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Review

Role of anti-apoptotic pathways activated by BCR/ABL in the resistance of chronic myeloid leukemia cells to tyrosine kinase inhibitors

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Chronic myeloid leukemia (CML) is a hematological stem cell disorder characterized by the excessive proliferation of the myeloid lineage. In its initial chronic phase, the myeloid progenitor cells expand and demonstrate apparently normal differentiation. The disease may then transform into the accelerated phase, usually associated with resistance to therapy, and finally, into acute leukemic progression phase — blast crisis. Abnormal myeloid cells produce progenitors, which have lost their ability to differentiate, but retain the capacity to proliferate. The molecular hallmark of CML is the Philadelphia chromosome, resulting from reciprocal chromosome translocation, t(9;22)(q34;q11), and containing the BCR/ABL fusion gene, producing the BCR/ABL protein with a constitutive tyrosine kinase activity. BCR/ABL-positive cells have faster growth and proliferation over their normal counterparts and are resistant to apoptosis. Introduction of imatinib (IM), a tyrosine kinase inhibitor, revolutionized the therapy of CML, changing it from a fatal disease into a chronic disorder. However, some patients show a primary resistance to IM, others acquire such resistance in the course of therapy. Therefore, a small number of leukemic stem cells retains self-renewal capacity under IM treatment. Because BCR/ABL is involved in many signaling pathways, some of them may be essential for resistance to IM-induced apoptosis. The PI3K/AKT, Ras and JAK/STAT signaling pathways are involved in resistance to apoptosis and can be activated by BCR/ABL. Therefore, they can be candidates for BCR/ABL-dependent pro-survival pathway(s), allowing a fraction of CML cells to withstand treatment with tyrosine kinase inhibitors.

Key words: BCR/ABL, chronic myeloid leukemia, apoptotic signaling, tyrosine kinase inhibitor, imatinib, drug resistance

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INTRODUCTION

Chronic myeloid leukemia (CML), the first described type of leukemia (Bennett, 1845; Craige, 1845), is a clonal myeloproliferative disorder of the multipotent hematopoietic stem cells (HSCs). It is frequently detected in its early stage, termed chronic phase, followed by the progression to an intermediate — accelerated phase, and finally to a terminal stage, called blast crisis. The first clue to the pathogenesis of CML was the discovery of an acquired genetic abnormality — an abnormal shortened chromosome present in the pluripotent stem cells within the bone marrow, designated as the Philadelphia (Ph) chromosome (Nowell & Hungerford, 1961). Ph results from a balanced reciprocal translocation between the long arms of chromosomes 9 and 22 t(9;22)(q34;q11) (Nowell & Hungerford, 1960). During this translocation, most of the Abelson *c-ABL* proto-oncogene is transposed from its location on chromosome 9 to the breakpoint cluster region (*BCR*) gene, located on chromosome 22, forming the *BCR/ABL* fusion gene (Rowley, 1973). For the majority of CML patients, the breakpoints in the *ABL* gene occur in its intron 1 or 2 and between exons 13 and 14, or 14 and 15 of the *BCR* gene (Groffen *et al*., 1984). The new *BCR/ABL* fusion gene encodes a chimeric 8.5 kb mRNA, which is translated into a hybrid 210 kDa oncoprotein, p210 BCR/ABL (Lugo *et al*., 1990). Experimental evidence supports the central role of p210 BCR/ABL in inducing and sustaining CML (Clark *et* al., 1989). It was shown in *in-vitro* studies on cell line model (Laneuvilleet *al*., 1992) and in animal models (Gishizky *et al*., 1993), that the presence of p210 is crucial and sufficient to induce malignant transformation resulting in CML (Gishizky *et al*., 1993). It was also shown that transplantation of p210 BCR/ABL-transduced HSCs or its transgenic expression leads to leukemia, lymphomas and CML-like syndromes (Li *et al*., 1999), proving the direct connection to CML induction. The expression of p210 BCR/ABL leads to an increased proliferation of hematopoietic cell lines *via* induction of growth factor independence (Jiang *et al*., 2000), changes in adhesion properties (Bhatia *et al*., 1999), and increased resistance to apoptosis (Cortez *et al*., 1995).

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Abbreviations: Apaf-1, apoptotic protease activating factor-1; Ara-C, cytosine arabinoside; Bcl-xL, B-cell lymphoma-extra large protein; *BCR*, the breakpoint cluster region gene; *c-ABL*, the Abelson proto-oncogene; CCyR, complete cytogenetic response; CHR, complete hematologic response; CML, chronic myeloid leukemia; ER, endoplasmic reticulum; FOXO, forkhead box O transcription factor; GATA-2, GATA binding protein 2; GRB2, growth factor receptor-bound protein 2; HSC, hematopoietic stem cell; IGF, insulin-like growth factor; IL-3, interleukin 3; IM, imatinib mesylate, imatinib; IKK, I-κB kinase; INF-α, interferon-α; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; MCyR, major cytogenetic response; MDR/P1, multi-drug resistance protein (P-glycoprotein) 1; MDM2, mouse double minute 2 homolog; Nox-4, NADPH oxidase 4; PDGF, platelet derived growth factor; PDGF-R, platelet derived growth factor receptor; PERK, PKR-like ER-resident kinase; Ph chromosome, the Philadelphia chromosome; PHLPP, PH domain leucine rich repeat protein phosphatase; PI3K, phosphatidylinositol-3-kinase; PKB, protein kinase B; PKR, double-stranded RNA-dependent protein kinase (PKR); PMRD16, polymyxin resistance protein *16;* PP1α, protein phosphatase alpha; PtdIns, phosphatidylinositol; SH2, Src homology domain 2; STAT, signal transducer and activator of transcription; STI, signal transduction inhibitors; TK, tyrosine kinase; UPR, unfolded protein response

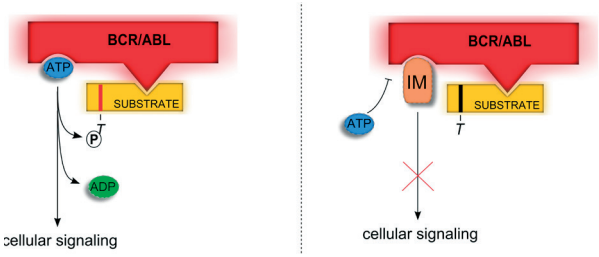

Figure 1. Imatinib (IM) inhibits BCR/ABL activity by blocking the site of ATP binding in the active center

INHIBITION OF BCR/ABL TYROSINE KINASE ACTIVITY WITH IMATINIB

Standard treatment options for chronic CML included hydroxyurea, allogenic stem cell transplantation and interferon-α (INF-α). Since the *BCR/ABL* gene, its mRNA as well as the BCR/ABL fusion protein are typical for CML progenitors, they constitute a suitable target for therapy. New therapeutic options were focused on three main areas — the inhibition of *BCR/ABL* gene expression by antisense strategies, stimulation of the immune system to recognize and destroy leukemic cells, and the use of specific signal transduction inhibitors in order to modify certain protein functions. The latter proved to be the most promising.

The central role of BCR/ABL tyrosine kinase activity in leukemic transformation was the reason why the inhibition of the enzyme activity became such an attractive therapeutic target for CML patients (Oda *et al.*, 1995). Chemicals displaying the ability to inhibit the BCR/ABL kinase, belong to a new class of anticancer drugs – signal transduction inhibitors (STI).

Imatinib mesylate (IM) (Gleevec, STI571 by Novartis Pharma AG, Switzerland) is a selective inhibitor of BCR/ABL, and its introduction led to a significant change in CML treatment. IM functions by binding to the highly conserved ATP-binding pocket of the ABL catalytic domain, thereby preventing the phosphorylation of the tyrosine residue, which in turn leads to the inhibition of cellular signaling (Talpaz *et al.*, 2002) (Fig. 1). It was shown to inhibit the proliferation of leukemic cells and restore interleukin 3 (IL-3) dependent growth and differentiation of BCR/ABL positive cells with practically no effect on normal cells (Deininger *et al.*, 1997). IM downregulates anti-apoptotic proteins such as B-cell lymphoma-extra large protein (Bcl-xL), signal transducer and activator of transcription 5 (STAT5), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and protein kinase B (PKB, AKT) (Deininger *et al.*, 2000). It was found to inhibit all ABL tyrosine kinases (Druker *et al.*, 2002), as well as the SCF, c-KIT tyrosine kinase (Savage & Antman, 2002) and cellular processes activated by PDGF and SCF (Sawyers *et al.*, 2002). This broad range of activity of IM and inhibiting the BCR/ABL tyrosine kinase in *in vivo* and *in vitro* studies was the reason for its introduction to clinical trials for patients resistant to INF-α therapy. We showed that the IM anti-leukemic mechanism of IM action might include not only the inhibition of BCR/ABL tyrosine kinase, but also induction of BCR/ABL-specific DNA damage (Czechowska *et al.*, 2005; Majsterek *et al.*, 2006).

Randomized study was conducted in chronic phase CML patients resistant to IFN-α or intolerant to therapy with this agent. Complete hematologic response (CHR), manifested by the normalization of the white blood cell counts, was observed in 93% of patients, 60% of patients showed major cytogenetic response (MCyR) and 42% showed complete cytogenetic response (CCyR) (Silver *et al.*, 2004). Another study reported that 53 out of 54 patients had CHR following 4 weeks of IM treatment, most of whom retained the response for over 1 year (Druker *et al.*, 2001). IM used in the advanced stages of CML is significantly less effective, and the responses obtained are usually short termed (Druker *et al.*, 2001).

MECHANISMS OF RESISTANCE TO IMATINIB

Despite the breakthrough in CML treatment associated with IM use, resistance to this drug became an emerging problem. There are two basic types of resistance to IM therapy: primary and secondary (acquired). Primary resistance occurs when after 3 months of treatment CHR is not achieved, when a 6-month treatment fails to induce any cytogenetic response (Cyr), or when following 1 year of treatment, no MCyR was achieved. Secondary resistance occurs when the CHR or cytogenetic response gained earlier is lost. IM resistance was observed much more frequently in advanced stages of CML, especially in the blast crisis (70% of patients) (Hochhaus & La Rosee, 2004). A low effectiveness of IM treatment in the advanced stages of CML might be a result of various molecular events accumulating simultaneously with the disease progression (Skorski, 2008). We showed that BCR/ABL kinase stimulates the production of ROS, which results in oxidative DNA damage, leading to mutations in the BCR/ABL kinase. Inhibition of ROS in leukemia cells by the use of antioxidants decreased the mutagenesis rate and frequency of IM resistance (Koptyra *et al.*, 2006). Since BCR/ABL kinase induces genomic instability, IM should impede the accumulation of further genetic changes in CML cells. Indeed, IM reduced the accumulation of reactive oxygen species (ROS), oxidative DNA damage, point mutations, and other genetic aberrations in BCR/ABL cells (Koptyra *et al.*, 2006). However, point mutations and chromosomal aberrations continue to accumulate in IM-treated cells (Nowak *et al.*, 2010). We showed that BCR/ABL kinase disturbed DNA repair by inhibiting the mismatch repair (MMR) system in CML cells, which may be one of the direct causes for point mutations generation in *BCR/ABL* and other genes, including *p53* and *Rb,* leading to IM resistance as well as to the malignant progression of CML (Stoklosa *et al.*, 2008).

Mutations are one of the most prevalent mechanisms leading to primary resistance in patients with CML and are observed in 50–90% of cases (Deininger *et al.*, 2005). A sequencing study of patients in blast phase CML showed mutations in almost 77% of cases (Grossmann *et al.*, 2011). Point mutations in *BCR/ABL* can lead to a change in the amino acid sequence directly involved in the interaction with IM, or to conformational changes in the tyrosine kinase activation loop (Gorre *et al.*, 2001) (Fig. 2A). IM binds to the ABL kinase domain in its inactive form and induces various types of conformational changes, while binding the protein substrate (Schindler *et al.*, 2000) (Fig. 2B). Mutations in the amino acid sequence directly interacting with the drug, prevent the conformation which enables binding of the drug (Shah *et al.*, 2002) (Fig. 2C).

Over 40 different point mutations associated with resistance to IM were identified (Al-Ali *et al.*, 2004). A number of BCR/ABL mutants were allocated outside of the ABL kinase domain — in the Src homology domain 2 and 3 (SH2 and SH3) of ABL. Those domains are cru-

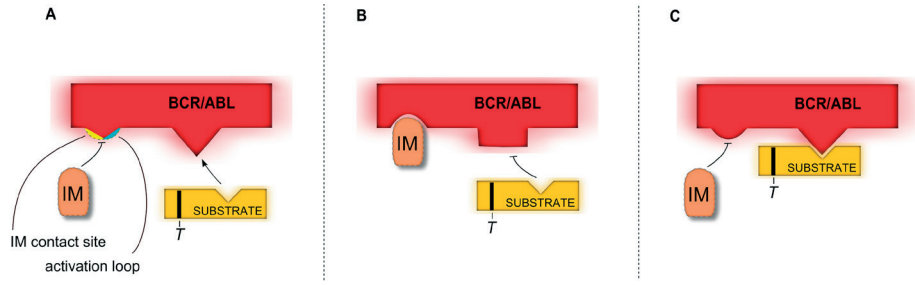

Figure 2. Schematic representation of resistance to imatinib (IM)

cial for the conformational change from the inactive to active form of ABL. Since IM binds to the inactive conformation of ABL, mutations occurring in these domains may impede the functioning of the drug. The most frequently observed point mutations are T315I, E255K and M351T (Deininger *et al.*, 2005). It was shown that some CML patients, with activated TK domain in BCR/ABL, had no mutations in the ABL kinase domain (Schindler *et al.*, 2000).

In vitro studies in human and murine BCR/ABL positive cell lines resistant to IM revealed that a frequent mechanism of resistance is the amplification and overexpression of the *BCR/ABL* gene (Mahon *et al.*, 2000). Amplification of *BCR/ABL* constitutes about 10% of resistance cases (von Bubnoff *et al.*, 2003). It allows the lymphoid cells to maintain sufficiently high level of cellular signaling, allowing cell survival even in the presence of IM. Dosage increase is usually successful in overcoming this type of resistance.

Clonal evolution is yet another mechanism of IM resistance. The emergence of genetic aberrations leads to the activation of new cellular signal transduction pathways, avoiding the inhibitory effect of IM. Genetic aberrations associated with the progression of CML into the blast crisis may play a role in IM resistance (Lahaye *et al.*, 2005). Mutations in GATA binding protein 2 (GATA-2), partial deletions of RUNX1 and polymyxin resistance protein 16 (PMRD16), as well as expression of RUNX1/PMRD16, detected in the CML blast phase, may result in the disturbance of myelomonocytic cell differentiation, indicating their involvement in disease transformation and drug resistance (Kim *et al.*, 2010).

Another cause of IM resistance is a mechanism leading to the reduction in the intracellular concentrations of IM. This can occur following the expression of the alpha1-acid glycoprotein, resulting in the reduction of drug influx into the cell (Peng *et al.*, 2005), or by the expression of the multi-drug resistance protein (P-glycoprotein) 1 (MDR/P1), which in turn increases drug efflux out of the cell (Breedveld *et al.*, 2006).

The frequency of additional chromosomal aberrations is about 7% in the chronic phase CML and rises to 40-70% in the advanced blastic phase of the disease (Bacher *et al.*, 2005). Approximately 70-80% of patients with CML show additional non-random chromosomal abnormalities (Deutsch *et al.*, 2001) involving chromosomes 8, 17, 19 and 22, the most frequent being the duplication of the Ph chromosome (Mitelman, 1993) or its trisomy (Dubrez *et al.*, 1998). The low efficacy of IM treatment may be due to its insufficient dosage in the light of an increased number of Ph chromosome-containing cells. The higher dose (800 mg/d) of IM, however, proved to be toxic, which unavoidably limits the suitability of this drug for treatment. IM is was tested in combination with other drugs in BCR/ABL-positive cells (O'Dwyer, 2002).

SIGNIFICANCE OF REDUCED APOPTOSIS FOR LEUKEMOGENESIS

Chronic phase CML cells were known to exhibit decreased apoptosis and activate several hematopoietic signaling pathways (Galbraith & Abu-Zahra, 1972). Acute phase of CML is characterized by the rapid increase of immature myeloid blast cell number in peripheral blood. Once fully committed to differentiation, all HSCs have finite lifespans and undergo programmed cell death at fixed times depending on the lineage and environmental factors (Squier *et al.*, 1995). There are numerous reports demonstrating that apoptosis is inhibited under a variety of conditions in cell lines expressing p210 BCR/ABL, e.g. following activation of Ras-dependent signaling pathway and a pathway leading to *myc* RNA induction. (Scheid *et al.*, 1998).

BCR/ABL activity facilitates the accumulation of various molecular and chromosomal aberrations, leading directly or indirectly to reduced apoptosis susceptibility of CML blast-crisis cells. Those aberrations include duplication of Ph chromosome, trisomy 9, trisomy 19 (Chopra *et al.,* 1999), point mutations in the coding sequences of *RAS* (Cogswell *et al.,* 1989), *p53* (Feinstein *et al.*, 1991), *MYC* (Sawyers, 1993) or rearrangements of *Rb and p16* (Towatari *et al.*, 1991). This in turn contributes to emergence of an increasing number of additional genetic alterations and favors the generation of more aggressive molecular clones, creating a vicious circle. In CML self-renewing cell lines, multiple copies of the *BCR/ABL* gene were identified (Keating, 1987). The majority of previous studies on *BCR/ABL* expressing cell lines determined that the *BCR/ABL* expression prolongs cell survival by inhibiting apoptotic cell death, induced by physical and chemical stresses (Nishii *et al.*, 1996). Hence, it appears to stimulate the development of CML and resistance to various chemotherapeutics, including IM (Horita *et al.*, 2000).

The characteristics of chronic phase CML – expansion and premature circulation of the malignant myeloid population, can be partially explained by the disruption of key cellular processes, such as apoptotic pathways. The enhanced TK activity of p210 BCR/ABL was shown to result in phosphorylation of numerous cellular substrates and in autophosphorylation, which in turn led to the recruitment and binding of a number of molecules. Signaling pathways instructing cells to undergo apoptosis are multiple and complex, hence a relatively large number of proteins were found to be tyrosine-phosphorylated in cells expressing *BCR/ABL*.

BCR/ABL-DEPENDENT ANTI-APOPTOTIC SIGNAL TRANSDUCTION PATHWAYS

Activation of apoptotic pathways occurs in order to eliminate cells carrying unrepaired and potentially mutagenic DNA aberrations (Shuai *et al.*, 1996). BCR/ABL-mediated protection from apoptosis (Skorski, 2002), leads to leukemic cells resistant to the damaging effects of cytotoxic drugs in contrast to normal cells. The TK activity of p210 BCR/ABL leads to constitutive phos-

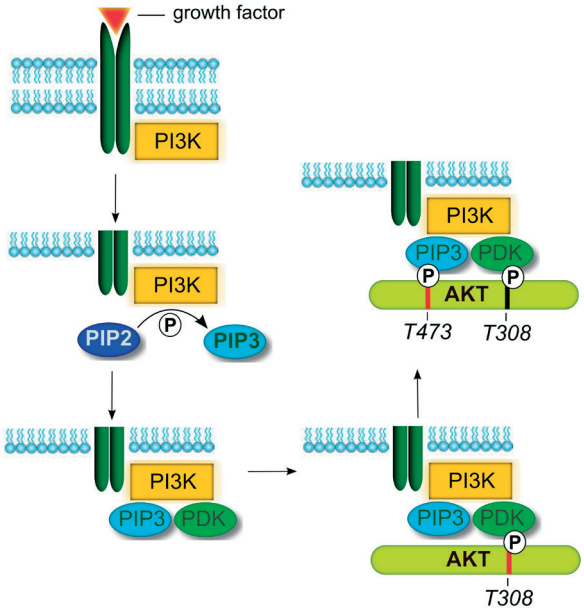

Figure 3. Overview of the PI3K/AKT signaling pathway in normal cells.
PI3K, phosphatidylinositol-3-kinase; PIP2, phosphatidylinositol (3,4)-disphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PDK, phosphoinositide-dependent kinase; AKT, protein kinase B

phorylation of the intracytoplasmic substrate proteins crucial for the transduction of mitogenic signals and affects anti-apoptotic pathways (Lugo *et al.*, 1990).

The PI3K/AKT pathway

BCR/ABL causes the activation of the PI3K/AKT pathway (Jain *et al.*, 1997), which is constitutively active in CML cells (Skorski *et al.*, 1997) and is the major pathway by which BCR/ABL exerts its anti-apoptotic effect (Fig. 3). Overexpression of AKT was reported in a variety of human cancers, and it was shown that cells expressing elevated levels of AKT are less responsive to apoptosis (Cicenas *et al.*, 2005). Hyperactivation of AKT is also associated with intensified cell growth, proliferation, metastasis, angiogenesis, and cellular energy metabolism (Harrington *et al.*, 2005).

The PI3K/AKT signaling pathway involves 4 major components: phosphatidylinositol-3-kinases, phosphoinositide-dependent kinase, phosphatidylinositol and protein kinase B.

Phosphatidylinositol-3-kinases

Phosphatidylinositol-3-kinases (PI3Ks) are a family of intracellular signal transducing enzymes associated with various cellular functions involved in cancer transformation. One of those functions is inhibition of apoptosis (Yao & Cooper, 1996). Activation of PI3Ks requires their translocation to the plasma membrane and binding to an activated receptor tyrosine kinase or its substrates (Wymann & Pirola, 1998). PI3K is activated by signals, such as members of the insulin-like growth factor (IGF) signal proteins family, transduced by various transmembrane receptors, having protein kinase cytosolic domains. PI3Ks are capable of phosphorylating the 3-position hydroxyl group of the inositol ring of phosphatidylinositol (PtdIns).

Three mammalian PI3K gene classes have been identified. PI3K proteins contain an N-terminal domain, interacting with regulatory protein subunits, a domain binding a small G protein Ras, an accessory PIK domain and a C-terminal catalytic subunit. Class I PI3K is the most studied class of PI3K, being the most significant in signaling in HSCs, since only the class I isoform was implicated in the regulation of hematopoiesis (Polak & Buitenhuis, 2012). PI3K is regulated by the activation of growth factor receptors. Class I PI3K are composed of a regulatory and a tightly associated catalytic subunit. They are further subdivided into subclasses IA and IB, depending on sequence similarity, the former being the predominant subgroup triggered by activated tyrosine kinases, such as BCR/ABL (Kharas & Fruman, 2005). Class IA PI3K is composed of a p85 regulatory subunit and a p110 catalytic subunit (Carpenter *et al.*, 1990). There are five isoforms of the p85 regulatory subunit, designated p85α, p55α, p50α, p85β, and p85γ. Among them, p85α appears to be essential for the survival of CML cells. The p85α subunit of PI3K forms a complex with oncogenic tyrosine kinases such as BCR/ABL, which results in activation of the p110 catalytic subunit of PI3K. Point mutations in the SH2 domain and SH3 domain of p85α prevented their interaction with BCR/ABL as well as binding of Src homology 2 domain containing (Shc) protein, c-Cbl adaptor protein, and GRB2-associated binding protein 2 (Gab2), which led to inhibition of BCR/ABL-dependent activation of PI3K/AKT signaling pathway (Ren *et al.*, 2005). There are three variants of the p110 catalytic subunit designated p110α, β, or δ, all expressed by separate genes, *PIK3CA*, *PIK3CB*, and *PIK3CD,* respectively. The IB PI3K subclass comprises the p110 regulatory and p110γ catalytic subunits, each encoded by a single gene. The regulatory subunit contains SH2 and SH3 domains, which take part in stabilizing the catalytic subunit (Yu *et al.*, 1998). The SH2 domain also allows binding to phosphorylated tyrosine residues in cell receptors and other cellular molecules, directing the heterodimer to membrane-associated signaling complexes. The catalytic subunit phosphorylates Ptdlns, which leads to the production of Phosphatidylinositol 3-phosphate (PI(3)P), Phosphatidylinositol (3,4)-disphosphate (PI(3,4)P2), and Phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P3) (Hawkins *et al.*, 1997), and those then pass a signal along in a cell signal cascade.

Phosphatidylinositol

Ptdlns are signaling components of eukaryotic cell membranes. The inositiol ring in their structure can be phosphorylated on any of the free hydroxyl groups (Fruman *et al.*, 1998). Ptdlns are precursors to many secondary messenger molecules.

Phosphoinositide-dependent kinase

Phosphoinositide-dependent kinase (PDK) requires the Ptdlns product of PI3K for activation. PDK consists of two domains: a C-terminal pleckstrin homology (PH) domain and an N-terminal kinase domain. PDK is ubiquitously expressed in human tissues and localizes to the cytosol (Currie *et* al., 1999).

Protein kinase B

The downstream target of PDK is AKT/PKB. PDK is able to phosphorylate threonine 308 of AKT, but not serine 473, which is required for complete activation of AKT (Alessi *et al.*, 1996). The prerequisite for the phosphorylation of serine 473 is a conformational change in AKT caused by its association with phosphoinositides (Walker *et al.*, 1998). On activation, AKT phosphorylates

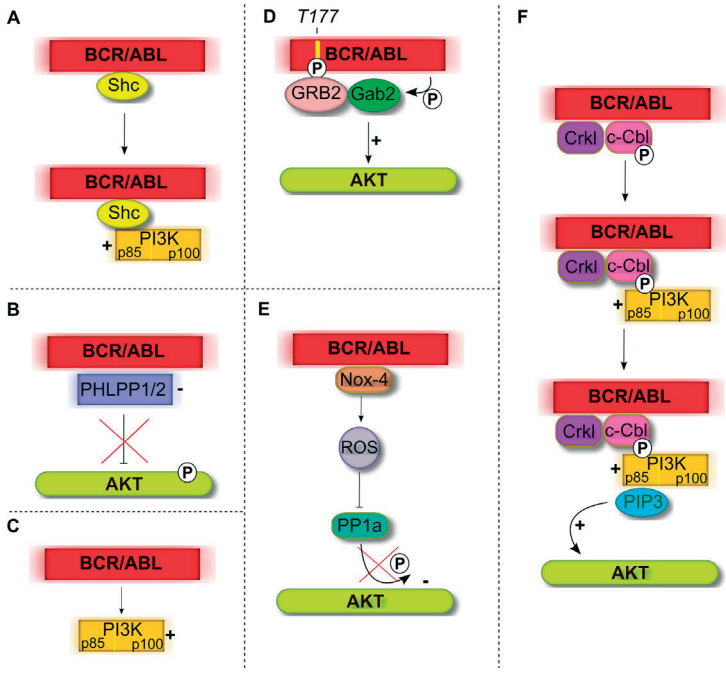

Figure 4. Various routes of BCR/ABL-mediated activation of the PI3K/AKT signaling pathway.
BCR/ABL, hybrid p210 oncoprotein; Shc, Src homology 2 domain containing protein; PI3K, phosphatidylinositol-3-kinase; PHLPP1/2, PH domain leucine rich repeat protein phosphatase 1/2; AKT, protein kinase B; GRB2, growth factor receptor-bound protein 2; Gab2, GRB2-associated binding protein 2; Nox-4, NADPH oxidase; ROS, reactive oxygen species; PP1α, serine/threonine protein phosphatase alpha; Crkl, adaptor protein; c-Cbl, adaptor protein

key survival proteins, resulting in decreased cell susceptibility to apoptosis (Maurer *et al.*, 2006). AKT activation disorders were observed in many human diseases, especially cancers (Chin & Toker, 2009).

BCR/ABL mediated activation of the PI3K/AKT pathway

BCR/ABL can activate the PI3K/AKT pathway directly by multiple mechanisms, or by the induction of autocrine cytokines, which lead to its activation. It was reported that BCR/ABL associates with Shc (Harrison-Findik *et al.*, 1995), which subsequently bound to the p85 regulatory subunit of PI3K (Ren *et al.*, 2005), resulting in its stimulation (Fig. 4A). In addition, expression of BCR/ABL was shown to decrease the levels of PH domain leucine rich repeat protein phosphatases – PHLPP1 and PHLPP2, which are negative regulators of AKT phosphorylation (Hirano *et al.*, 2009) (Fig 4B), and increase the level of p110 catalytic domain of PI3K (Hickey & Cotter, 2006) (Fig. 4C).

BCR/ABL also contains binding sites for growth factor receptor-bound protein 2 (GRB2) at a phosphorylated tyrosine 177 of BCR (Ruibao, 2005). GRB2 constitutes a scaffold for Gab2, which is then phosphorylated by the fusion kinase (Sattler *et al.*, 2002). Subsequently, Gab2 protein phosphorylation leads to the activation of the PI3K/AKT signaling pathway (Ren, 2005) (Fig. 4D).

Activation of the PI3K/AKT pathway by BCR/ABL can also take place by binding of Crkl and c-Cbl adapter proteins to the ABL part of the fusion kinase (Hochhaus *et al.*, 2002). Phosphorylation of c-Cbl protein leads to the recruitment of the p85 subunit of PI3K and its subsequent activation (Jain *et al.*, 1997), binding of the PIP3 molecule, and finally the activation of the serine-threonine AKT kinase (Skorski *et al.*, 1997) (Fig. 4E).

It was shown that BCR/ABL could indirectly induce the activity of the PI3K/AKT pathway by promoting the production of reactive oxygen species (ROS) by NADPH oxidase 4 (Nox-4) (Naughton *et al.*, 2009). The action of ROS then leads to inhibition of serine/threonine protein phosphatase alpha (PP1α) (Rao & Clayton, 2002; O'Loghlen *et al.*, 2003), which, normally *via* dephosphorylation of AKT (Millward *et al.*, 1999), serves as a negative regulator of the PI3K/AKT signaling pathway (Naughton *et al.*, 2009) (Fig. 4F).

Downstream anti-apoptotic effects of the PI3K/AKT pathway

It is known that AKT may exert anti-apoptotic effects both directly and indirectly. Direct impact is associated with phosphorylation of pro-apoptotic proteins, which results in their inactivation, degradation or change in their location. AKT affects apoptosis indirectly by modulating the phosphorylation of transcription factors, which, in response to apoptotic stimuli, modulate the transcription of certain genes involved in apoptosis (Parcellier *et al.*, 2008).

The first identified anti-apoptotic effect of PI3K/AKT was inactivation of Bcl-2-associated death promoter (Bad) protein — a pro-apoptotic member of the B-cell lymphoma 2 (Bcl-2) family (Neshat *et al.*, 2000), which in its non-phosphorylated state promotes apoptosis. AKT phosphorylates Bad at serine 136, allowing it to interact with 14-3-3 proteins, promoting cell survival (Andreeff *et al.*, 1999). Furthermore, the interaction of Bad with 14-3-3 proteins suppresses the capacity of Bad to associate with Bcl-2 and Bcl-xL proteins. This allows Bcl-xL to bind to pro-apoptotic Bcl-2 associated X (Bax) molecules, preventing their activation (Steelman *et al.*, 2004). Bad and Bcl-xL proteins were found to be expressed in normal and leukemic HSCs (Andreeff *et al.*, 1999). It was shown that affecting PI3K kinase activity by BCR/ABL, results in the increased expression of apoptosis inhibitory protein Bcl-2 (Skorski *et al.*, 1997).

AKT inhibits the expression of Bcl-2 proteins, regulating the activity of the family of forkhead box O transcription factors (FOXO) playing very diverse functions (Fu & Tindall, 2008). AKT phosphorylates FOXO1, FOXO3a and FOXO4 at threonine 24, serine 256 and serine 319 (Van Der Heide *et al.*, 2004). Phosphorylation of FOXO proteins located in the cell nucleus results in increased binding affinity for the 14-3-3 protein (Shah *et al.*, 2001), which allows the complex to be exported from the nucleus and retained in the cytoplasm (Medema *et al.*, 2000). By this mechanism, AKT inhibits the FOXO-mediated transcription of genes that promote apoptosis and cell cycle arrest (Fu & Tindall, 2008). An important target of FOXO transcription factors is a gene encoding a pro-apoptotic Bim protein, which, after cytokine removal, contributes to the death of HSCs (Fu & Tindall, 2008) as well as others, such as cyclin-dependent kinase inhibitor (CKI), p27 (KIP1) (Capellini *et al.*, 2003), Fas ligand (Brunet *et al.*, 1999), and Bcl-6 (Tang *et al.*, 2002).

Caspase 9 is synthesized as an inactive proenzyme – procaspase 9. During apoptosis, cytochrome *c* released from mitochondria into the cytoplasm, binds to apoptotic protease activating factor-1 (Apaf-1), and contributes to the activation of procaspase 9. Active caspase 9 leads to proteolytic cleavage and resulting activation of caspases 3 and 7, which in turn degrade many nuclear and cytoplasmic proteins. It was found that AKT phosphorylates procaspase 9 at serine 196, and this modification inhibits its proteolytic maturation (Parcellier *et al.*, 2008). It was shown that overexpression of AKT inhibits cytochrome c-induced caspase activation (Cardone *et al.*, 1998) and that BCR/ABL kinase activity leads to inhibition of caspase 9 (Deming *et al.*, 2004).

The transcription factor NF-κB promotes cell survival by inducing the transcription of genes encoding proteins related to inhibition of apoptosis, such as caspase inhibitors — cellular inhibitor of apoptosis 1 and 2 (c-IAP1 and c-IAP2). Binding of the NF-κB inhibitor (I-κB) to NF-κB leads to arrest of this factor in the cytoplasm and prevents its participation in transcription. AKT activates transcription of anti-apoptotic genes through regulation of NF-κB (Ozes *et al.*, 1999). It was shown that, in cells stimulated with platelet-derived growth factor, AKT temporarily binds to the IKK kinase and activates it (Romashkova & Makarov, 1999). AKT-mediated phosphorylation of I-κB by I-κB kinase (IKK) contributes to the degradation of the inhibitor. NF-κB can then freely translocate to the nucleus and induce transcription.

Another protein taking part in the PI3K/AKT pathway is Yes-associated protein (YAP). In normal conditions, YAP phosphorylation at serine 127 causes binding to the 14-3-3 protein in the cytoplasm, resulting in YAP being translocated to the nucleus, where it can act as a coactivator of transcription factors such as p73. YAP is also a substrate of AKT, which inhibits its ability to promote p73-mediated gene transcription of various pro-apoptotic proteins, such as Bax (Downward & Basu, 2008).

AKT kinase also phosphorylates mouse double minute 2 homolog (MDM2) protein, which in this form translocates into the nucleus, then interacts with p300 protein (Zhou *et al.*, 2001). The p300 protein then dissociates from p19ARF, which ultimately leads to degradation of p53 protein and cell cycle progression (Welsh *et al.*, 2005). Both IL-3 and BCR/ABL kinase increase cellular levels of MDM2 protein, in this way prolonging cell survival (Goetz *et al.*, 2001).

THE Ras/Raf/MEK/ERK PATHWAY

The PI3K/AKT pathway can also be activated by the protein Ras — a member of the Ras/Raf/MEK/ERK pathway, which leads to abnormal cell proliferation.

Ras are small (21 kDa) GTP-binding and membrane-associated proteins (Boguski & McCornick, 1993). They convey signals from ligand-activated tyrosine kinase receptors to downstream effectors (Bokoch & Der, 1993). Ras protein is an important regulator of cell growth and one of its functions is the direct regulation of PI3K (Rodriguez-Viciana *et al.*, 1994).

BCR/ABL autophosphorylation at tyrosine 177 brings new regulatory domains to ABL, such as an adapter GRB2 protein (Ruibao, 2005), containing two SH3 domains and one SH2 domain, where the latter ensures binding to ABL. Simultaneously, GRB2 binding can lead to the recruitment of Shc and Crkl adapter molecules, which can be involved in mediating the activation of Ras (Deininger *et al.*, 2000). These molecules bind to the ABL part of the fusion protein *via* their SH2 and SH3 domains, respectively (Pelicci *et al.*, 1995). The BCR/ABL-GRB2 complex recruits Son of Sevenless protein (SOS), which associates with the SH3 domain of GRB2 (Cortez *et al.*, 1997). The result of joining the SOS protein to the complex is twofold. It stimulates the transformation of the inactive GDP-bound form of Ras to the GTP-bound active form (Ren, 2005), leading to activating the p110 subunit of PI3K independently of p85, and results to activation of the scaffold adapter GRB2-associated binding protein 2 (GAB2) (Sattler *et al.*, 2002). Subsequently, the GRB2-GAB2-SOS complex activates PI3K, which leads to constitutive activation of the PI3K/AKT downstream pathway (Skorski *et al.*, 1995) (Fig. 5).

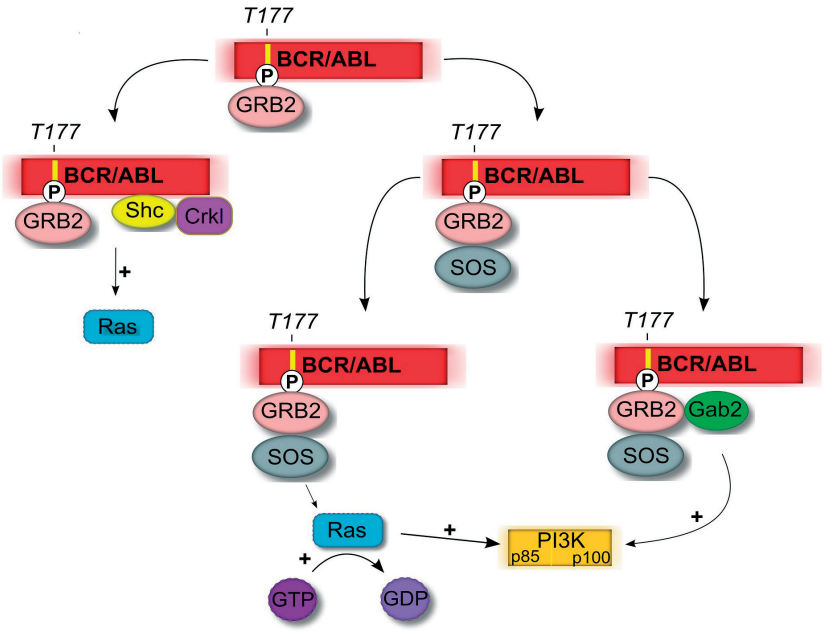

Figure 5. Role of Ras in BCR/ABL-mediated activation of the PI3K/AKT signaling pathway. BCR/ABL, hybrid p210 oncoprotein; GRB2, growth factor receptor-bound protein 2; Shc, Src homology 2 domain containing protein; Ras, small GTP-binding protein; SOS, son of sevenless protein; GTP, guaonosine triphosphate; GDP, guanosine diphosphate; Gab2, GRB2-associated binding protein 2; PI3K, phosphatidylinositol-3-kinase

THE JAK/STAT PATHWAY

The mammalian proteins from Janus protein tyrosine kinase (JAKs) family are associated with cytokine and growth factor receptors and play a major role in cytokine signaling (Liu *et al.*, 1998).

The STAT gene family were originally identified as mediators of cytokine-induced gene expression. Four domains can be distinguished in the structure of the STAT family proteins – the N-terminal oligomerization domain, an SH2 domain, a DNA-binding domain and a transactivation do-

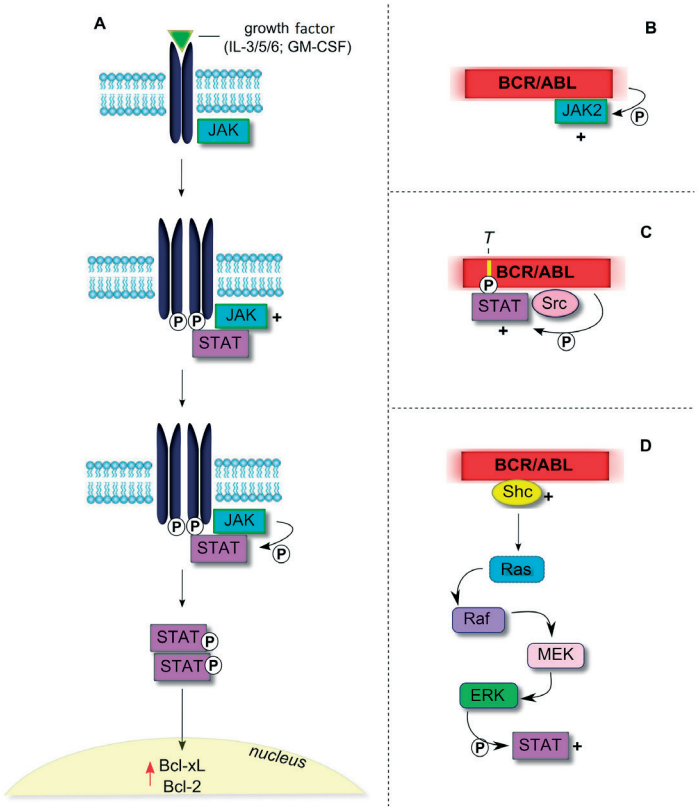

Figure 6. Overview of the JAK/STAT signaling pathway in normal cells and various routes of BCR/ABL-mediated activation.
JAK, Janus protein tyrosine kinase; STAT, Signal Transducer and Activator of Transcription; Bcl-xL, B-cell lymphoma-extra large protein; Bcl-2, B-cell lymphoma 2 protein; BCR/ABL, hybrid p210 oncoprotein; Src, non-receptor tyrosine kinase; Shc, Src homology 2 domain containing protein; Raf, proto-oncogene serine/threonine-protein kinase; MEK, mitogen-activated protein/extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinases

main, the latter being responsible for activation of transcription.

In case of normal cells, translocation of STATs to the nucleus occurs solely after cytokine binding to receptors and is preceded by activation of the receptor-associated JAK kinases. JAK is indirectly activated by cytokines like IL-3, IL-5, IL-6 as well as the granulocyte-macrophage colony-stimulating factor (GM-CSF) (Krempler *et al.*, 2004). Activation and trans-phosphorylation of cytokine receptors leads to activation of the associated JAK proteins, as well as binding of STAT (Eitel *et al.*, 2009). STAT then undergoes phosphorylation and dimerization, which facilitates its transport to the nucleus, binding to DNA, transcription factor activity, and increased protein stability.

The activation of STAT5 is, at least partially, responsible for the protection from apoptosis through the up-regulation of the anti-apoptotic genes Bcl-xL and Bcl-2 (Fig. 6A). We showed that expression of antiapoptotic protein Bcl-xL was enhanced in cells transformed by tyrosine kinases able to activate STAT5 (Slupianek *et al.*, 2002).

BCR/ABL signaling affects mainly the JAK2, STAT1, STAT3 and STAT5 proteins (Chai *et al.,* 1997; de Groot *et al.*, 2000), which were found to be constantly active in BCR/ABL-positive cell lines and in primary cells from CML patients, and are thought to contribute to the induction of cytokine independence (Wilson-Rawls *et al.*, 1996; Xie *et al.*, 2002). BCR/ABL was shown to abrogate the cytokine dependence of certain hematopoietic cell lines (Regimbeau *et al.*, 2004; Klein *et al.*, 2006; Saxena *et al.*, 2007; Galle, 2008). Studies conducted on a megakaryocytic cell line showed that expression of BCR/ABL induced tyrosine phosphorylation of JAK2 but not JAK1 (Wilson-Rawls *et al.*, 1996). These results were confirmed by a study on the 32D cell line transformed with BCR/ABL (Xie *et al.*, 2001). Detailed studies provided new insights on the topic. In contrast to normal cells, in CML, BCR/ABL appears to directly activate the JAK2 protein independently of the activation of STAT5 (Xie *et al.*, 2001, 2002). Hence, this type of JAK2 activation is different from the pathway involving IL-3. It occurs *via* binding the C-terminus of the ABL portion of BCR/ABL to JAK2 and phosphorylation of the tyrosine residue, which results in activatiion of the JAK2 tyrosine kinase (Xie *et al.*, 2001) (Fig. 6B). JAK2-BCR/ABL complex also includes several other proteins, all of which become tyrosine phosphorylated (Xie *et al.*, 2002). One of these associated proteins is involved in JAK2 activation and sustaining its activity (Rui & Carter-Su, 1999).

Overexpression of STAT is frequently observed in human cancers, however STAT5 seems to be the most involved (Chen *et al.*, 2013). Antibody-blocking studies show, that STAT proteins seem to be activated by BCR/ABL in a JAK2 independent manner (Chai *et al.*, 1997). In BCR/ABL transformed HSCs, this activation may occur by the action of BCR/ABL either in a direct or indirect manner (Klejman *et al.*, 2002).

The direct activation starts with a direct association of STAT SH2 domains with phosphorylated tyrosines on BCR/ABL (Carlesso *et al.*, 1996). However, an earlier study (Klejman *et al.*, 2002) showed that the Src kinase family proteins participate in BCR/ABL signaling and one of its members — Hck works in concert with BCR/ABL to phosphorylate STAT5 in myeloid leukemia cells (Fig. 6C). STAT activation contributes to growth factor independence of BCR/ABL expressing cell lines.

STAT may also be indirectly activated by BCR/ABL. The BCR/ABL tyrosine kinase targets adaptor proteins such as Shc. Shc is expressed in cells in three different forms, one of which is a target of phosphorylation by BCR/ABL (Puil *et al.*, 1994). Following activation, Shc itself leads to the activation of the Ras/Raf/MEK/ERK pathway. Extracellular-signal-regulated kinase (ERK) component is able to phosphorylate the threonine residue of STAT, further regulating its activity (Tannapfel *et al.*, 2003) (Fig. 6D).

Experiments involving the suppression cell proliferation, which proved to be resistant to IM, involved treating them with molecular inhibitors of components of PI3K, Ras and JAK/STAT pathways (Daley, 2003). Previously conducted studies showed that the PI3K/Akt pathway is not only involved in BCR/ABL-mediated leukemic transformation (Sonoyama *et al.*, 2002) but also in conferring resistance to other kinase inhibitors to the cells (Engelman *et al.*, 2005). Considering the ongoing problem with resistance to currently used kinase inhibi-

tors it seems promising to develop drugs targeting other components of survival pathways, thus reducing apoptosis and allowing the commence of terminal differentiation program.

CONCLUSIONS AND PERSPECTIVES

The first milestone in defining the molecular basis of CML was the discovery of chromosomal translocation, resulting in forming of the Philadelphia chromosome, containing the *BCR/ABL* fusion gene, being the central point in the pathogenesis of this disease. The second important event was the introduction of imatinib, an inhibitor of tyrosine kinases, being the breakthrough in the therapy of CML. However, the resistance to imatinib is an emerging problem. Because the induction of apoptosis in leukemic cells is the main mode of IM action, targeting the components of anti-apoptotic signaling pathways activated by BCR/ABL may assist IM-based therapy of CML. A recent study conducted by transfection of chronic myeloid leukemia cells with specific anti-STAT3, -STAT5A and -STAT5B siRNAs, showed that STAT expression was downregulated both at mRNA and protein levels, which resulted in leukemic cell apoptosis induction. These results suggest that siRNA may be considered in therapy of CML patients who developed resistance to treatment with IM (Kaymaz *et al*., 2013). Another study showed that targeting of INA-6, a human myeloma cell line, with INCB16562 — a novel, selective inhibitor of JAK1 and JAK2, led to the inhibition of STAT phosphorylation and influenced intracellular signaling pathways, proliferation and apoptosis. This study demonstrated that inhibition of JAK1/2 improved the antitumor activity of two myeloma therapies, melphalan and bortezomib *in vivo*. Hence, it suggests that targeting JAK1 and JAK2 could be beneficial in the treatment of myeloma patients, particularly in combination with other agents (Li *et al*., 2010). It was also shown that downregulation of Gab2 activity leads to increased sensitivity of cells to various BCR/ABL inhibitors, such as IM. Since Gab2 could be a potential therapeutic target for diseases resistant to tyrosine kinase inhibitors (Wöhrle *et al*., 2013). Furthermore, introducing MEK inhibitor PD184352, proved to enhance the ability of cytotoxic farnesyl transferase inhibitor BMS-214662 in the induction of apoptosis in CD34+ CML progenitor cells insensitive to tyrosine kinase inhibitors (Pellicano *et al*., 2011).

Some recent works point at the endoplasmic reticulum (ER) as a target of stress associated with CML and response to this stress in ER was suggested to be involved in leukemic progression (Piwocka *et al.,* 2006; Kriss *et al.,* 2012). Therefore, mechanism of this response may be important for leukemic therapy. It was supported by showing that inhibitors of PI3K and Src interacted synergistically with IM by inducing apoptosis and autophagy in BCR/ABL$^+$ cells by mechanism related to ER stress (Ciarcia *et al.,* 2013). To adopt to the stress ER developed a protective mechanism — the unfolded protein response (UPR) involving several signaling pathways, including the PKR-like ER-resident kinase (PERK) pathway (Higa & Chevet, 2012; Zanetti, 2013). It was shown that the PERK-eIF2α pathway, a part of PERK, was upregulated in CML and was associated with IM resistance suggesting a new, perspective target for CML therapy, including TKI-resistant cases (Kusio-Kobialka *et al.,* 2012).

As we mentioned, BCR/ABL is at the crossroad of many signaling pathways, including those involved in survival of BCR/ABL$^+$, like Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR (McCubrey *et al.,* 2008). Due to limited space of this review, it is not possible to mention all anti-apoptotic or pro-survival pathways, either BCR/ABL-dependent or independent, which may be associated with resistance of BCR/ABL$^+$ cells to TKIs.

Accumulating evidence suggests that not only apoptosis, but autophagy as well, may be targeted in CML therapy (Helgason *et al.,* 2011). As mentioned, PI3K/AKT and mTOR, which are essential for the formation of phagophores and their expansion to autophagosomes, may be activated in BCR/ABL signaling. It was shown that BCR/ABL$^+$ hematopoietic precursor cells were strongly dependent on autophagy, in spite of a low level of this process (Altman *et al.,* 2011). In addition, it was shown that BCR/ABL inhibition with TKIs results not only in CML cell apoptosis, but autophagy as well (Bellodi *et al.,* 2013). Autophagy protects CML stem cells against detrimental TKIs action. Therefore, inhibiting both BCR/ABL activity and autophagy in CML cell may enhance the effectiveness of therapy of this disease.

The discovery of new generations of TKIs has opened a new perspective in overcoming CML resistance to proapoptotic action of IM. Nilotinib (AMN107), a representative of this group of drugs, is over thirty-fold more potent than IM. The same concern dasatinib (BMS354825) as well as third generation bosutinib (SKI-606) and ponatinib (AP24534), which are especially effective in overcoming TKI-resistance underlined by point mutations in BCR/ABL (Amsberg & Schafthausen, 2013; Press *et al.,* 2013). Accumulating data suggest that the benefit of multiple treatment regimens may be blunted because of the activation of survival pathways, therefore further studies on the mechanism of survival the treatment with TKI by CML cells, both BCR/ABL-dependent and independent, are needed.

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Conflict of interest statement

The authors do not declare any conflict of interest.

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