proliferation of cells, but unable to induce specific damage to leukemic cells; thus, patients usually progressed to the accelerated and blast crisis phases [57].

4.1. Interferon-α

Interferon-α (IFNα) was the first drug capable of extending the chronic phase of the disease and retarding the evolution to the accelerated phase. IFNα is a nonspecific stimulant of the immune system that regulates T-cell activity and produces a complete hematologic response (CHR) in 40-80% of patients, and a complete cytogenetic response (CCR) in 6-10% of patients with a median survival of 89 months [58].

In vitro studies have indicated that IFNα might function via selective toxicity against the leukemic clone, since it is able to inhibit long-term cultures from patients with CML in chronic phase and reduces the percentage of Ph+ cells [59]. It also inhibits CML myeloid progenitors while sparing normal myeloid progenitors [60]. In vivo, IFNα enhances immune regulation through the activation of dendritic, natural killer, and cytotoxic T cells, all of them capable of generating anti-tumor responses. In Bcr-Abl+ cells, IFNα induces a state of tumor dormancy and delays progression to advanced phase [61], and is able to modulate hematopoiesis through enhanced adhesion of CML progenitor cells to stromal cells, whereas adhesion of normal progenitors was unaffected. This enhanced adhesion by CML progenitor cells has been associated with a reduction in neuraminic acid levels and by enhanced hematopoietic cell-microenvironmental cell interactions, which is achieved by the induction of molecules such as β2-Integrin, L-selectin, ICAM-1 and ICAM352 [58, 62].

Because IFNα is a nonspecific immunostimulant, it produces secondary symptoms and toxicities and many patients discontinue therapy. However there are evidence that a significant proportion of IFNα-treated patients in prolonged CCR were able to discontinue treatment without disease relapse [63], and it was recently reported that in a specific group of patients treated with monotherapy there are increased numbers of NK cells and clonal $\gamma\delta$ T cells [64].

4.2. Tyrosine kinase inhibitors

Having identified that tyrosine kinase activity of Bcr-Abl is a major factor in the pathophysiology of CML, it was clear that such a molecule was an attractive target for designing a selective kinase inhibitor. In 1996, Buchdunger et al, synthesized several compounds that inhibit the activity of platelet-derived growth factor receptor (PDGF-R) and ABL kinase. One of these was

the 2- phenylaminopyrimidine, which served as a starting point for the development of other related compounds [65]. The activity of the 2-phenylaminopyrimidine series was optimized and gave rise to STI571 (also named imatinib mesylate, CGP57148B or Gleevec®, Novartis Pharmaceuticals).

Imatinib

Imatinib is a highly selective inhibitor of the protein tyrosine kinase family, which includes BCR-ABL protein, PDGF-R and the c-kit receptor. It competitively binds to the ATP-binding site of BCR.ABL and inhibits protein tyrosine phosphorylation *in vitro* and *in vivo* [66]. In vitro studies had shown that Imatinib is capable to inhibit cell proliferation of cell lines expressing Bcr-Abl [67-69], effect accomplished through JAK5-STAT and PI3 kinase signaling inhibition [70, 71]. It has also been shown that STI571 can inhibit CML MNC, obtained both in chronic phase and blast crisis [71] and reduces the colony forming cells from Mobilized Peripheral Blood (MPB) and Bone Marrow from patients with CML in chronic phase [60]. Furthermore, Imatinib inhibits proliferation and cell cycle of stem (CD34+CD38-) and progenitor (CD34+CD38+) cells without altering the behavior of normal cells [72].

Studies in CML marrow by Holyoake and her colleagues have demonstrated the presence of a rare, highly quiescent, CD34+ cell subpopulation in which most of the cells are Ph+ with the ability to proliferate upon specific induction [11]. These cells are insensitive to the effects of STI571 and remain quiescent and viable even in the presence of growth factors [16]. This tumor resistance feature was also reported by Bathia, who mention that STI571 suppressed but does not eliminate primitive cells even after patients remain in CCR [73]. These primitive Ph+ cells could not be detected by nested PCR, when they are obtained from Imatinib-treated patients; however, when the cells are cultured in liquid cultures for a couple of weeks, the Ph+ population becomes detectable, indicating that they were able to remain even after Imatinib treatment [74].

In clinical trials, Imatinib has been shown remarkably effective as a single agent in IFNα-resistant CML chronic phase patients. It induces complete cytogenetic responses in more than 80% of newly diagnosed patients; however, the persistence of detectable leukemic cells in a quiescent state and the presence of patients with resistance or intolerance to Imatinib, lead to the development of a second generation of Tyrosine Kinase Inhibithors.

Nilotinib

Nilotinib (Tasigna, Novartis Pharmaceutical), is an oral aminopyrimidine that is a structural derivative of Imatinib. It was designed to be more selective against the Bcr-Abl tyrosine kinase than imatinib. Like imatinib, it acts through competitive inhibition of the ATP site in the kinase domain [75]. Clinically Nilotinib showed activity in imatinib-resistant patients in all phases of the disease. In chronic phase, it induced 92% of CHR and in accelerated phase and blast crisis the hematological responses were achieved in 72% of cases [76].

In vitro, Nilotinib is 20 times more potent than imatinib against cells expressing wild type Bcr-Abl, and similar results have been observed in studies of mutants cell lines, with the exception of the T315I mutation, which is resistant to both TKIs [77]. In primary CML CD34+ cells,

Imatinib-induced apoptosis is preceded by Bim accumulation; this effect was decreased when cells were cultured in a cytokine-containing medium [78]. In contrast to Imatinib, whose main effect on CML cells seems to be induction of apoptosis, the predominant effect of nilotinib seems to be antiproliferative -rather than apoptotic [17]. Indeed, it has been suggested that Nilotinib can induce a G_0/G_1 cell cycle blockade in cells expressing wild type Bcr-Abl, which could result in disease persistence [79].

Dasatinib

Dasatinib (Sprycel, Bristol-Myers Squibb) is a potent, orally bioavailable thiazolecarboxamide. It is structurally unrelated to imatinib; it has the ability to bind to multiple conformations of the Abl kinase domain and it also inhibits SRC family kinases. In vitro, Dasatinib demonstrated 325-fold greater activity against native Bcr-Abl, as compared with imatinib, and it has shown efficacy against all imatinib-resistant Bcr-Abl mutants with the exception of T351I. Dasatinib is also active against PDGFR, C-Kit and ephrin A receptor [75, 76].

Dasatinib is very effective at inducing apoptosis in CML cells –either, in the presence or absence of added growth factors- and in contrast to Imatinib, that kills those cells destined to move from G_0/G_1 cell cycle phases, but is unable to act on those cells destined to remain quiescent in culture, Dasatinib can act on quiescent CD34+ cells. As expected, based on its structure and mode of action, it has selective cytotoxic activity for leukemic cells over normal cells [80].

Other tyrosine kinase inhibitors

Several TKIs have been developed that exhibit a target spectrum similar to the approved drugs, although they are distinct in terms of off-target effects [81].

SKI-606 (Bosutinib)

Bosutinib (Wyeth) is a 4 anilino-3-quinolinecarbonitrile dual inhibitor of Src and Abl kinases without effect in c-Kit or PDGFR. It has 200-fold grater potency for Bcr-Abl than imatinib and has activity against a number of mutations, but not T315I [76]. In clinical trials, Bosutinib induced 73% of complete hematological response in patients pretreated with Imatinib followed by Dasatinib [82]. In vitro, Bosutinib effectively inhibits Bcr-Abl kinase activity and Src phosphorylation, and reduces the proliferation and CFC growth in CML CD34+ cells; however, it does not seem to induce apoptosis [19].

AP24534 (Ponatinib)

Ponatinib, is a mulitargeted kinase inhibitor that is active against all BCR-ABL mutants, including T315I. This drug also inhibits FLT3, FGFR, VEGFR, c-Kit, and PDGFR and is able to reduce the proliferation of different cell lines and prolong survival of mice that have been injected intravenously with BCR-ABL. Ponatinib showed significant activity in a phase I study of patients with Ph+ cells who had failed to other TKIs [81, 83].

4.3. Hematopoietic cell transplant

Although molecular therapy for CML is highly effective and generally non-toxic, it is unclear whether long-term outcomes with the different therapies (IFNα or TKIs) will be equivalent to

cases treated with allogeneic stem cell transplantation, which has shown the highest percentage of long-term disease-free survival of any therapy [75].

In patients younger than 50 years of age and who receive a transplant before 1 year after diagnosis, 5 years survival rates superior to 70% have been attained. However, the application of this procedure is limited by the availability of matched donors and by the toxicity of the procedure in older patients. Moreover, outcomes deteriorate with disease duration [76]. This information associated with the knowledge that quiescent leukemic stem cells remain in patients after treatment, several other agents has been reported.

4.4. Other agents

Danusertib (PHA 739358) is a small molecule with activity against BCR-ABL and aurora kinases and it is able to block the proliferation of leukemia cell lines as well as CD34+ cells from newly diagnosed CML patients including the mutation T315I. However, similarly to other tyrosine kinase inhibitors, no induction of apoptosis in quiescent hematopoietic stem cells could be achieved and resistant BCR-ABL positive clones emerged in the course of Danusertib treatment. This latter observation is related to Abcg2 proteins over-expression [84].

Lonafarnib (SCH66336) is an orally bioavailable non peptidomimetic farnesyl trransferase inhibitor with significant activity against Bcr-Abl+ cell lines and primary CML cells. It can enhance the toxicity of Imatinib in K562 cell line and can inhibit the proliferation of imatinib-resistant cells and increases imatinib-induced apoptosis. However it is unable to kill quiescent CD34+ leukemic cells [20]. In a clinical phase 1 study, it was shown that the combination of Lonafarnib and Imatinib is well tolerated in patients with CML who failed Imatinib, with some patients achieving a complete hematologic response and a complete cytogenetic response [85].

INNO 406 is a 2 phenylaminopyrimidine Bcr-Abl inhibitor with activity against PDGF, c-kit and Lyn that have shown to be 25-55 times more potent than Imatinib in Bcr-Abl+ cell lines. In contrast to other molecules INNO406 does not inhibit all SRC kinases, but it induces programmed cell death in chronic myelogenous leukemia (CML) cell lines through both caspase-mediated and caspase-independent pathways [86].

MK0457 is an aurora kinase inhibitor with activity against Bcr-Abl. This agent was observed to inhibit autophosphorylation of T315I mutant and demonstrate antiproliferative effects in CML cells derived from patients with this mutation, an event that may lead to its use as a combination partner with the approved and established TKI [76].

5. TKI resistance mechanisms

The knowledge of the central role of BCR-ABL in the pathogenesis of CML has allowed the development of several drugs that inhibit the constitutive activity of such an ABL tyrosine kinase. However, although the treatment with tyrosine kinase inhibitors has proven effective in about 80% of CML patients at any stage, the remaining 20% can't respond to it [87].

In CML, the criteria for successful response to treatment, as established by the European consortium LeukemiaNet and subsequently adopted by the National Comprehensive Cancer Network (NCCN) [88], include: complete hematologic remission (CHR), that is to say, a normal blood cell count and complete disappearance of signs and symptoms of the disease; complete cytogenetic response (CCR), which means the total absence of Ph+ metaphases; and complete molecular response, in which transcripts for BCR-ABL are no longer detectable. Using these response criteria, drug resistance is defined as the inability to achieve any of the following: a complete hematologic response (CHR) at 3 months, any cytogenetic response (CyR) at 6 months, partial cytogenetic response (PCyR) at 12 months, or a complete cytogenetic response (CCR) at 18 months of treatment with Imatinib [89].

Two types of resistance mechanisms to TKIs have been described: 1) Primary resistance, which occurs in less than 10% of cases and is defined as the failure of therapeutic effect during the chronic phase of CML without changing clones; and 2) secondary resistance, defined as the loss of the response initially obtained, and commonly occurs in accelerated phase (40-50%) and blast (80%) [90]

It is estimated that the probability of an individual to stay in CCR for 5 years after diagnosis, after treatment with Imatinib is approximately 63%; however, this percentage may represent a sub-estimation since in a significant proportion of cases there is discontinuation of treatment and this, of course, may underestimate the efficacy of the drug [91].

The molecular mechanisms of acquired drug resistance can be divided into two categories: BCR-ABL-dependent and BCR-ABL-independent.

5.1. Bcr-Abl-dependent resistance mechanisms

The inhibition of the activity of tyrosine kinase turned out to be an ideal target for molecular therapy in CML [67]. However, shortly after the introduction of Imatinib, in vitro studies demonstrated that some cell lines became refractory to the drug, suggesting a possible inherent or acquired resistance to therapy [92]. This was quickly followed by the clinical description of patients resistant to Imatinib.

BCR-ABL mutations

The most common mechanism against TKIs therapy are point mutations within the kinase domain, which make conformational changes that decrease the affinity of the TKIs to BCR-ABL kinase domain. These point mutations in the *BCR-ABL* kinase domain are a major cause of Imatinib resistance, and may be identified in approximately 50% or more of the cases. Many more than 100 different mutations affecting more than 70 amino acids have so far been identified, with varying degrees of clinical relevance [93].

The first point mutation reported in TKI resistance was in the region coding for the ATP-binding site of the ABL kinase domain resulting in a threonine to isoleucine substitution at amino acid 315 (Th315→Ile315; T315I) preventing the formation of a hydrogen bond between the oxygen atom provided by the side chain of threonine 315 and the secondary amino group of Imatinib. Moreover, isoleucine contains an extra hydrocarbon

group on its side chain, and this inhibits the binding of Imatinib [94]. T315I confers resistance to all currently approved BCR–ABL kinase inhibitors. Recent reports have shown that T315I mutation can be found in approximately 15% of patients after failure of imatinib therapy [85].

Other important TKI's resistant mutations are frequently mapped to the P-loop region (residues 244 to 256) of the kinase domain, which serves as a docking site for phosphate moieties of ATP and interacts with imatinib through hydrogen and van der Waals bonds. These mutations modify the flexibility of the P-loop and destabilize the conformation required for Imatibib binding [95]. Clinical relevance of P-loop mutations is that imatinib treated patients who harbor them have been suggested to have a worse prognosis than those with non-P-loop mutations [96]. Another study identified BCR/ABL mutations in CD34+ cells from CML patients in CCR following Imatinib treatment and suggested that these mutations could lead to imatinib resistance in a small population of progenitors, which consequently could expand and cause the relapse [97].

Several additional mutations that disrupt the interaction between TKIs and BCR-ABL have been characterized, including the P-loop, C-helix, SH2 domain, substrate binding site, A-loop, and C-terminal lobe, some even prior to the initiation of therapy [98]. Most of the reported mutants are rare, however seven mutated sites constitute two thirds of all detected mutations: G250, Y253, E255 (P loop), T315I (gatekeeper), M351, F359, and H396 (activation loop or activation loop backbone) and are frequently evident in the later disease stages [99]. Recently a pan-BCR-ABL inhibitor active against the native enzyme and all tested resistant mutants, including the uniformly resistant T315I mutation has been developed [100].

BCR-ABL kinase domain mutations are not induced by the drug, but rather, just like antibiotic-resistance in bacteria, arise through a process whereby rare pre-existing mutant clones are self-selected due to their capacity to survive and expand in the presence of the drug thus gradually outgrowing drug-sensitive cells [101].

BCR-ABL gene amplification

Overexpression of Bcr-Abl leads to resistance by increasing the amount of target protein needed to be inhibited by the therapeutic dose of the drug. Amplification of the BCR–ABL gene was first described in resistant CML cell lines generated by serial passage of the cells in Imatinib containing media and demonstrated elevated Abl kinase activity due to a genetic amplification of the Bcr–Abl sequence [102, 103].

Cells expressing high amounts of Bcr-Abl in CD34+ CML cells, as in blast crisis, are much less sensitive to Imatinib and, more significantly, take a substantially shorter time for yielding a mutant subclone resistant to the inhibitor than cells with low expression levels, as in chronic phase [104]. However overexpression and amplification of the *BCR-ABL* gene itself accounts for Imatinib failure in a smaller percentage of patients with an overall percentage of 18% [94].

5.2. BCR-ABL-independent resistance mechanisms

Drug efflux

HSC are characterized by their ability to pump-out fluorescent dyes, and this led to isolation of stem cells based on this property. In fact, such an efflux capacity has become one of the most efficient methods to purify stem cells from different sources [105]. In this regard, ATP-binding cassette (ABC) transmembrane transporters have shown to be responsible for most of the efflux of the fluorescent dyes in HSCs [106].

In cancer cell lines, multidrug resistance is often associated with an ATP-dependent decrease in cellular drug accumulation, which is attributed to the overexpression of ABC transporter proteins [107]. The first studies on imatinib-resistance showed increased levels of the multi-drug resistance protein MDR1 (ABCB1) in Imatinib resistant BCR-ABL+ cell lines [108]. Later on, it was confirmed that Imatinib is a substrate of membrane ABC transporters, such as ABCB1 (MDR1, P-gp), and that variations in the activity or expression of P-gp affects the pharmacokinetics of Imatinib, reducing or increasing its bioavailability [109]. P-gp-positive leukemic cells have low intracellular levels of Imatinib; decreased Imatinib levels, in turn, were associated with a retained phosphorylation pattern of the Bcr-Abl target Crkl and loss of effect of Imatinib on cellular proliferation and apoptosis. The modulation of P-gp by Ciclosporin A readily restored imatinib cytotoxicity in these cells [110].

Another drug efflux pump, the breast cancer resistance protein BRCP encoded by ABCG2, has also been implicated in Imatinib resistance. Imatinib has been variably reported to be a substrate and/or an inhibitor for the BCRP/ABCG2 drug efflux pump, which is overexpressed in many human tumors and also found to be functionally expressed in CML stem cells [111, 112].

CML stem cells have been shown to express the ATP dependent transporter cassette protein ABCG2, which could decrease the intracellular accumulation of Imatinib in CML LSC [103]. Thus, overexpression of ABC transporters gives protection to tumor cells from TKIs [114].

Drug intake

Inversely to the drug efflux pump proteins, the human organic cation transporter 1 (OCT1) mediates the active transport of Imatinib into cells, and inhibition of OCT1 decreases the intracellular concentration of Imatinib [115]. OCT1 was also found to be expressed in significantly higher levels in patients who achieved a CCR to Imatinib than in those who were more than 65% Ph chromosome positive after 10 months of treatment [116]. Tyrosine Kinase Inhibitor Optimization and Selectivity (TOPS) trial suggested that patients with lower hOCT1 levels had reduced MMR rates at 12 months when receiving the standard dose of Imatinib, compared with high-dose Imatinib [117].

Recently Engler and cols. found that the intracellular uptake and retention (IUR) of imatinib, OCT-1 activity and OCT-1 mRNA expression are all significantly lower in CML CD34+ cells. However, no differences in IUR or OCT-1 activity were observed between these subsets in healthy donors. Low Imatinib accumulation in primitive CML cells, mediated through reduced OCT-1 activity may be a critical determinant of long-term disease persistence [118].

Differential interactions between drug efflux/influx pumps and kinase inhibitors might be a possible means to tailor drug selection for individual patients, because OCT-1 expression is a key determinant of intracellular availability of Imatinib but not of Nilotinib [119]. Other TKIs, such as Dasatinib and, as just mentioned, Nilotinib, do not appear to be substrates for hOCT1, but whether this difference alone will lead to reduced resistance rates with these second-generation TKIs remains unknown [120]. An adequate balance between influx (hOCT1) and efflux (MDR1, ABCG2) transporters may be a critical determinant of intracellular drug levels and, hence, resistance to Imatinib.

Quiescence

One feature of CML is the presence of a population of highly quiescent primitive cells [11], which, as their normal counterparts, is capable of regenerating hematopoiesis and reconstitutes the disease in immunocompromised mice [121]. These stem cells are Ph+, express high levels of CD34 and do not express CD38, CD45RA and CD71, and may spontaneously exit the G_0 phase and enter a state of constant proliferation [122]. Several reports have documented that quiescent cells from CML patients are insensitive to in vitro treatment with Imatinib and Dasatinib [16, 123].

A possible cause of insensitivity to TKIs is that BCR-ABL mRNA transcript levels are 300-fold higher in the most primitive $CD34^+CD38^-Lin^-$ population than in terminally differentiating $CD34^-Lin^+$ CML cells [124]. It has been reported that elevated levels of Bcr-Abl confer reduced sensitivity to Imatinib [125]. Moreover, the quiescent state of CML stem cells allows them to evade chemotherapy treatments, which are designed to eliminate metabolically active cell population as well as targeted therapies, thus contributing to relapse when treatment with tyrosine kinase inhibitors is discontinued.

Activation of BCR-ABL alternative signaling

BCR-ABL activates different signaling pathways that promote the growth and survival of hematopoietic cells, thus inducing cell transformation. These pathways include Ras, mitogen activated protein kinase (MAPK), c-jun N-terminal kinase (JNK), stress-activated protein kinase (SAPK), nuclear factor kappa B(NF-kB), signal transducers and activators of transcription (STAT), phosphoinositide 3- (PI-3) kinase, and c-Myc [126]. A well characterized pathway involves the Src Family Kinases (SFKs), which are activated by BCR-ABL and the subsequent inhibition of BCR-ABL by Imatinib may not result in the complete inhibition of Src family kinases elucidating a Bcr-Abl independent mechanism of imatinib resistance [127]. Phosphorylation of the Bcr-Abl SH2 and SH3 domains by the SFK may increase the activity of the Abl kinase and may alter its susceptibility to Imatinib [128].

Activation of the Janus kinase (Jak) and subsequent phosphorylation of several Signal Transducer and Activator of Transcription (STAT) family members has been identified in both Bcr–Abl–positive cell lines and in primary CML cells and may contribute to the transforming ability of Bcr–Abl [129].

The tyrosine residue at position 177 within the BCR portion is essential for the binding of adaptor proteins, including Growth Factor Receptor-Bound Protein 2 (GRB2) GRB10, 14-3-3,

and the SH2 domain of ABL1 [130]. Bcr-Abl protein is able to activate the Ras/Raf/Mek kinase pathway and the phosphatidylinositol 3′ kinase (PI3K)/Erk pathways through GRB2 [131, 132].

Autocrine loops could contribute to resistance. It has been demonstrated that IL-3 and granulocyte-colony G-CSF are produced within primitive CD34+ cells from patients with CML-CP, both of these cytokines stimulate cellular proliferation in an autocrine manner and protect cells from Imatinib-induced apoptosis [122].
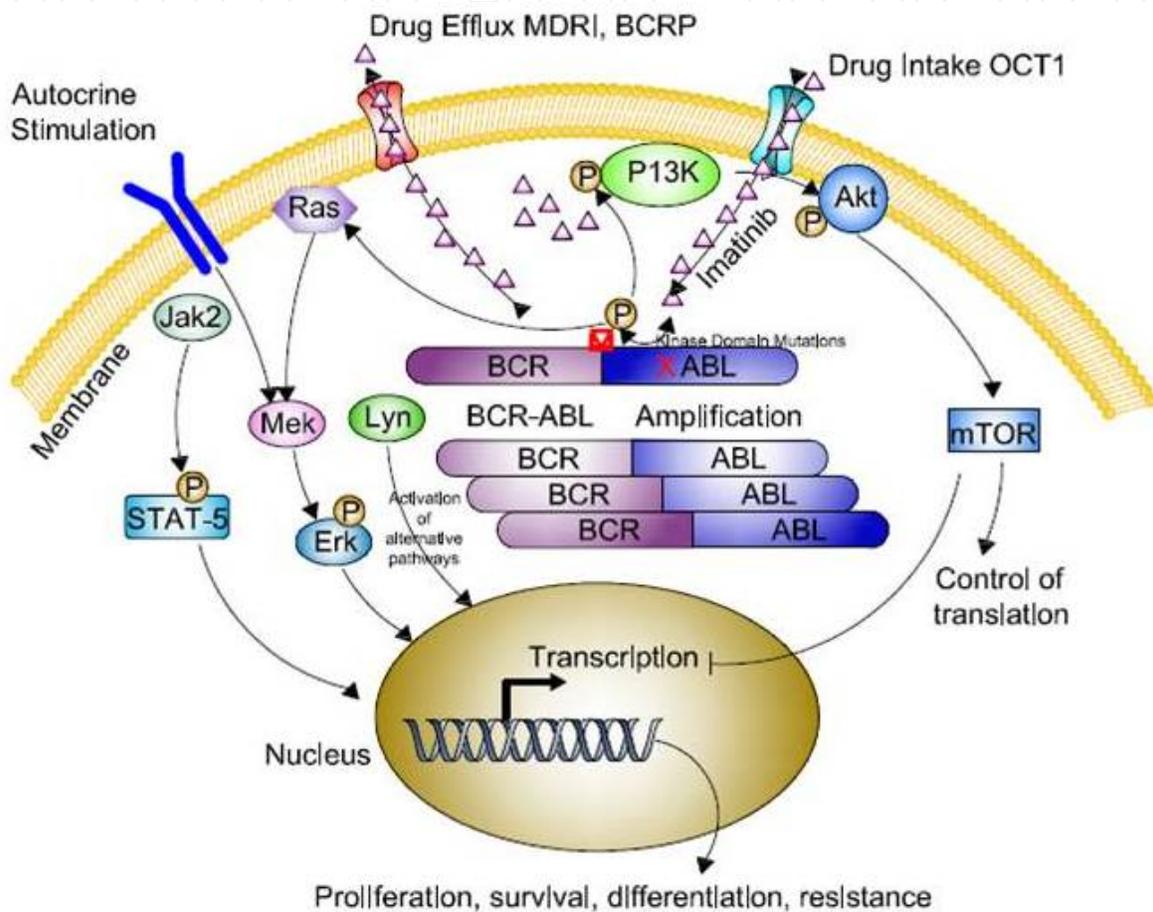
Figure 3. Resistance mechanism in Chronic Myeloid Leukemia. Principal mechanisms involved in dependent and independent BCR-ABL mechanisms are shown (modified to [99]).

6. Concluding remarks

The presence of a rare population of cells capable of initiating and sustaining leukemia in CML (LSC) has major implications for the biology of the disease and the development of new and more effective treatments. As recognized by several investigators, LSC are key players in the origin and progression of CML, as well as in the reappearance of the disease after treatment. Thus, it is evident that novel therapies must be directed towards the elimination of such cells. However, since their numbers within the marrow microenvironment are extremely low, as

compared to the bulk of the malignant cells, and their biology is quite different from that of the rest of the CML cells, the task of finding solutions to this problem is a rather difficult one. It is a great challenge, but significant advances will surely be achieved in the years to come.

Acknowledgements

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