Co-operating STAT5 and AKT signaling pathways in chronic myeloid leukemia and mastocytosis: possible new targets of therapy

Siham Bibi,[1] Melis Dilara Arslanhan,[1] Florent Langenfeld,[1,2] Sylvie Jeanningros,[1] Sabine Cerny-Reiterer,[3,4] Emir Hadzijusufovic,[3,4,5] Luba Tchertanov,[2] Richard Moriggl,[6] Peter Valent,[3,4] and Michel Arock[1,7]

[1]Molecular Oncology and Pharmacology, LBPA CNRS UMR8113, Ecole Normale Supérieure de Cachan, France; [2]Bioinformatics, Modelisation and Molecular Dynamics, LBPA CNRS UMR8113, Ecole Normale Supérieure de Cachan, France; [3]Department of Internal Medicine I, Division of Hematology and Hemostaseology, Medical University of Vienna, Austria; [4]Ludwig Boltzmann Cluster Oncology, Medical University of Vienna, Austria; [5]Internal Medicine, Small Animal Clinic, Department for Companion Animals and Horses, University of Veterinary Medicine Vienna, Austria; [6]Ludwig Boltzmann Institute of Cancer Research, Vienna, Austria; and [7]Laboratory of Hematology, Groupe Hospitalier Pitié-Salpêtrière, Paris, France

ABSTRACT

Chronic myeloid leukemia and systemic mastocytosis are myeloid neoplasms sharing a number of pathogenetic and clinical features. In both conditions, an aberrantly activated oncoprotein with tyrosine kinase activity, namely BCR-ABL1 in chronic myeloid leukemia, and mutant KIT, mostly KIT D816V, in systemic mastocytosis, is key to disease evolution. The appreciation of the role of such tyrosine kinases in these diseases has led to the development of improved therapies with tyrosine kinase-targeted inhibitors. However, most drugs, including new KIT D816V-blocking agents, have failed to achieve long-lasting remissions in advanced systemic mastocytosis, and there is a similar problem in chronic myeloid leukemia, where imatinib-resistant patients sometimes fail to achieve remission, even with second- or third-line BCR-ABL1 specific tyrosine kinase inhibitors. During disease progression, additional signaling pathways become activated in neoplastic cells, but most converge into major downstream networks. Among these, the AKT and STAT5 pathways appear most critical and may result in drug-resistant chronic myeloid leukemia and systemic mastocytosis. Inhibition of phosphorylation of these targets has proven their crucial role in disease-evolution in both malignancies. Together, these observations suggest that STAT5 and AKT are key drivers of oncogenesis in drug-resistant forms of the diseases, and that targeting STAT5 and AKT might be an interesting approach in these malignancies. The present article provides an overview of our current knowledge about the critical role of AKT and STAT5 in the pathophysiology of chronic myeloid leukemia and systemic mastocytosis and on their potential value as therapeutic targets in these neoplasms.

Introduction

Several new targets have recently been identified in neoplastic mast cells (MCs), and various targeted drugs have been examined for their effectiveness in malignant MC disorders. However, most drugs, including new KIT D816V-blocking agents, such as midostaurin (PKC412),[1] and various BCR-ABL1 inhibitors known to block KIT-activity, such as imatinib or dasatinib,[2] have failed to achieve long-lasting remissions in aggressive systemic mastocytosis (ASM). There is a similar problem in Ph$^+$ CML, where imatinib-resistant patients do not reach molecular remission even when second- or third-line BCR-ABL1 tyrosine kinase inhibitors (TKIs) are applied, particularly in patients exhibiting the BCR-ABL1 T315I mutant.[3] During disease progression, additional signal transduction pathways become activated in neoplastic cells. Among these, AKT and STAT5 are critical downstream signaling molecules constitutively phosphorylated imatinib-resistant chronic myeloid leukemia (CML) and *KIT* D816V$^+$ SM.[4,5] This has been demonstrated *in vitro* using *KIT* D816V$^+$ and *BCR-ABL1*$^+$ imatinib-resistant cell lines, where inhibition of phosphorylation of these targets has shown their crucial role in cell proliferation.[6,7] It has also been reported that STAT5 and AKT remained activated in neoplastic myeloid cells, even after inhibition of BCR-ABL1 by TKIs.[8] Moreover, during disease progression, the levels of STAT5 mRNA and protein increase, and STAT5 production and activation is triggered not only by BCR-ABL1 or mutant KIT, but also by other pro-oncogenic pathways relevant to disease progression and resistance.[9] Taken together, these observations strongly suggest that STAT5 and AKT are key drivers in drug-resistant CML and SM, and thus represent potential therapeutic targets. Small molecules targeting STAT5 or AKT may indeed be effective in these malignancies, especially in TKIs-resistant patients. However, inhibitors available today, such as pimozide or BP-1-108 for STAT5,[10,11] or perifosine for AKT,[12] are neither specific nor potent enough to be applicable in clinical practice. Therefore, it seems important to develop compounds that specifically and potently target STAT5 and AKT.

Mast cells and mastocytosis

KIT and cytokine signaling in normal mast cells

MCs originate from bone marrow (BM)-derived hematopoietic stem cells (HSCs) that enter the peripheral tissues *via* the bloodstream and undergo maturation under the

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Correspondence: arock@ens-cachan.fr

influence of stem cell factor (SCF), a major cytokine-ligand of KIT (CD117).[13] KIT is a transmembrane receptor with intrinsic tyrosine kinase (TK) activity.[14] SCF-binding to KIT leads to dimerization and activation of the receptor.[15] During KIT phosphorylation on specific tyrosines, the resulting phosphotyrosine (PT) residues become docking sites for signal transduction molecules. Activated KIT also catalyzes the phosphorylation of substrate proteins and triggers multiple signal transduction pathways.[14] Once activated, KIT recruits the phosphatidylinositol 3-kinase (PI3K), which in turn activates AKT.[16] Activated KIT also recruits the RAS/RAF and JAK/STAT signaling pathways.[17,18] These pathways are involved in survival, proliferation, migration and differentiation of MCs.[19]

Pathogenesis of mastocytosis

Mastocytosis is a term collectively used for a heterogeneous group of disorders defined by expansion of MCs in one or more organs.[20] Clinical symptoms result from MC-derived mediators, and from the destructive infiltration of neoplastic MCs in various organs.[21] Mastocytosis can affect children and adults.[21] However, mastocytosis developing in childhood is usually restricted to the skin and may resolve during or shortly before puberty, whereas in adult-onset mastocytosis, the disease is usually chronic, and defined by systemic involvement, with or without skin lesions.[22]

The World Health Organization (WHO) classification describes several different categories of mastocytosis, including cutaneous and systemic variants (SM) (Table 1).[23] SM can further be divided into indolent SM (ISM), aggressive SM (ASM), MC leukemia (MCL), and SM with associated hematologic non-MC-lineage disease (SM-AHNMD).[21] In the latter case, the AHNMD is frequently a myeloid neoplasm, such as an acute myeloid leukemia (AML) or a CML, a myelodysplastic syndrome (MDS) or a chronic myelomonocytic leukemia (CMML), whereas association of SM with a myeloma or a non-Hodgkin's lymphoma is a rare event (Table 1).[21] The *KIT* D816V mutation is found in up to 90% of all patients with SM (Figure 1).[24] By contrast, pediatric patients usually harbor *KIT* mutations in other exons (Figure 1).[25] KIT with the D816V mutation is constitutively activated, leading to the recruitment of major pro-oncogenic signaling cascades, such as the RAS/RAF-, STAT-, or PI3K-AKT-signaling pathways (Figure 2).[19]

Treatment of systemic mastocytosis

Treatment of non-advanced mastocytosis is based on pharmacological agents targeting symptoms caused by MC mediators.[22] The most frequently used drugs are H1 and H2 antihistamines and glucocorticoids.[22] Epinephrine is reserved for life-threatening episodes of anaphylaxis.

In advanced SM, additional drugs are required to control MC expansion. However, so far, no standard anti-neoplastic therapies for patients with ASM, SM-AHNMD or MCL have been developed. Interferon alpha (IFN-α) showed variable efficacy, but also side effects, limiting its use.[26] Cladribine (2-CdA) induces major clinical responses in a smaller group of patients with ASM.[27] Both IFN-α and 2CdA can also induce cytopenia and immunosuppression.[26] The same also holds true for other conventional anti-neoplastic drugs, like hydroxyurea, or chemotherapy, used to treat patients with ASM. All these agents also have a certain mutagenic potential. More recently, approaches to treat ASM and MCL have focused

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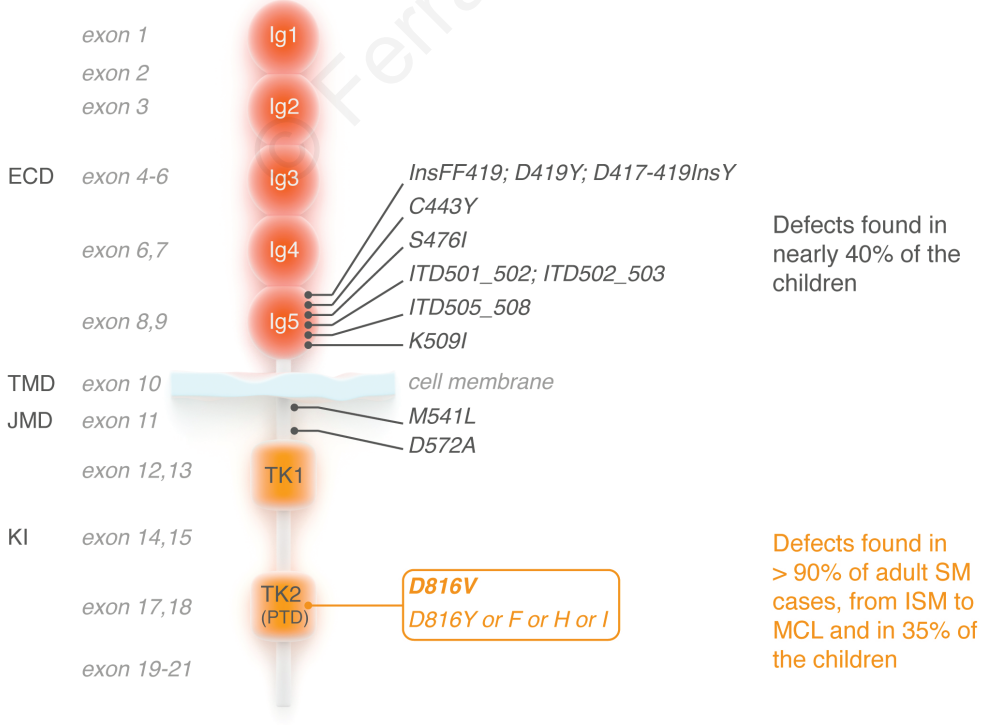

Figure 1. Representation of the structure of KIT, illustrating the known function of its domains and the localization of the more frequently observed mutations in the *KIT* sequence in pediatric or adult patients with mastocytosis. The receptor is presented under its monomeric form, whereas its wild-type counterpart dimerizes upon ligation with SCF before being activated in normal cells. The KIT D816V PTD mutant is found in up to 90% of the adult patients, whereas the ECD mutants are found in nearly 40% of the affected children. ECD: extracellular domain; Ins: Insertion; ITD: internal tandem duplication; JMD: juxtamembrane domain; KI: kinase insert; TK1: tyrosine kinase domain 1 (ATP binding site); TK2: tyrosine kinase domain 2 (activation loop) = PTD (phosphotransferase domain); TMD: transmembrane domain.

on KIT and KIT TKIs, because of the ubiquity of *KIT* mutations detected in these patients.[28] Imatinib is usually not indicated, because the *KIT* D816V mutation confers resistance.[2] Nevertheless, imatinib can reduce the MC burden and symptoms in SM patients exhibiting *KIT* mutations in other regions of the receptor.[21] Other TKIs, such as dasatinib or PKC412 (midostaurin), are capable of inhibiting the KIT D816V activity *in vitro*.[29] However, with the exception of PKC412, these drugs have very low efficacy in ASM.[26] Therefore, alternative drugs and approaches using combinations of targeted drugs have been proposed for the treatment of patients with ASM and MCL.[30]

Chronic myelogenous leukemia

Chronic myelogenous leukemia (CML) is a myeloid neoplasm characterized by the presence of the BCR-ABL1 oncoprotein and the expansion and prominent granulocytic differentiation of neoplastic myeloid cells.[31] A low rate of apoptosis is considered to lead to accumulation of neoplastic myeloid cells over time in these patients. As a result, CML patients usually present with marked leukocytosis, thrombocytosis, and often also anemia.[32] The natural course of CML can be divided into 3 distinct phases: 1) a chronic phase (CP); followed by 2) an accelerated phase (AP); and eventually 3) the blast phase (BP) of CML. If untreated, CP inevitably evolves to AP and finally to BP, which resembles an acute leukemia.

Pathogenesis of CML

Chronic myelogenous leukemia is defined by the presence of a fusion gene acquired in an early hematopoietic progenitor, the *BCR-ABL1* oncogene, which leads to the synthesis of the fusion protein BCR/ABL1 in leukemic cells (Figure 3).

The *c-ABL* gene has 11 exons located on chromosome 9q34, and it encodes a weak TK of 140 kDa.[33] It displays an alternative exon I (a or b), with the exon Ib located upstream of the other exons (Ia and 2-11).[33] The translocation breakpoint occurs between exon Ib and Ia in approximately 90% of the cases, resulting in a fully functional *C-ABL* coding sequence (exons Ia and 2-11) to be recombined to the *BCR* gene.[34] The leukemogenic effect of BCR-ABL1 is mediated through activation of several downstream signaling pathways, including the RAS/RAF-, PI3K/AKT- and STAT-signaling pathway (Figure 3).[4,35,36]

Treatment of chronic myelogenous leukemia

The first clinically used drugs in CML that showed a survival benefit were busulfan in 1953,[37] and hydroxyurea from 1972.[38] Cytogenetic responses were first seen in patients treated with IFN-α.[39] Despite these advances, however, many patients progressed to AP and BP. A milestone in the history of the treatment of CML remains allogenic hematopoietic stem cell transplantation (SCT), which was first introduced in the mid-1970s.[40]

In the early 2000s, imatinib, which binds the ATP-binding site of the chimeric BCR-ABL1 TK in a competitive

Table 1. WHO Classification of mastocytosis.

Variant - Term	Abbreviation	Subvariants	Major characteristics	Prognosis
Cutaneous mastocytosis	CM	Urticaria pigmentosa (UP) Diffuse CM (DCM) Mastocytoma of skin	No systemic involvement (most patients are children)	Good
Indolent systemic Mastocytosis	ISM	Smoldering SM Isolated bone marrow mastocytosis	No C-Findings (mostly seen in adults)	Good
Systemic Mastocytosis with an associated clonal hematologic non-MC lineage disease	SM-AHNMD	SM-AML SM-MDS SM-MPN SM-CEL SM-CMML SM-NHL SM-Myeloma	often associated with MDS, MPN, MPN/MDS, less frequently with AML or NHL	Often depending on that of the AHNMD
Aggressive systemic mastocytosis	ASM	Lymphadenopathic SM with eosinophilia	Organ failure secondary to MC infiltration: Bone marrow (BM) Hepatic failure with ascites Splenomegaly with hypersplenism Large osteolysis with pathological fractures Digestive tract involvement with malabsorption and weight loss	Poor
Mast cell leukemia	MCL	Aleukemic MCL Chronic MCL (cMCL)	Atypical MCs (multilobulated nucleus, multinucleated cells) More than 20% of MCs found in BM smears	Very Poor
Mast cell sarcoma	MCS		Malignant tumor destroying soft tissue MCs are highly atypical	Very Poor
Extracutaneous mastocytoma			Rare benign tumor made of mature cells	Good

Clinical Findings (C-Findings): correspond to the presence of one or more organ failure secondary to mast cell infiltration, among the following: bone marrow failure, hepatic failure with ascites, splenomegaly with hypersplenism, large osteolysis with pathological fractures, digestive tract involvement with malabsorption and weight loss; MCs: mast cells; AML: acute myeloid leukemia; MDS: myelodysplastic syndrome; MPN: myeloproliferative neoplasm; CEL: chronic eosinophilic leukemia; CMML: chronic myelomonocytic leukemia; NHL: non-Hodgkin's lymphoma.

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manner, was introduced in clinical trials. It was soon proven to improve the survival rates in patients with CML. Since then, the drug is considered first-line standard therapy in CP CML.[41] During the last ten years, two others TKIs have been approved for the treatment of CML. Dasatinib binds BCR-ABL1 as well as other major oncogenic kinases, such as SFK (Src-Family Kinases), in both their active and inactive states.[42] This rather specific drug effect leads to broader inhibition of ABL independent of the protein conformation, making dasatinib more potent in advanced CML. Nilotinib (AMN107) exhibits a higher binding affinity and selectivity than imatinib. Both nilotinib and dasatinib have been approved for the treatment of imatinib resistant and newly diagnosed patients.[43] More

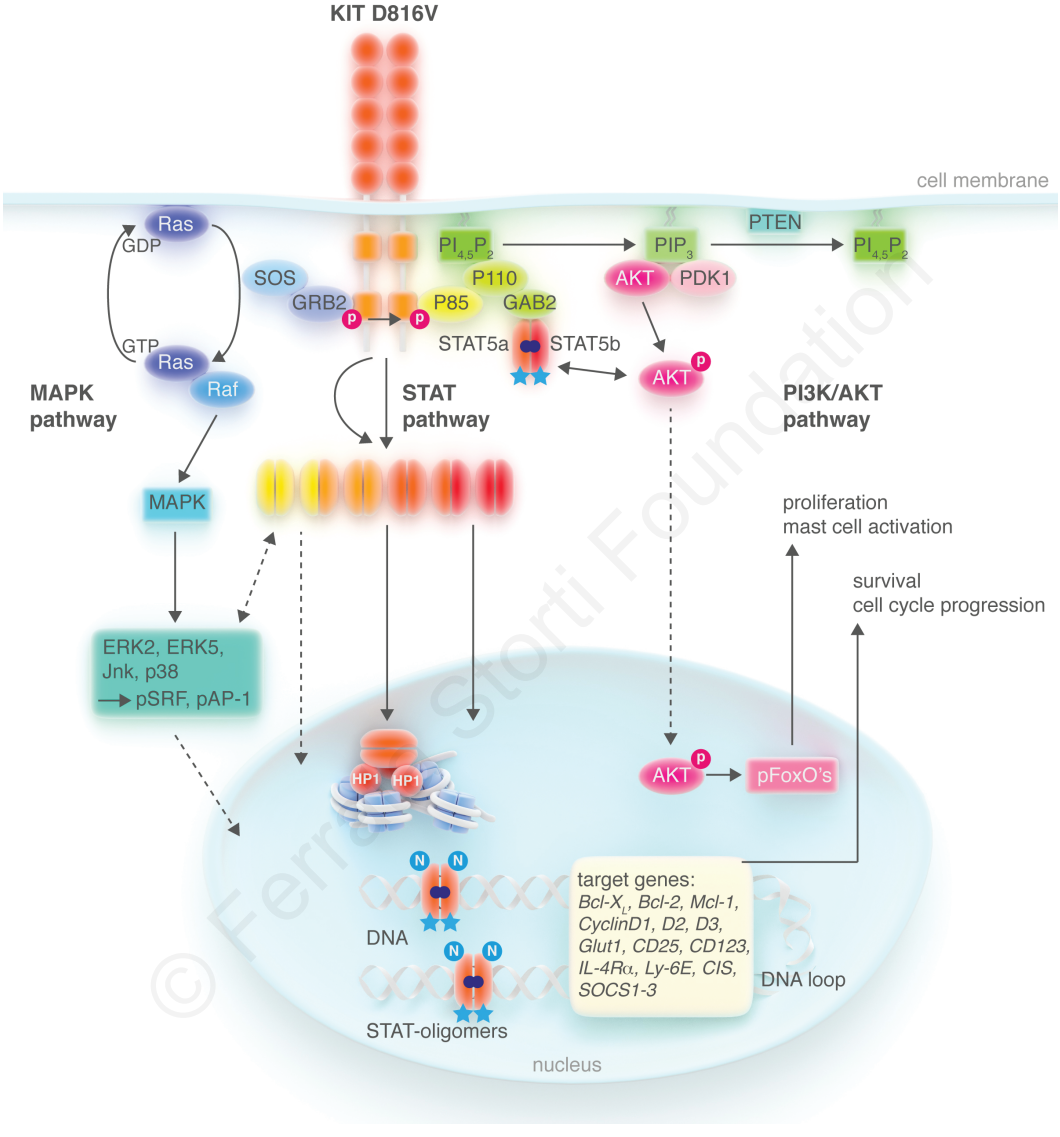

Figure 2. Major signal transduction pathways activated by the mutant KIT D816V receptor. The mutant receptor is represented under its monomeric form and at the cell membrane, although it remains unclear if it is present mainly in the cytoplasm or at the cell membrane in malignant mast cells, and if it is active under monomeric and/or dimeric form. Note the prominent role of STAT5 and AKT signaling in the proliferation induced by constitutive activation of the KIT receptor. It has been recently postulated that STAT5 can be directly activated by the mutant KIT receptor in the absence of JAK proteins (blue curved arrow). Moreover, STAT1 and STAT3 homo- and heterodimers were shown to be activated by mutant KIT and these can interact on higher order chromatin structures with STAT5 complexes so called STAT-Oligomers. STAT5 is efficiently dimerized and translocated to the nucleus upon full phosphorylation, which requires both tyrosine and serine phosphorylation as indicated by small orange circle or asterix. The cytoplasmic retention of pSTAT5 *via* the GAB2 scaffold protein was demonstrated to control PI3K-AKT signaling and this mechanism is important for survival of neoplastic mast cells, but it also slows down the transcription through nuclear pSTAT5. Furthermore, the lipid phosphatase PTEN (3´-inositol phosphatase and tensin homolog) further regulates negatively AKT signaling by dephosphorylation of PIP3. Other prominent signaling pathways triggering transcription factor changes in the nucleus such as forkhead family member phosphorylation which then leaves the nucleus upon AKT phosphorylation or RAS-RAF-MAPK signaling and their influence on transcription factor activation by phosphorylation events such as Serum response factor (SRF), AP-1 (Jun/Fos/ATF-1 homo- or heterodimers) is also illustrated. Moreover, unphosphorylated STAT5a was recently shown to interact with heterochromatin protein 1 (HP1α) and this mechanism protects DNA from noxias through keeping the densely packed chromatin, but pSTAT5 opens up chromatin most likely by its ability to induce oligomers *via* the N-terminus (black circles labeled with N and a dimerizer itself) and loop formation on DNA. This culminates into STAT5 target gene induction and most prominent genes known to be under control of STAT5 signaling in MC are exemplified, which leads to survival and growth.

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recently, bosutinib (SKI-606), which is more specific for BCR/ABL1 than imatinib or nilotinib, received approval as second-line drug for the treatment of imatinib-resistant Ph+ CML.[44] Finally, the third-generation TKI ponatinib (AP24534) has shown strong anti-leukemia activity in Ph+ CML patients, including those with the highly resistant *BCR-ABL1* T315I mutation.[45] It has entered clinical trials for the treatment of T315I+ patients and other drug-resistant patients, but early analysis of interim data evidenced a high occurrence of arterial thrombotic events, which led to the recent discontinuation of these trials.

Tyrosine kinase inhibitors have greatly improved survival rates and remission in CML, allowing higher major molecular response rates at five years, as reported, for instance, in the International Randomized Study of Interferon and STI571 (IRIS) trial.[46] However, approximately 20-30% of patients with CML develop either primary or secondary resistance to imatinib.[3] Several different mechanisms underlie TKI resistance in CML, such as increased BCR-ABL1 expression, overexpression of drug-efflux proteins, secondary mutations in *BCR-ABL1* that reduce drug affinity or drug effects, or upregulation and activation of downstream signaling molecules, including PI3K/AKT and STAT5 (Figure 3).[9,48] However, mutations in the kinase domain of BCR-ABL1 are thought to be a primary cause of resistance in patients with CML, seen in up to 40-60% of cases of secondary resistance.[3] These mutations can be detected usually in separate subclones, or rarely as compound mutations in the same clones. The most commonly detected mutation is T315I (20% of the retrieved mutations), which is resistant to almost all currently approved TKIs.[49]

Another challenging point in CML is the eradication of leukemic stem cells (LSC) which is a prerequisite for the development of curative therapies.[50,51] There is growing evidence that TKIs fail to eliminate all primitive CML LSC in most patients.[51] A better understanding of the biology and target expression profiles, as well as of the relationship between *BCR-ABL1*+ LSC and their specific microenvironment in the bone marrow (niche), has paved the way for the development of new treatment approaches.[51,52] These strategies involve drug combinations, such as the pharmacological silencing of BCR-ABL1 with simultaneous inhibition of crucial signal transduction pathways,[53] which may lead to the elimination, or at least suppression, of all CML LSC subsets.

The phosphoinositide 3-kinase (PI3K)-AKT pathway

Phosphoinositide 3-kinase (PI3Ks) represent a family of cytosolic, intracellular signaling proteins involved in the regulation of several cellular functions including proliferation and differentiation, survival, and malignant transformation.[54] The primary enzymatic activity of these kinases is the phosphorylation of the 3-OH of inositol head groups of phosphoinositide (PI) lipids.[55] PI3Ks can be divided into three main classes (I-III). These classes are based on their *in vitro* substrate specificity, structure, and probable mode of regulation. There are four isoforms of the catalytic subunit of class I PI3Ks: p110α, p110β, p110γ and p110δ.[56] Whereas α and β isoforms are expressed ubiquitously, γ and δ isoforms are expressed mainly in lymphocytes. Interestingly, increased expression of p110γ

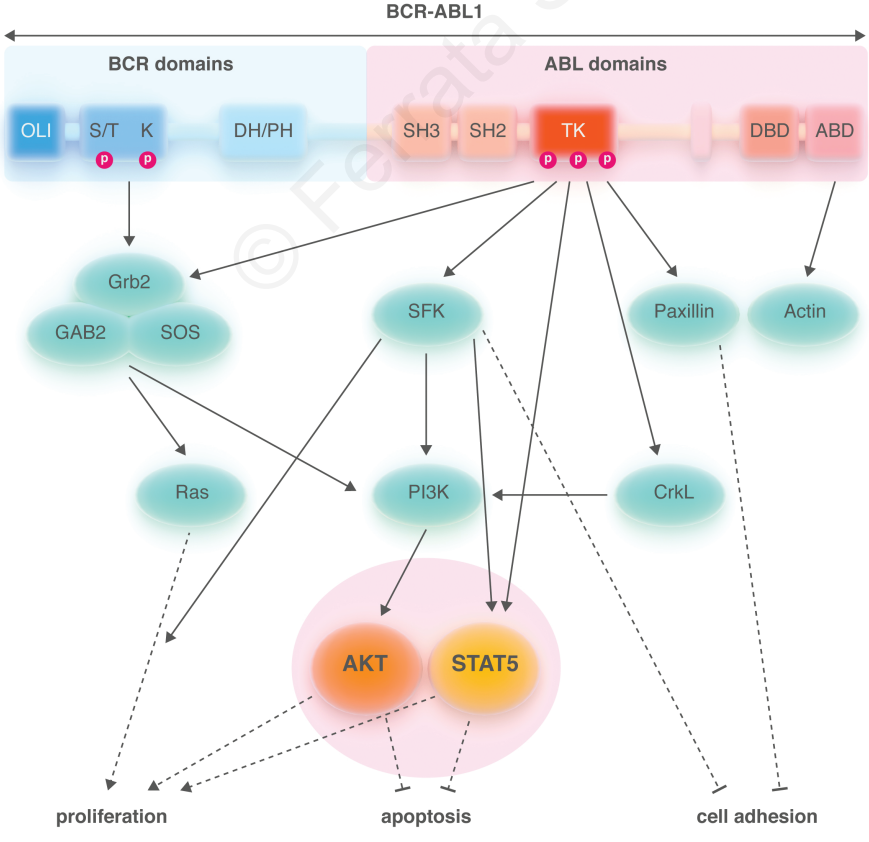

Figure 3. Effects of BCR-ABL1 (p210) functional domains on downstream signaling effectors. BCR-ABL1 signaling leads to enhanced proliferation, reduced apoptotic potential, and altered cell adhesion. Contributions from both BCR and ABL domains on downstream signaling are illustrated. OLI: oligomerization domain; S/T kinase: serine/threonine kinase; DH: Dbl homology; PH: Pleckstrin homology; SH2 or SH3: Src homology 2/3; TK kinase: tyrosine kinase; DBD: DNA binding domain; ABD: actin binding domain. Note the central place occupied by both AKT and STAT5 (red circle) in the signal transduction pathways leading to altered apoptosis and enhanced proliferation of CML cells.

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is seen in CML and might account for resistance to treatment.[57]

AKT, also known as protein kinase B (PKB), is the major signaling arm of PI3K. In mammals, AKT is present in three different isoforms, AKT1 (or PKBα), AKT2 (PKBβ), and AKT3 (PKBγ). These isoforms are products of distinct genes, but are highly related and exhibit greater than 80% homology. Each isoform possesses an N-terminal pleckstrin homology (PH) domain, followed by the kinase domain (KD), which shows a high degree of similarity to those found in PKA and PKC (Figure 4A).[58] Also present in this region is a threonine residue (T308 in PKBα/AKT1) whose phosphorylation is necessary for activation of AKT. Following the KD is a hydrophobic C-terminal tail containing a second regulatory phosphorylation site (S473 in PKBα/AKT1).

Following activation of receptor tyrosine kinases (RTKs), or of other cell surface receptors, the p85 adaptor subunit of PI3K associates with the receptor, leading to the activation of the p110 catalytic subunit. Activated p110 phosphorylates phosphatidylinositol 4-phosphate (PI4P), phosphatidylinositol 5-phosphate (PI5P) or phosphatidylinositol-4,5-bisphosphate (PIP2), generating thus the second messenger, phosphatidylinositol-3,4,5-triphosphate (PIP3) at the inner side of the plasma membrane (Figure 5). The interaction of the AKT PH domain with PIP3 induces conformational changes in AKT, resulting in the exposure of its two main phosphorylation sites at T308 and S473. Phosphorylation of these two sites by the S/TK3'-phosphoinositide-dependent kinases 1 and 2 (PDK1 and PDK2), which are also recruited by PIP3, is required for maximal activation of AKT. Phosphorylated AKT mediates the activation of various targets like Foxo, NF-κB and CREB transcription factors, the proapoptotic protein BAD, and cyclin D regulation through activating transcription factors downstream of mTOR/FRAP signaling. Overall, this results in anti-apoptotic effects, cell growth and proliferation (Figure 5).[59] Through these interactions, AKT

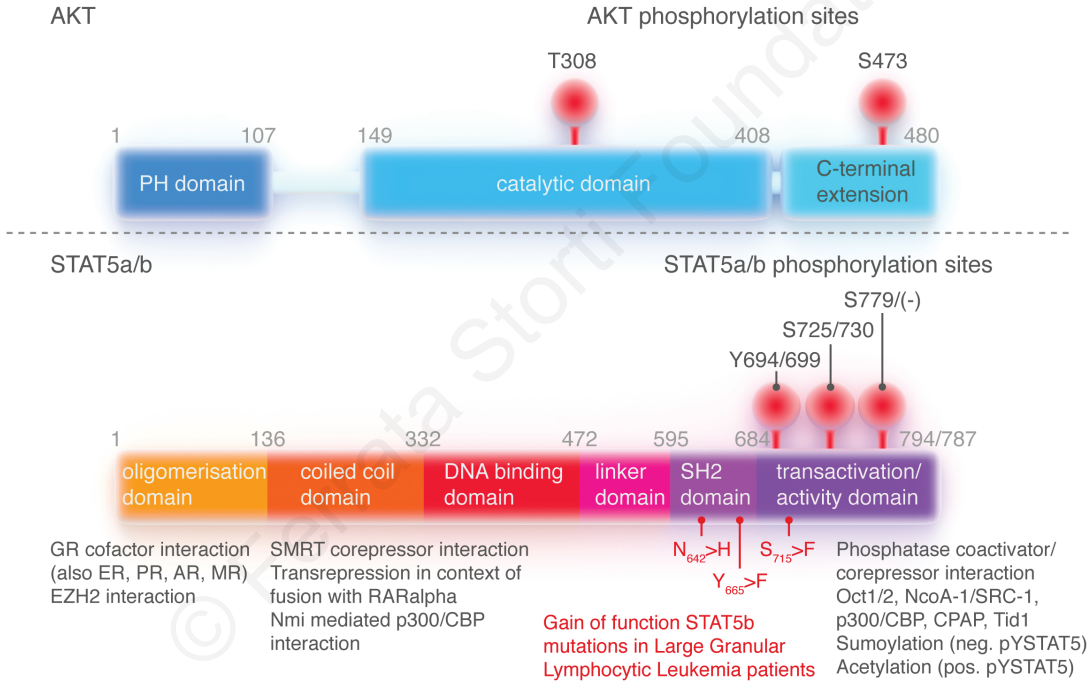

Figure 4. General structure of the PKB/AKT and STAT5a/b proteins. (A). AKT. All the PKB isoforms possess the kinase domain in the central region of the molecule. The PH (pleckstrin homology) domain acts as a phosphoinositide-binding module. The hydrophobic motif (HM) is located at the carboxyl-terminus adjacent to the kinase domain. Phosphorylation sites in the activation loop of the kinase domain and the hydrophobic motif are indicated. Length of the protein and positions of the phosphorylation sites may vary depending on the isoform. (B). Domain structure of the STAT5a/b isoforms. The N-terminal domain (ND), which prevents autoactivation and is the docking domain to cytokine receptors is known to interact with nuclear hormone receptors such as the glucocorticoid receptor (GR; similarly progesterone (PR), estrogen (ER), androgen (AR) and mineralocorticoid receptor (MR) might follow similar rules of docking to the STAT5 N-terminus, which is postulated due to close homology to the GR, but experimentally largely not precisely mapped). Moreover, the N-terminus of STAT5 is a dimerization domain that can execute higher order chromatin structures by STAT oligomerization and co-factor recruitment, where particular interaction with a histone methylase involved in many cancers called EZH2 is of significance. The coiled-coil dimerization domain (CCD) was reported to be involved in repression together with the extreme C-terminus and here SMRT co-repressor interaction was mapped. Moreover, it is crucial for transrepression in context of a RARalpha fusion oncoprotein found in AML patients and Nmi-mediated histone acetyl transferase activity through p300/CBP interaction was also linked to this domain. Moreover, p300/CBP are also bound by the C-terminus, which is also called transactivation and protein activity domain since tyrosine phosphatases recognize the end domain around the critical pY. The C-terminus binds also other proteins which were mapped and it is a domain that also undergoes acetylation, sumoylation and serine phosphorylation plus splicing or proteolytic processing. The SH2 domain (SD) is important for stable parallel dimer formation and efficient nuclear transport and DNA binding complex formation and the laboratory of Patrick Gunning used this domain for specific STAT inhibitors successfully.[11] The DNA binding domain (DBD) is in the middle and separated by a linker domain (LD) from the SH2 domain. Apart from critical tyrosine phosphorylation, also Serine phosphorylation (shown in red) is important for myeloid transformation with regard to STAT5a and STAT5b proteins. Of further importance are findings of gain of function mutations in STAT5b found in patients with large granulocytic leukemia (N642H or Y665F) both present in the SH2 domain. Finally, gain of function mutations like the STAT5a/b S710/S715F oncogenic variants were described elsewhere[47] and originate from retroviral screens to make IL-3-dependent myeloid cells factor independent.

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may contribute to malignant cell growth and disease evolution in *BCR-ABL1*$^+$ CML and *KIT* D816V$^+$ SM.[60,61]

Signals from PI3K are mainly antagonized by the 3´-inositol phosphatase and tensin homolog (PTEN) (Figure 5). PTEN is a tumor suppressor gene phosphatase that negatively regulates signaling through the PI3K pathway, inhibiting proliferation and survival.[62] Interestingly, using an animal model in which PTEN can be deleted in an inducible way, Furumoto *et al*. have recently shown that this deletion caused MCs hyperplasia in various organs, which was associated with increased phosphorylation of STAT5 and AKT.[63] Moreover, in another study, Peng *et al*. have reported that PTEN is down-regulated by BCR-ABL1 in an *in vivo* model of *BCR-ABL1*-induced CML and that overexpression of PTEN delayed the development of CML and prolonged survival of leukemia.[64] In the same study, it was shown that PTEN suppressed leukemia stem cells and induced cell-cycle arrest of leukemia cells.[64]

AKT and mastocytosis

Recently, AKT activation has been identified as a key signaling molecule involved in KIT-dependent growth of neoplastic MCs harboring oncogenic *KIT* mutants.[61] AKT was found constitutively phosphorylated in neoplastic MCs isolated from patients suffering from *KIT* D816V$^+$ SM and in the HMC-1 cell line, a human *KIT* D816V$^+$ leukemia MC line, raising the hypothesis that AKT activation plays a critical role in the pathogenesis of mastocytosis.[61] In line with this hypothesis, abrogation of AKT activity is associated with growth inhibition of neoplastic MCs expressing the oncogenic KIT D816V mutant.[61]

AKT and CML

Downstream signaling cascades in *BCR-ABL1*-transformed cells include the STAT-, RAS/RAF- and the PI3K/AKT/mTOR pathways, all of which affect cell viability, cell-cycle progression and leukemogenesis.[65] Activation of PI3K has emerged as one essential signaling step in ABL-mediated leukemogenesis. In line with this assumption, PI3K enzyme activity can be detected in BCR-ABL1 immunoprecipitates, which led to the initial assumption that activation of PI3K occurred mainly from a direct association of ABL with PI3K.[60]

The essential role of AKT in *BCR-ABL1*-mediated leukemogenesis was established by experiments demonstrating that the kinase-deficient dominant-negative Akt K179M mutant inhibits BCR-ABL1 induced transformation of BM cells *in vitro* and suppressed leukemia development in mice.[36] The residual leukemogenic potential of wild-type (wt) BCR-ABL1 in the presence of the dominant-negative Akt mutant is most likely due to Akt-independent mechanisms of transformation, although one cannot exclude incomplete suppression of Akt activation in cells co-expressing wt BCR-ABL1 and the Akt K179M mutant.[36] Consistent with the important role of AKT in *BCR-ABL1*-driven leukemogenesis, the constitutively active Akt E40K mutant rescued the defective transformation mediated by BCR-ABL1 SH2 mutants (delta SH2 and R1053L) *in vitro*.[36]

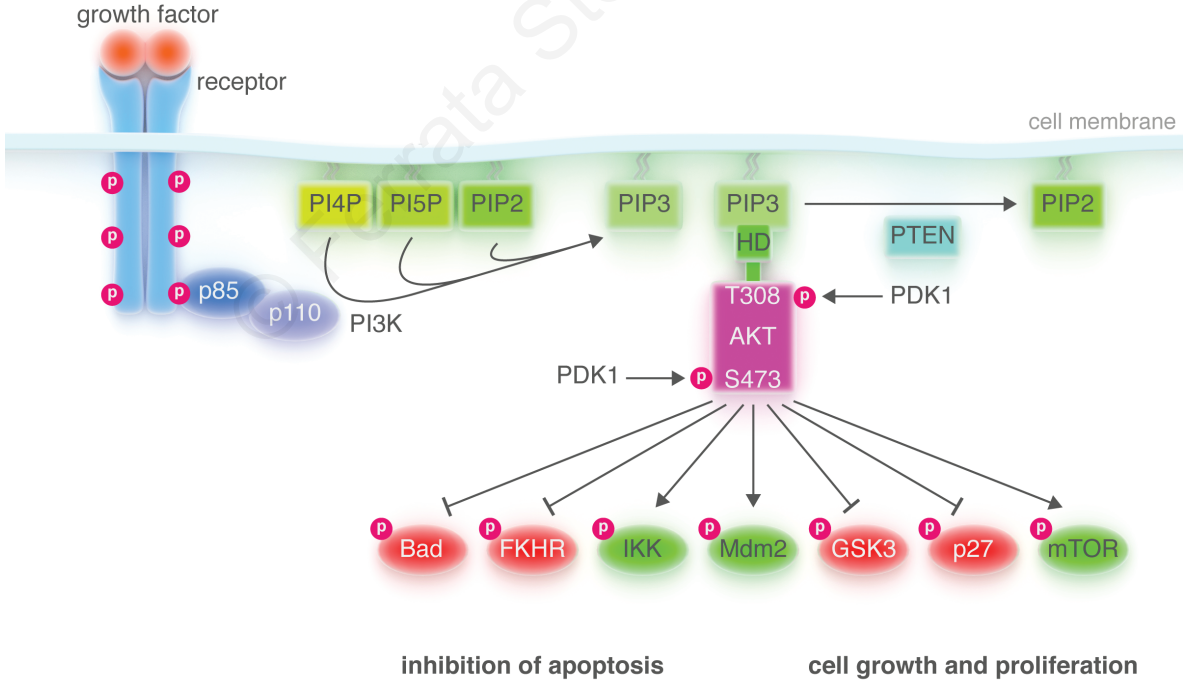

Figure 5. Activation of growth factor receptor protein tyrosine kinases results in autophosphorylation on tyrosine residues. PI3K is recruited to the membrane by directly binding to phosphotyrosine consensus residues of growth factor receptors or adaptors through one or both SH2 domains in the adaptor subunit. This leads to allosteric activation of the catalytic subunit. Activation results in production of the second messenger phosphatidylinositol (PtdIns)-3,4,5-trisphosphate (PIP3). The lipid product of PI3K, PIP3, recruits a subset of signaling proteins with pleckstrin homology (PH) domains to the membrane, including PDK1 and AKT. PTEN (a PI 3,4,5-P3 phosphatase) negatively regulates the PI3K/AKT pathway. Once activated, AKT mediates the activation and inhibition of several targets, resulting in cellular survival, growth and proliferation through various mechanisms.

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The importance of Akt as a signal transducer from the SH2 domain of BCR-ABL1, established by *in vitro* experiments, was confirmed *in vivo* using retrovirally infected BM cells injected into SCID mice.[36]

Compared with wt BCR-ABL1, cells expressing ΔSH2 BCR-ABL1 have markedly decreased leukemic potential as demonstrated by decreased tumor burden, only occasional involvement of non-hematopoietic organs, and diminished frequency of blastic transformation.[36] Co-expression of the constitutively active Akt E40K restored the leukemogenic properties of ΔSH2 BCR-ABL1 *in vivo*.[36] In addition, Chu *et al.* have demonstrated that, in CD34$^+$ cells from CML patients and human CD34$^+$ cells ectopically expressing the *BCR-ABL1* gene, cytoplasmic p27 levels were increased, allowing increased cell cycling and expansion in culture.[66] Interestingly, cytoplasmic relocation of p27 in CML progenitors was related to signaling through BCR-ABL1 Y177, activation of AKT kinase and phosphorylation of p27 on Thr-157 (T157).[66] These observations underline the importance of AKT-mediated p27 phosphorylation in altered p27 localization and enhanced proliferation and expansion of primary CML progenitors.

On the other hand, all the BCR-ABL1 mutants capable of activating PI3K can also activate AKT, as demonstrated for the T315I mutation in the KBM-5 cell line.[67] Thus, AKT appears to be the primary target of PI3K in the signaling pathway activated from the SH2 domain of BCR-ABL1, and is required for the *BCR-ABL1*-mediated leukemogenic transformation of hematopoietic cells.[36]

AKT inhibitors

AKT is viewed as an attractive target for cancer therapy and inhibition of AKT by targeted drugs is currently being evaluated in pre-clinical and clinical studies. AKT

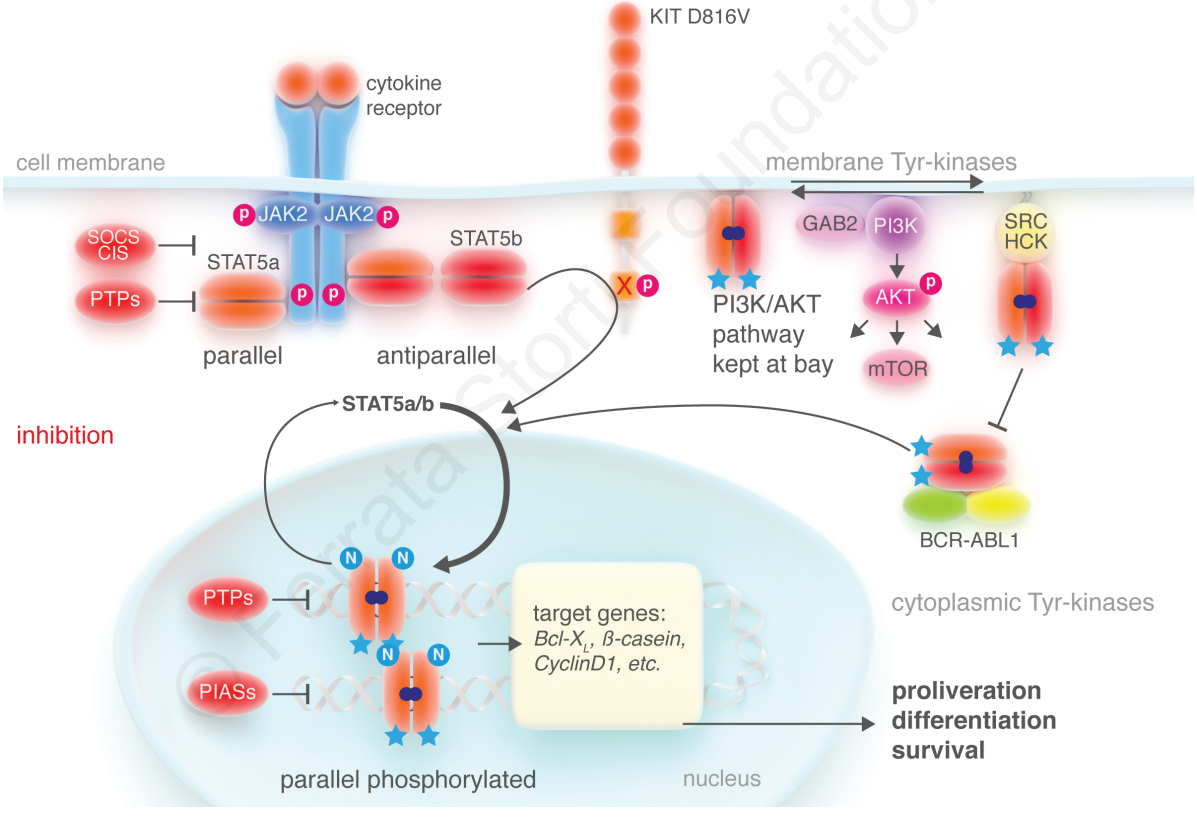

Figure 6. The canonical and non-canonical JAK-STAT pathway and its subversion by BCR-ABL or mutant KIT. Cytokine receptors, devoid of any tyrosine kinase activity, are constitutively linked to members of the JAK protein family. JAK kinases are cytoplasmic kinases where many cancer driver mutations of constitutively active JAK kinases have been mapped acting in a similar fashion like cytoplasmic fusion kinases such as BCR-ABL1 or the transforming membrane TK mutant KIT D816V. Upon activation of the cytokine receptor by ligand binding, conformational changes occur in the intracytoplasmic tail of the receptor, leading to activation of JAKs by auto- and transphosphorylation. Activated JAKs phosphorylate many substrates, among which are also the essential STAT proteins. Parallel STAT dimers are thought to be efficiently transported into the cell nucleus *via* associating with importins, bind then to specific DNA sequences and to activate the transcription through many cell type specific protein partners on target gene regulatory loci. This canonical pathway can be diverted in the presence of mutant KIT receptors (particularly, but not only KIT D816V), or by BCR-ABL1, both oncogenic proteins being able to phosphorylate STAT5 without JAK involvement (red arrows) by direct tyrosine phosphorylation *via* their kinase domain. This cascade of signalization is negatively regulated by at least three different families of proteins: protein tyrosine phosphatases (PTPs: SHP, CD45, PTP1B/TC-PTP and overall poorly studied), SOCSs (suppressors of cytokine signaling: CIS, SOCS1-SOCS3) or PIAS proteins (protein inhibitors of activated stats). PTPs dephosphorylate and recycle STAT5, while SOCS and CIS proteins bind either to the STAT5 activating receptors or they bind rather to the TKs. STATs are also negatively regulated by protein inhibitors of activated STAT (PIASs), which act in the nucleus through several mechanisms. For example, PIAS1 and PIAS3 inhibit transcriptional activation by STAT1 and STAT3, respectively, by binding and blocking access to the DNA sequences they recognize. Moreover, two non-canonical pSTAT5 cytoplasmic retention systems were found to be present in CML and/or neoplastic MCs, namely pSTAT5 retention by SRC and HCK kinases or docking to the p85 regulatory subunit of PI3K *via* the GAB2 scaffold protein that controls then AKT signaling. These cytoplasmic retention systems might block nuclear accumulation of pSTAT5, keeping STAT5-mediated transcription at bay.

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inhibitors include MK-2206, Tricibine (API-2), GSK690963, GSK2141795, KP372-1, Perifosine, Enzasturin (LY317615), PBI-05204, Erucylphosphocholine (ErPC), Erucylphosphohomocholine (ErPC3) and RX-0201.[68]

GSK2141795 is an AKT inhibitor with activity against various neoplastic cells, including blood and solid cancers. The drug has also been investigated in clinical trials with some success.[68] KP372-1 induces mitochondrial dysfunction and apoptosis in AML cells at concentrations ranging between 0.5 μM and 1.0 μM, but did not induce apoptosis in normal hematopoietic progenitor cells below 1.0 μM.[69]

Effects of perifosine have been evaluated in different tumor types, resulting in an IC50 between 8 and 20 μM after 24 h on T-acute lymphoblastic leukemia cells,[70] and between 1.25 and 6 μM (after 72 h) in human endometrial cancer cell lines.[71]

MK-2206, an allosteric AKT inhibitor, decreases the auto-phosphorylation of both AKT T308 and AKT S473. In addition, MK-2206 decreases T-acute lymphocytic leukemia cell viability by arresting the cells in the G0/G1 phase of the cell cycle, and by inducing apoptosis with IC50 values ranging between 1.7 and 5.1 μM.[72] It has also been shown that inhibition of PI3K/AKT signaling by MK-2206 affects the growth of both *JAK2* V617F- or *MPL*W515L-expressing primary neoplastic cells and cell lines *via* reduced phosphorylation of AKT and inhibition of its downstream signaling molecules.[73] In the same study, MK-2206 alleviated hepato-splenomegaly and reduced the megakaryocyte burden in the BM, liver and spleen of mice with *MPL* W515L-induced MPN.[73] However, most of these effects were only observed at concentrations of MK-2206 above 1.0 μM.[73] All in all, the currently available AKT inhibitors exhibit IC50 values at the micromolar range, suggesting that there is a need to develop inhibitors of AKT acting at the nanomolar range. In addition, the use of AKT inhibitors in combination may increase their effects on cell proliferation. For instance, it has been recently demonstrated in the human leukemic MC line (HMC-1.2) expressing mutant *KIT* that treatment with STAT5-shRNA and LY294002 (PI3K inhibitor) resulted in an 80% inhibition of proliferation, which was superior to that induced by either STAT5-shRNA alone (60%) or Ly294002 treatment alone (55%).[74] Moreover, the effects of STAT5 and PI3K/AKT inhibition on cell cycle are additive.[74] Thus, the simultaneous targeting of PI3K/AKT and STAT5 signaling pathways may even better inhibit malignant cell proliferation in CML and SM.

JAK-STAT signaling

Seven mammalian STAT proteins have been identified, namely STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6.[75] All STAT proteins share the ability to transmit a cellular signal from the cell membrane to the DNA, by steering transcriptional regulation of important genes relevant for normal or neoplastic cell growth or cell survival. STAT transcription factors are activated by various ligands and act together with cell type-specific co-factors or co-repressors which may explain in part their cell-specificity.[76] Likewise, essential tyrosine and serine/threonine phosphorylation sites related to the transforming function of STATs have been mapped (Figure 4B).[77]

Although non-phosphorylated STAT5 (non-pSTAT5) may epigenetically suppress tumor growth by promoting heterochromatin formation, acting thus as a "tumor-suppressor",[78] the major role of STAT5 (like other STATs) is to promote the transcription of different genes. To fulfill this role, STAT5: i) undergoes an activation consisting in a tyrosine-phosphorylation step; ii) dimerizes through reciprocal interaction mediated by the phosphor-tyrosyl residue and the SH2 domain of the STAT monomers; iii) is internalized into the cell nucleus *via* associating with importins; iv) binds a specific DNA sequence; and v) activates the transcription through recruitment of protein partners. In addition, heterochromatin protein 1 (HP1), a conserved chromatin binding protein involved in heterochromatin assembly and gene silencing, and acting as a tumor suppressor in leukemogenesis, is inhibited by pSTAT5 protein, leading to enhanced cell proliferation activity (Figure 2).[79]

Several STAT activators have been described of which the canonical JAK-STAT pathway is best known (Figure 6).[80] Thus, activating mutations in JAK2 may lead to STAT5 phosphorylation. Notably, the *JAK2* V617F mutation is present in over 95% of patients with polycythemia vera (PV), and in approximately 50% of patients with primary myelofibrosis (PMF) or essential thrombocythemia (ET).[81] Interestingly, this mutation induces increased expression of Stat5 in mice.[82] There are differences in the recruitment of different STATs between *JAK2* V617F^{+} MPNs, but both mice and humans with PV have a very significant activation of STAT5.[83] Furthermore, in mice, it has been shown that activation of STAT5 was essential to the initiation and maintenance of PV after introduction of the JAK2 V617F mutant.[82]

Besides, the implications of the TK receptors and transforming intracellular TKs as STATs activators have been extensively reported.[84,85] Among the STAT proteins, particularly activated STAT5a/b proteins (Figure 6) are thought to be of importance in MPNs, including SM and CML.[5,7] However, SM or CML are often slowly progressing diseases, and it is thus not surprising that there are cytoplasmic pathways that maintain high pSTAT5 levels in the cytoplasm. Direct docking of pSTAT5 *via* the scaffold molecule GAB2 to the regulatory p85 subunit of PI3K is a prominent mechanism of cytoplasmic retention (Figures 2 and 6).[86]

In myeloproliferative neoplasms (MPN), important target genes of activated STAT5 are survival genes such as Mcl-1, Bcl-2, Bcl-X_L, proliferation genes such as Cyclin D1 to D3, C-Myc, or cytokine receptor chains such as IL-4Ralpha chain, CD25 and CD123, both of which are expressed on CML LSC, the lymphocyte antigen Ly-6E,[87] or negative regulators such as Cis or Socs1-3 (Figure 2).[88]

Thus, STAT5 is a potential major target in MPNs for which so far there are no specific and potent pharmacological inhibitors. However, Page *et al*. could show that the SF-1-088 salicylic acid-containing inhibitor binds the SH2 domain of STAT5, decreasing the binding of STAT5 to its phosphorylating partner, thus inducing a lower phosphorylation level and diminished transcription through it.[11]

STAT5 and mastocytosis

The involvement of STAT5 in growth and survival of normal and neoplastic MCs is well known.[89] Consequently, as the uncontrolled cell growth is one feature of tumors, several teams studied the implication of STAT5 in neoplastic MC growth, survival and transforma-

tion, and some light was shed on its implication in tumor growth downstream of *KIT* D816V.

A first study published by Gouilleux and colleagues showed that pSTAT5 is found in the cytoplasm of MCs from patients with SM.[90] The study also further emphasized the molecular interactions between STAT5 and PI3K *via* the GAB2 scaffold protein interaction bridging p85 and pSTAT5 interaction (Figures 2 and 6). Moreover, knockdown of STAT5 (or AKT) led to cell growth inhibition.[61] Thus, STAT5 function in MC neoplasms is linked to PI3K-AKT signaling and intrinsic cytokine signaling by IL-3/-4 will further boost their synergism.[61,91]

A second study has further explored this non-canonical STAT pathway, and has shown that neoplastic MCs express cytoplasmic and nuclear pSTAT5.[91] Furthermore, the same team showed that KIT D816V promotes direct STAT5-activation, and that it contributes to growth of neoplastic MCs.[91] Finally, despite STAT1/3 activation, the expression levels of STAT5 seem to be critical for transcriptional regulation in HMC-1 and P815 MC lines, and for neoplastic cell growth and survival.[7]

Altogether, these results strongly suggest that STAT5 is one major cellular effector in mastocytosis by controlling the mutant KIT-mediated aberrant growth signaling. However, the pharmacological inhibition of STAT5 remains challenging, and new STAT5 inhibitors active at pharmacological doses on both indolent and aggressive forms of SM are still needed.

STAT5 and CML

In CML, BCR-ABL1 was shown to directly phosphorylate STAT5 (Y694/Y699; Figure 6) that then dimerizes in a parallel fashion to allow rapid nuclear translocation and oligomerization on chromatin to regulate gene transcription, which subsequently promotes myeloid cell survival and growth.[92] However, pSTAT5 appears mostly retained in the cytoplasm in *BCR–ABL1*-positive cells, this retention being linked to binding to GAB2 or to Src family kinases (Figure 6).[90,93] Whatever mechanism underlies cytoplasmic retention of pSTAT5, more recent studies on STAT5 in CML cells have proven that this molecule is necessary for both transformation and cell cycle progression.[4] In addition, STAT5a and STAT5b suppression by siRNA transfection mediated CML cell apoptosis,[94] and STAT5a suppression induced a higher sensitivity of imatinib-sensitive K562 cells to imatinib, and sensitized imatinib-resistant K562 cells to imatinib.[95] Interestingly, high levels of pSTAT5 are correlated to TKI resistance *in vitro* and *in vivo*, and to CML progression.[9] Furthermore, a recent publication described a highly significant correlation between the level of *STAT5a* mRNA and the occurrence of *BCR-ABL1* mutations in a cohort of 50 CML patients, possibly mediated by the enforced production of reactive oxygen intermediates.[96] Moreover, using a mouse model with a conditional null mutation in the *Stat5a/b* gene locus, Waltz *et al.* have determined the requirement for STAT5 in MPNs induced by *BCR-ABL1* in a retroviral transplantation model of CML.[82] They provided evidence that the loss of one Stat5a/b allele results in a decrease in *BCR-ABL1*-induced CML-like MPN and the appearance of B-cell acute lymphoblastic leukemia, whereas complete deletion of Stat5a/b prevented the development of leukemia in primary recipients.[82] However, the specific contributions of the two related genes, *Stat5a* and *Stat5b*, to growth and survival of CML cells were not clarified in this report. In a recent study using an RNAi-based strategy, Casetti *et al.* showed that *STAT5a/STAT5b* double-knockdown triggers CML cell apoptosis and suppresses the long-term clonogenic potential of normal and CML progenitor cells.[97] In addition, the same authors reported that STAT5a attenuation alone was ineffective at impairing growth of normal and CML CD34$^+$ cells isolated at diagnosis. In contrast, STAT5a attenuation was reported to be sufficient to enhance basal oxidative stress and DNA damage of normal CD34$^+$ and CML cells and to inhibit growth of CML CD34$^+$ cells from patients with acquired resistance to imatinib.[97] These data are in line with those reported by Rousselot *et al.* who have demonstrated that targeting expression of STAT5a and b using pioglitazone, a peroxisome proliferator-activated receptor (PPARs)-agonist, resulted in an improvement in molecular response in patients with CP CML treated with imatinib.[98]

All in all, the above-mentioned reports provide solid evidence that targeting STAT5 may be an attractive therapeutic approach in CML. A complete loss of STAT5 might not be beneficial because of the important biological roles this molecule plays in physiological tissues. Rather, interfering with the extra production or activation of (too much) pSTAT5 in neoplastic cells might be the right way to go. This should be done by direct targeting of the molecule or by targeting distinct STAT5-controlled survival proteins such as BCL-2/BCL-X_L or to interfere with cytoplasmic control *via* STAT5 on AKT/mTOR signaling.[99] Finally, targeting STAT5 or related signals activated by this molecule could not only overcome drug resistance as well as disease progression, but also might open opportunities to eradicate the most primitive and TKI-resistant CML LSC populations.

STAT5 inhibitors

Cell lines or CD34$^+$ cells from CML patients treated with pimozide revealed decreased pSTAT5 levels.[10] Furthermore, pimozide showed major effects on cell survival and induced cell cycle arrest and apoptosis in CML cells. In addition, pimozide showed synergistic anti-leukemic effects together with imatinib, presumably through decreased STAT5 phosphorylation.[10] Finally, pimozide also exhibits inhibitory effect on CD34$^+$ CML cell growth, whereas non-CML cells are only slightly affected,[10] suggesting that a STAT5-targeted therapy may act rather specifically on leukemic cells over-expressing activated STAT5. However, the concentrations of pimozide required to elicit anti-leukemic effects were rather high.[10]

Two other classes of STAT5 inhibitors have also been reported recently.[11,100] These drugs share common features in their inhibition-profiles: suppression of STAT5 activation and induction of apoptosis. Although no results on imatinib-resistant or primary CML cells are available for acid-salicylic-containing molecules,[11] indirubin derivatives have shown anti-neoplastic activity in imatinib-sensitive, T315I-positive-imatinib-resistant and CD34$^+$ primary CML cells.[100] However, these effects were obtained at an IC50 of around 5 μM, which might be difficult to achieve during administration to humans. Further side group modifications or screening of new small molecular weight compound libraries could improve selectivity and efficacy in a next generation of STAT5 isoform inhibitors.

Conclusions and future perspectives

Multiple lines of evidence suggest that the STAT and the PI3-K/AKT pathways are crucial for disease evolution and progression in CML and advanced SM. Indeed, in both types of neoplasm, these two effector molecules are activated and may act together to trigger proliferation and survival as well as drug resistance in neoplastic (stem) cells. Moreover, chemical inhibition or gene silencing experiments have shown that both AKT and STAT5 are required for oncogenesis and disease evolution, and that co-inhibition of both STAT5 and AKT cells may elicit synergistic effects on leukemic cell growth and proliferation in these two malignancies. As a result, STAT5 and AKT are currently regarded to be the most attractive potential targets of therapy in advanced CML and SM, and there is hope that their simultaneous pharmacological inhibition could lead to improved anti-neoplastic effects. Whether such an approach will indeed overcome drug resistance in neoplastic (stem) cells in these malignancies remains to be determined in forthcoming pre-clinical and clinical trials. In addition, besides CML and SM, there is substantial interest in targeting PI3-K/AKT and STAT5 molecules in other MPNs such as PV, ET or PMF whereas JAK2 inhibitors have failed to provide substantial therapeutic effects, being unable to lead to complete remission of these diseases.

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