

Molecular Pathways: BCR-ABL

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Abstract

Aberrant tyrosine kinase activity plays a critical role in many hematologic disorders, including chronic myeloid leukemia characterized by the constitutive activity of BCR-ABL. ABL therefore represents a crucial target for new therapeutic strategies. Here, we summarize the molecular pathways that are abnormally activated by the oncoprotein. Such pathways may provide additional opportunities to develop new drugs to overcome the resistance to tyrosine kinase inhibitors. In particular, the phosphoinositide 3-kinase (PI3K)/AKT pathway can be effectively blocked by mTOR inhibitors, and several compounds can hit the RAS pathway and the resulting mitogen-activated protein (MAP) extracellular signal-regulated kinase (ERK)1/2 (MEK) and MAP kinase activation. Furthermore, mitotic kinases can be blocked by Aurora kinase inhibitors, and Pim kinases can be blocked by selective serine-threonine kinase inhibitors. Finally, the abnormal pathways that sustain the self-renewal of leukemic stem cells are described as possible targets to completely eradicate leukemic clones. Such pathways include the Hedgehog pathway, which can be blocked by Smoothed inhibitors, and the CXCR4/SDF1 axis, which can be targeted by specific antagonists. *Clin Cancer Res*; 18(4); 930–7. ©2011 AACR.

Background

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder characterized by the Philadelphia (Ph) chromosome, which results from t(9;22)(q34;q11) balanced reciprocal translocation (1). The molecular consequence of the Ph chromosome is the generation of the *BCR-ABL* oncogene that encodes for the chimeric BCR-ABL oncoprotein, with constitutive kinase activity that promotes the growth advantage of leukemic cells (2).

The deregulated tyrosine kinase activity of BCR-ABL has been shown to be necessary and sufficient to maintain the leukemia phenotype of CML (3–5). Activation of the ABL tyrosine kinase is a primary event in the genesis of CML, as shown by the retrovirally mediated insertion of a human *BCR-ABL* gene into murine hematopoietic stem cells and the creation of *BCR-ABL* transgenic mice (3). This represents a critical issue in the effort to design molecular therapies.

BCR-ABL oncogenetic pathway

The ABL protein physiologically shuttles between the nucleus and the cytoplasm; however, when fused to BCR, the oncoprotein loses this property and is mainly retained within the cytoplasm, where it interacts with the majority of proteins involved in the oncogenic pathway. ABL tyrosine

kinase activity is constitutively activated by the juxtaposition of BCR, thus favoring dimerization or tetramerization and subsequent autophosphorylation. This increases the number of the phosphotyrosine residues on BCR-ABL and, as a consequence, the binding sites for the SH2 domains of other proteins (6, 7).

Abnormal interactions between the BCR-ABL oncoprotein and other cytoplasmic molecules lead to the disruption of key cellular processes. Examples include the perturbation of the Ras–mitogen-activated protein kinase (MAPK) leading to increased proliferation, the Janus-activated kinase (JAK)–STAT pathway leading to impaired transcriptional activity, and the phosphoinositide 3-kinase (PI3K)/AKT pathway resulting in increased apoptosis (8). The amino-terminal BCR-encoded sequences of BCR-ABL contain a tyrosine-phosphorylated site that binds the SH2 domain of the adaptor protein GRB2 (6). It is now evident that the phosphorylation of BCR Tyr177 is essential for BCR-ABL-mediated leukemogenesis (9), and its mutation largely abolishes GRB2 binding and diminishes BCR-ABL-induced Ras activation (7).

The latter results from the interaction of BCR-ABL with other cytoplasmic proteins, which function as adaptor molecules, thus creating multiprotein signaling complexes. The BCR-ABL/GRB2 complex recruits Son of Sevenless (SOS), which is constitutively associated with the GRB2 SH3 domain (10). In turn, the BCR-ABL/GRB2/SOS complex stimulates conversion of the inactive GDP-bound form of Ras to its active GTP-bound state (11) and the activation of the scaffold adapter GRB2-associated binding protein 2 (GAB2; ref. 12). As a consequence, the GRB2/GAB2/SOS complex causes constitutive activation of the RAS downstream pathway, thereby activating mitogen-activated protein (MAP) extracellular signal-regulated kinase (ERK)1/2

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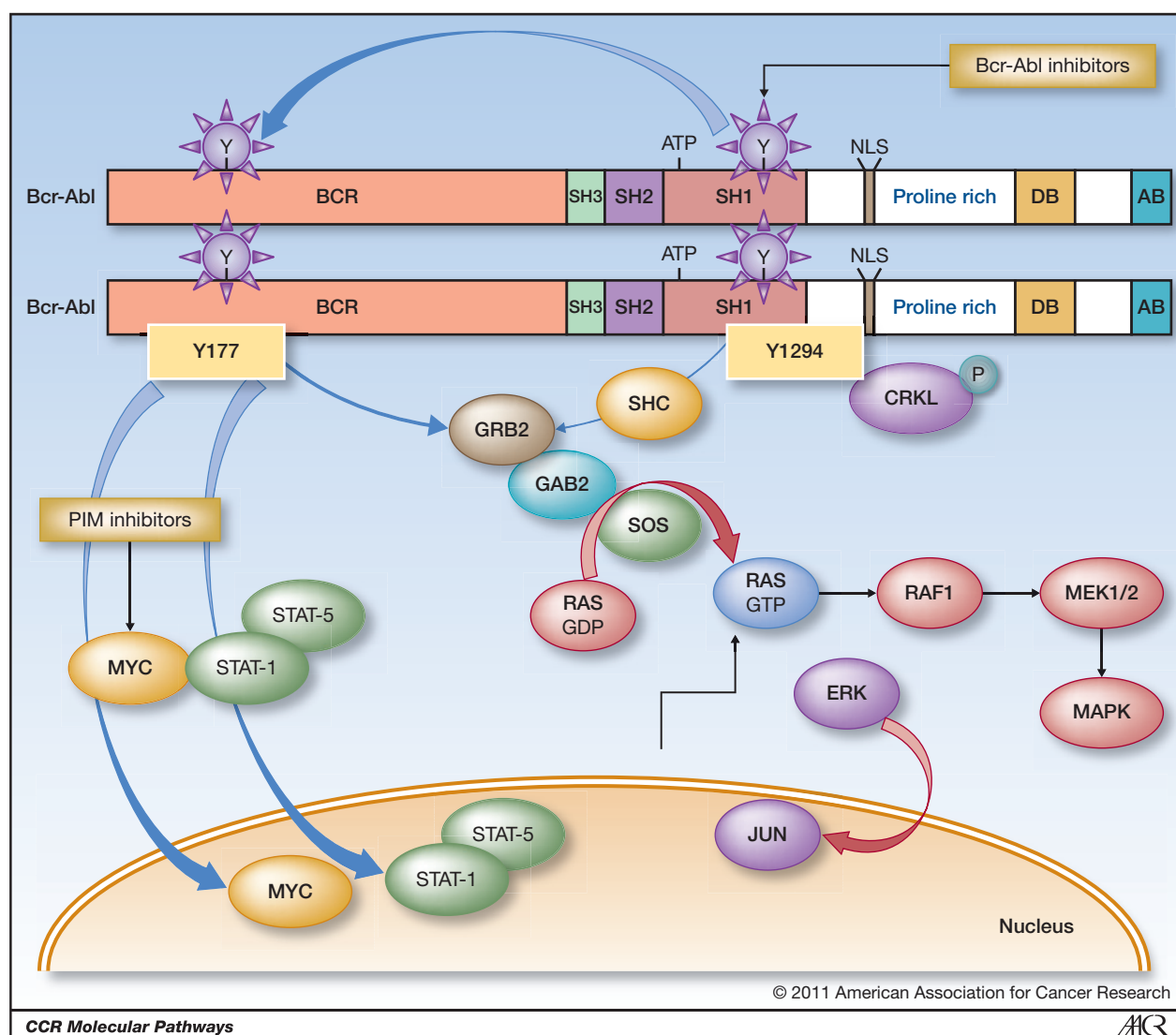


Figure 1. Schematic representation of the molecular pathway activated by BCR-ABL. Bcr-Abl phosphorylation of BCR Tyr177 is essential for BCR-ABL-mediated leukemogenesis. The BCR-ABL/GRB2 complex recruits SOS, which is constitutively associated with the GRB2 SH3 domain. The BCR-ABL/GRB2/SOS complex stimulates conversion of the inactive GDP-bound form of Ras to its active GTP-bound state, and activation of the scaffold adapter GAB2. As a consequence, the GRB2/GAB2/SOS complex causes constitutive activation of the RAS downstream pathway, thereby activating MEK1/2 and MAPK proteins and resulting in abnormal cell proliferation. In addition, this complex activates the PI3K/AKT pathway (see Fig. 2B).

(MEK) and MAP kinase proteins and resulting in abnormal cell proliferation. In addition, this complex activates the PI3K/AKT pathway (ref. 13, Fig. 1), which promotes survival by suppressing the activity of the forkhead O (FOXO) transcription factor, and enhances cell proliferation by inducing p27 proteasomal degradation and by mTOR activation.

In addition, BCR-ABL, through PI3K/AKT/FOXO4 and finally through upregulation of mTOR, potently blocks important cellular processes, such as autophagy. BCR-ABL may activate PI3K by more than one pathway, because Crk and Crkl have also been shown to connect BCR-ABL with PI3K (14, 15). Once activated, PI3K activates AKT kinase, which serves as a key downstream effector by exerting many cellular effects through the

phosphorylation of downstream substrates that regulate the apoptotic machinery [e.g., Bad, caspase 9, Mdm2, and Ask1 (16)], resulting in prolonged survival and expansion of the abnormal clone.

Key transcription factors are involved in BCR-ABL signaling. Among these a key role is played by *STAT1* and *STAT5* (signal transducer and activation of transcription), which are constantly active in BCR-ABL-positive cell lines and in primary cells from CML patients, contributing to the induction of cytokine independence (17).

In normal cells, nuclear translocation of STATs occurs exclusively after cytokine binding to receptors and is mediated by activation of the receptor-associated JAK kinases. By contrast, in CML, STATs seem to be activated

in a JAK-independent manner through a direct association of STAT SH2 domains with phosphorylated tyrosines on BCR-ABL (18).

Activation of STAT5 is at least partially responsible for protection from programmed cell death through the upregulation of the antiapoptotic molecule BCL-xL together with the inactivation of the proapoptotic molecule BAD by AKT (8).

Another postulated nuclear target of the transforming activity of the BCR-ABL protein is the protooncogene MYC, which is expressed at a high level in CML cells. MYC activation seems to be independent of the RAS pathway but directly upregulated by the ABL SH2 region (19). Several lines of evidence indicate that Myc is often overexpressed in blast crisis compared with the chronic phase, thus linking MYC to progression (19). *In vitro* inhibition of c-Myc with antisense oligonucleotides or dominant-negative constructs can inhibit BCR-ABL transformation or leukemogenesis (19).

All reported activated signaling pathways converge into a unique terminal point: loss of control of proliferation and expansion of the leukemic clone. Defining the relative contribution of each signal transduction pathway to the leukemic process is an important area of research because the combination of a tyrosine kinase inhibitor (TKI) with a downstream inhibitor may prove to be a clinically successful strategy.

Despite the seemingly endless expansion of the list of pathways that are activated by BCR-ABL, and the increasing complexity that is being revealed in these pathways, it seems that all of the transforming functions of BCR-ABL depend on its tyrosine kinase activity (20). This precondition has an incredible intrinsic clinical potential with regard to the development of more-sophisticated targeted therapies.

Clinical-Translational Advances

Kinase inhibitors

Imatinib, a small-molecule TKI, was the first drug to be developed that was able to directly target BCR-ABL tyrosine kinase activity and to be tested in CML (21). In a short time, it has become the standard first-line therapy for all CML patients in early chronic phase based on the response rates and the good tolerability shown (22).

Despite the exciting results obtained with imatinib, after 8 years of follow-up, the cumulative complete cytogenetic response (CCyR) rate for first-line imatinib-treated patients was 83%, the event-free survival rate was 81%, and the estimated overall survival rate was 85%.

However, it should be considered that if a CCyR is not achieved after 12 months of imatinib therapy, the probability of progression or loss of response increases to 38%. Indeed, after 8 years of follow-up, it was concluded that early cytogenetic response is predictive of long-term outcome, and the cytogenetic response is therefore considered a prognostic indicator of lack of events, whereas nonoptimal responders had a poorer prognosis (23). In addition, the degree of response is critical. The IRIS study (22) also

confirmed that the achievement of a major molecular response at 12 months predicts a low risk of events or progression, thus emphasizing the value of achieving a molecular response early during treatment.

On the basis of these data, it appears clear that for at least one third of patients, the potential exists to improve on what can be achieved with standard imatinib treatment (400 mg daily). Therefore, researchers have investigated the possibility of inhibiting BCR-ABL activity in a more potent manner to improve the results of first-line therapy. The therapeutic strategies assessed include modified imatinib-based regimens and first-line administration of next-generation TKIs.

Several highly potent next-generation BCR-ABL inhibitors have been developed to overcome imatinib resistance and improve the prognosis of patients with CML. These include novel and more potent multi-TKIs such as dasatinib, an orally bioavailable dual BCR-ABL and Src inhibitor, and potent selective BCR-ABL inhibitors such as nilotinib. Both nilotinib and dasatinib induce significant clinical responses. Dasatinib blocks BCR-ABL at low concentrations but is less selective than imatinib. Similarly to imatinib, it inhibits BCR-ABL, Kit, and platelet-derived growth factor receptor (PDGFR), but in contrast, it also blocks Src, Tec, and Eph kinases, as well as many other kinases. Nilotinib blocks BCR-ABL at lower concentrations than does imatinib, but, like imatinib, it appears to be more selective than dasatinib in targeting tyrosine kinases.

In phase II trials of patients with chronic-phase CML, dasatinib and nilotinib were associated with 2-year CCyR rates of 45% and 41%, respectively, in a group of imatinib-resistant patients (24). Following the impressive results obtained in patients with imatinib failure, dasatinib and nilotinib were assessed in phase II studies of patients with newly diagnosed CML. The rate of CCyR at 12 months was 80%, and the rate of major molecular response was 44%, thus showing superiority over imatinib standard treatment and a favorable toxic profile (25). The results of these trials seem to indicate a superior efficacy of the more-potent second-generation TKIs with respect to imatinib, particularly in terms of higher rates of CCyR and major molecular response, which, of more importance, also seem to lead to a decreased number of disease progressions. Although these results still need to be fully evaluated after a longer period of follow-up, the use of next-generation agents in the first-line therapy of CML is likely to become a key area of clinical research during the next few years.

Considering the impressive results obtained with second-generation TKIs in first-line treatment, there are only a few fields of intervention we can still envisage that may optimize treatment of CML.

The first is to target what is commonly defined as the Achilles' heel, which is represented by the emergence of BCR-ABL mutant clones, including T315I, that are completely insensitive to second-generation TKIs (26). Although these insensitive clones occur very rarely, the

small fraction of patients who develop them fail to respond to treatment and experience disease progression.

An additional step forward would be to completely eradicate the leukemic stem cells that appear to be resistant to TKIs and represent a reservoir of the leukemic pool that could eventually favor reappearance of the disease when TKIs are stopped (27).

Very rarely, resistant patients develop mutated clones that are particularly aggressive and resistant to all available drugs (28). Resistance to BCR-ABL inhibitors may arise from different mechanisms, including BCR-ABL amino acid mutations, gene amplification, and mechanisms that are independent of BCR-ABL (29).

The T315I mutation at the gatekeeper residue occurs frequently in advanced phases of the disease and serves as one of the main causes of resistance by disrupting important contact points between the inhibitors and the enzyme. At least theoretically, we may be able to completely eradicate resistant clones by making use of 2 different approaches: In patients who develop mutations that are still sensitive to TKIs, a more potent inhibition of tyrosine kinase activity seems to overcome the resistance. This can be achieved by switching to second-generation TKIs (24) or by increasing the imatinib dose (30). For patients with mutants that are insensitive to the available drugs, a different and more complex approach is required. This could be achieved by targeting downstream pathways activated by BCR-ABL, or by a less selective inhibition of BCR-ABL by drugs that block multiple kinases, including the mutants.

AP24534 (ponatinib), a potent, orally available, multi-targeted kinase inhibitor, is active against pan-BCR-ABL and the mutated form, and against additional kinases such as VEGF receptor, fibroblast growth factor receptor, and PDGFR- α , LYN, and FLT3 (31). A phase I study showed the tolerability of the drug in all phases of CML and acute lymphoblastic leukemia.

A novel TKI in the preclinical phase of development is represented by DCC-2036 (Deciphera; ref. 32). It belongs to a class of TKIs with a novel mechanism of action, in that it blocks the switch pocket rather than the ATP pocket. This compound has been shown to inhibit, at least *in vitro*, all of the mutant variants of BCR-ABL.

Molecular targets downstream of BCR-ABL

An alternative approach would be to target molecules downstream of BCR-ABL. One important target is the PI3K/AKT/mTor pathway, which is activated by BCR-ABL and additional mechanisms, leading to impaired apoptosis of Ph-positive cells (Fig. 2A).

mTOR lies downstream of AKT in the PI3K kinase pathway. It is a serine/threonine kinase made of 2 complexes: mTORC1, which controls transit from G₁-phase to S-phase of the cell cycle, and mTORC2, which phosphorylates AKT, leading to its full activation (33). Rapamycin (sirolimus) has emerged as a potent inhibitor of mTORC signaling (34). Several rapamycin analogues with improved pharmaceutical properties but similar biologic effects in comparison

with rapamycin [CCI-779 (34), RAD-001 (35), and WYE-132 (36)] are currently undergoing clinical trials. Rapamycin and rapalogs are not able to inhibit mRNA translation and protein synthesis in different models of disease (37). The direct inhibition of protein synthesis represents a new emerging field of therapy. Therefore, investigators have developed small molecules (Torin1, PP242, and PP30) to target the mTOR kinase domain in order to inhibit both mTORC1 and mTORC2 signaling pathways.

It was shown that BCR-ABL inhibition results in a reactivation of autophagy (38). This process takes place through inhibition of the BCR-ABL/PI3K/AKT/FOXO4/mTOR pathway. Inhibition of mTor may therefore potentiate imatinib-induced autophagy.

In addition, the role of Pim proteins in mediating resistance to mTor inhibition was recently shown (39). Pim inhibition is therefore an attractive therapeutic approach, especially in combination with PI3K/AKT/mTor inhibition. Pim inhibitors have shown a high level of *in vitro* activity and are in the preclinical phase of development (40). Pim inhibitors act by reducing the expression of MYC. Furthermore, Pim inhibitors result in inhibition of cyclin-dependent kinase 2 activity, presumably regulated by translocation of p27 to the nucleus. It was shown that Pim inhibitors markedly increase the levels of p27, which is consistent with the G₁ arrest observed after treatment of leukemic cell lines.

Additional selective targets that could be exploited to eliminate mutant clones are the mitotic kinases. The Aurora kinases are a conserved family of serine/threonine kinases that play a critical role in the cell cycle. Investigators have identified 3 members of the Aurora family [Aurora A, B, and C (41)], each of which has a different localization and function. Aurora A is primarily associated with the centrosomes and the microtubules in close proximity to the centrosomes beginning in late S–G₂ phase. Aurora B acts as a chromosomal passenger protein whose expression peaks at the G₂–M transition, with maximum kinase activity in mitosis. Thus far, very few studies have addressed the exact role of Aurora C.

Promising results have been obtained with antitumor tubulin drugs, such as vinca alkaloids and taxanes, for the treatment of cancer. By inhibiting microtubules, these drugs result in mitotic arrest and cell death. However, microtubules are also required for adequate molecular transport in normal cells. Therefore, investigators are searching for specific targets to more selectively kill leukemic cells, and have developed different Aurora kinase inhibitors (42), including hesperidin, MK-0457, ZM447439, MLN8054, and AZD1152. These inhibitors act by blocking enzymatic activity by occupying the catalytic ATP binding site (42). Many of these compounds have been shown to be effective in inhibiting T315I mutants.

Stem cell pathways

An emerging concept in cancer biology is that a rare population of cancer stem cells exists in the heterogeneous cell mass that constitutes a tumor (43). This concept also applies to CML. Normal and leukemic hematopoietic stem

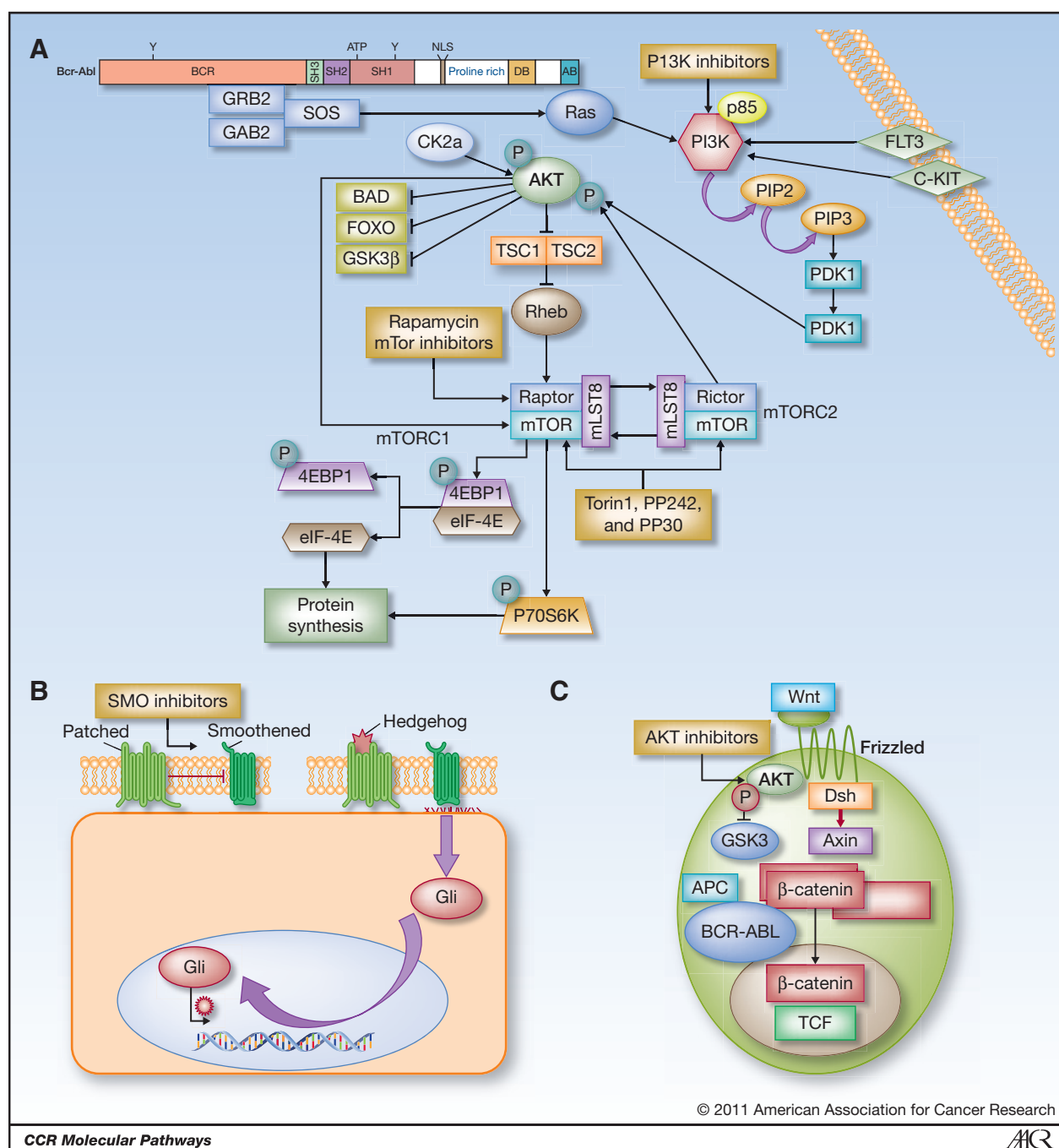


Figure 2. BCR-ABL-related pathways. **A**, schematic representation of RAS/PI3K signaling pathway and AKT pathway downstream of BCR-ABL. The PI3K/AKT pathway promotes survival by suppressing the activity of FOXO transcription factor and enhances cell proliferation by inducing p27 proteasomal degradation and mTOR activation. PI3K activates AKT kinase, which serves as a key downstream effector by exerting many cellular effects through the phosphorylation of downstream substrates that regulate the apoptotic machinery (e.g., Bad, caspase 9, Mdm2, and Ask1), resulting in prolonged survival and expansion of the abnormal clone. mTOR lies downstream of AKT in the PI3K kinase pathway. It is a serine/threonine kinase made of 2 complexes: mTORC1, which controls transit from G₁-phase to S-phase of the cell cycle, and mTORC2, which phosphorylates AKT, leading to its full activation. **B**, schematic representation of the Hedgehog (Hh) pathway. Hh pathway activation is initiated when Hh ligand binds to PTCH, which moves away from a second transmembrane protein called Smoothened (Smo). Smo signals to a cytoplasmic complex that releases a transcription factor (Gli) that translocates to the nucleus, where it activates the Hh target genes. **C**, schematic representation of the Wnt pathway. The major effector of the Wnt pathway is β-catenin. In the absence of Wnt signaling, β-catenin is retained in the cytoplasm through a direct interaction with Axin, APC, and GSK3. β-catenin phosphorylation by GSK3 induces its rapid proteasome-mediated degradation. By binding ubiquitinated β-catenin, Bcr-Abl stabilizes it against ubiquitination, resulting in increased β-catenin levels. After Wnt binds to a receptor complex composed of members of the Frizzled family, the Axin/APC/GSK3 complex is inhibited, leading to a block in β-catenin phosphorylation by GSK3. Hypophosphorylated β-catenin accumulates in the cytoplasm and is translocated to the nucleus, where it regulates target gene expression.

cell functions are defined by a common set of critical stemness genes that regulate self-renewal (44). Hematopoietic stem cells (HSC) and leukemic stem cells (LSCs) share common features, including self-renewal, the capacity to differentiate, resistance to apoptosis, and limitless proliferative potential (44). Despite these similarities, however, several stemness factors, such as Notch, BMI-1, and Wnt show differential activation in HSC versus LSC. Such differences could be exploited therapeutically (45).

It is important to consider that stemness in leukemia is linked to self-renewal. It is extremely likely that LSCs undergo self-renewal and are capable of recapitulating leukemia, and thus maintenance of the LSC pool would play a critical role in the success of any therapeutic intervention. Targeting stemness factors could be the key factor for a successful therapy.

HSCs reside mainly in specialized bone marrow microenvironments called HSC niches. The niche provides appropriate signals that maintain the balance between self-renewal and differentiation of stem/progenitor cells. Hedgehog (Hh) is one of the major regulators of the cell-fate decision. The Hh signal is critical for HSC and progenitor differentiation (Fig. 2B). Hedgehog pathway activation is initiated when Hh ligand binds to Patched (PTCH), which moves away from a second transmembrane protein called Smoothened (Smo). Smo signals to a cytoplasmic complex that releases a transcription factor (Gli) that translocates to the nucleus, where it activates the Hh target genes (46). Leukemic cells are believed to rely on autocrine signaling, and the Hh ligand produced by leukemic cells acts on neighboring leukemic cells to stimulate their growth or survival.

This model is supported by *in vitro* evidence that proliferation of tumor cell lines is accelerated by the addition of Hh ligand (47) and inhibited by the addition of Hh neutralizing antibody or by cyclopamine, an Hh pathway antagonist. Finally, Hh also acts through a paracrine mechanism. Hh ligand secreted by neoplastic cells signals to the microenvironment, and the stromal cell compartment signals back to the leukemic cells (48).

The clear link between the Hh pathway and human leukemias led to an effort to identify small molecules to block the pathway. Investigators have identified different classes of small-molecule Hh antagonists through cell-based screens using an Hh reporter assay (46).

One of these molecules, CUR61414, an aminoproline Hh antagonist, can block elevated Hh signaling activity resulting from oncogenic mutations within the Patched-1 sequence (49). In addition, a novel series of Hh pathway inhibitors, 1-amino-4-benzylphthalazines (Novartis, Institutes for Biomedical Research, Cambridge, MA), has been identified and confirmed to act via antagonism of the Smo receptor.

Wnt-mediated signaling has been shown to regulate cell-fate determination, proliferation, adhesion, migration, and polarity during development (Fig. 2C). In addition to their crucial role in embryogenesis, Wnt proteins and their downstream signaling molecules have been implicated in leukemogenesis. Wnt signaling has been implicated in maintaining and amplifying stem cells, as well as in defin-

ing the stem-cell fate. The major effector of the Wnt pathway is β -catenin. In the absence of Wnt signaling, β -catenin is retained in the cytoplasm through a direct interaction with Axin, APC, and GSK3. The phosphorylation of β -catenin by GSK3 was shown to induce its rapid proteasome-mediated degradation (50). After Wnt binds to a receptor complex composed of members of the Frizzled family, Axin/APC/GSK3 complex is inhibited, leading to a block in β -catenin phosphorylation by GSK3. Hypophosphorylated β -catenin accumulates in the cytoplasm and is translocated to the nucleus, where it regulates target gene expression (50). Deregulation of the Wnt signaling pathway is a hallmark of several types of leukemias (51). Different molecular mechanisms have been implicated in abnormal activation of the Wnt pathway, including the functional loss of Wnt antagonists resulting in leukemogenesis through deregulation of cell proliferation and differentiation. Although the Wnt signaling pathway is now recognized as one of the major players in the genesis of leukemia (52), it remains to be determined whether this pathway can be targeted by drugs.

The niche is anatomically and functionally defined, and has an endosteal and perivascular compartment within the bone marrow. Within the niche, critical bidirectional signals ensure the regulation of normal HSC numbers and maintenance of the quiescent long-term HSC pool. Adventitial reticular cells, which are of mesenchymal origin, have been shown to alter stem-cell function. These cells express high levels of the CX chemokine ligand12 [CXCL12; also called stromal-derived factor 1 (SDF1)] and are located between vessels and bone (53). Targeted deletion of CXCR4 (the ligand for CXCL12) led to a severe reduction in HSC numbers and increased chemosensitivity (53). Interactions between CXCR4 and SDF1 are important for the localization and retention of HSC and progenitor cells within the niche. In addition, they play a critical role in colonization of the bone marrow by HSCs during early development, as revealed by the observation that SDF1-deficient embryos have severely reduced HSC numbers and function (54).

The interaction between CXCR4 and CXCL12 appears to be critical for leukemic cell maintenance. The leukemic cells seem to have the unique capacity to directly modulate the niche at the expense of normal hematopoietic stem and progenitor cells (55) by downregulating CXCL12 levels in the areas of leukemia infiltration. Stem cell factor, which is secreted by the leukemic cells, is a niche regulator that leads to abnormal engraftment of normal HSCs in the tumor-infiltrated microenvironment. Emerging evidence indicates that we may be able to target the complex interactions between LSCs and their niche to selectively deplete the repopulating ability of LSCs and favor the normal HSC counterparts. To achieve a selective eradication of LSCs, a niche-targeted therapy would require a high degree of selectivity toward the aberrant interaction between the leukemic clone and the microenvironment (56). These could include self-renewal pathways such as Notch and Wnt, homing mechanisms, and cell adhesion molecules. Published data support the role of the Notch pathway in the maintenance of

leukemic stem cells and Wnt signaling in blast crisis CML stem cells (57). Of importance, evidence indicates that both of these pathways are at least partially regulated by the niche. Adhesion molecules are attractive candidate targets for LSC-specific therapies. Direct targeting of CXCR4 with chemical compounds may represent another developing strategy (58). The CXCR4 antagonist AMD3465 has been shown to overcome the protective effects of stromal cells toward chemotherapeutic agents. Further studies have also shown that treatment with AMD3100, another CXCR4 antagonist that is currently used mainly to mobilize normal HSCs, results in

mobilization of leukemic cells and increased chemosensitivity (59). Furthermore, targeting CXCR4 appears to have beneficial effects against leukemic clones.

Disclosure of Potential Conflicts of Interest

G. Saglio has received honoraria from the Speakers Bureau and is a consultant to Novartis and Bristol-Myers Squibb. D. Cilloni disclosed no potential conflicts of interest.

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