

The Important Role of STAT3 in Chronic Lymphocytic Leukaemia Biology

Důležitá role STAT3 v biologii chronické lymfocytární leukemie

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Summary

Background: Signal transducer and activator of transcription (STAT) proteins are cytoplasmic transcription factors that transmit the signal of cytokines, hormones and growth factors. STAT proteins control fundamental cellular processes including survival, proliferation and differentiation. Inappropriate activation of STATs might contribute to cellular transformation and leukaemogenesis. About 70% of all solid and haematological tumours exhibit aberrant STAT3 expression and/or activation, highlighting its essential role in tumourigenesis. Aberrant STAT3 activation has been found in several solid tumours and haematologic malignancies. Importantly, constitutive activation of STAT proteins has been found in several leukaemias including acute myeloid leukaemia, acute promyelocytic leukaemia, acute lymphoblastic leukaemia, chronic myeloid leukaemia and chronic lymphocytic leukaemia (CLL). Constitutively activated STAT3 plays an important role in CLL biology. CLL cells harbour constitutive phosphorylation on S727 and acetylation on K685 and transient phosphorylation on Y705 residues. Moreover, STAT3 messenger RNA expression is significantly higher in CLL cells compared to healthy B-lymphocytes. Interestingly, STAT3 inhibition is disclosed as an important by-product of ibrutinib treatment in CLL patients. **Purpose:** The purpose of this review is to describe the consequences of STAT3 dysregulation in CLL cells. Here, we discuss aberrantly modified processes by STAT3 activation in CLL cells such as proliferation, apoptosis, B cell receptor signalling, cytokine secretion, immune checkpoint regulation, microRNA regulation, free fatty acid metabolism and electron transport chain in the mitochondria.

Key words

STAT3 – STAT – chronic lymphocytic leukaemia – therapy – microenvironment – ibrutinib – constitutive activation – leukaemia

Souhrn

Východiska: Proteiny STAT (signal transducer and activator of transcription) jsou cytoplazmatické transkripční faktory, které přenášejí signál cytokinů, hormonů a růstových faktorů. Proteiny STAT kontrolují základní buněčné procesy vč. přežití, proliferace a diferenciaci. Nadměrná aktivace proteinů STAT může přispět k transformaci buněk a vzniku leukemie. Okolo 70 % všech solidních a hematologických nádorů vykazuje aberantní expresi a/nebo aktivaci STAT3, což dokumentuje zásadní roli STAT3 v tumorigenezi. Aberantní aktivace STAT3 byla popsána u několika solidních nádorů a hematologických malignit. Důležité je, že konstitutivní aktivace proteinů STAT byla detekována u několika typů leukemie vč. akutní myeloidní leukemie, akutní promyelocytární leukemie, akutní lymfoblastické leukemie, chronické myeloidní leukemie a chronické lymfocytární leukemie (CLL). Konstitutivně aktivovaný STAT3 hraje důležitou roli v biologii CLL. Buňky CLL jsou konstitutivně fosforylované na S727 a acetylované na K685, navíc může dojít i k fosforylaci na Y705. Expresí mediátorové RNA STAT3 je výrazně vyšší v buňkách CLL ve srovnání se zdravými B lymfocyty. Zajímavé je, že inhibice STAT3 byla popsána jako důležitý vedlejší produkt léčby ibrutinibem u pacientů s CLL. **Cíl:** Účelem tohoto přehledu je popsat důsledky deregulace STAT3 u buněk CLL. V práci jsou popsány procesy ovlivněné nadměrnou aktivací STAT3 jako proliferace, apoptóza, signalizace BCR (B cell receptor), sekrece cytokinů, regulace kontrolních bodů imunitního systému, regulace mikroRNA, metabolismus mastných kyselin a elektronový transportní řetězec v mitochondriích.

Klíčová slova

STAT3 – STAT – chronická lymfocytární leukemie – terapie – mikroprostředí – ibrutinib – konstitutivní aktivace – leukemie

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Introduction

Signal transducer and activator of transcription (STAT) proteins are intracellular transcription factors involved in cellular immunity, proliferation, apoptosis and differentiation. Aberrantly expressed STAT proteins were found to play an important role in cancer promotion [1]. Importantly, constitutive activation of STAT proteins have been found in several leukaemias including acute myeloid leukaemia [2–4], acute promyelocytic leukaemia [5], acute lymphoblastic leukaemia [6], chronic myeloid leukaemia [7] and chronic lymphocytic leukaemia (CLL) [8,9]. Importantly, in CLL STAT3 was constitutively activated in all analysed samples regardless of genetic aberrations, disease stage or other factors [8,10]. Therefore, it plays a crucial role in CLL. In this review we focus on the role of STAT3 in CLL biology.

STAT3

STAT3 belongs to the STAT family of signal responsive transcription factors which are kept in an inactive form in the cytoplasm of non-stimulated cells [11]. STAT

proteins are cytoplasmic transcription factors that convey signals from cytokine and growth-factor receptors to the nucleus (Fig. 1) [12]. STAT proteins regulate processes that are acquired by tumour cells such as uncontrolled proliferation, resistance to apoptosis, sustained angiogenesis and escape from the immune system [12,13]. STAT3 is activated by phosphorylation of tyrosine residues (Y705) or serine residues (S727) [8,11]. STAT3 activation is most commonly mediated by members of the Janus kinase (JAK) family tyrosine kinases. Then it dimerises and translocates to the nucleus, where it binds specific DNA sequences in the promoters of target genes. STAT proteins (especially STAT3 and STAT5) are constitutively activated in a surprisingly large number of cancers [12]. Remarkably, STAT3 activation is required for the survival and proliferation of a number of cancer cells [11]. STAT3 activation in cancer cells is often the result of chronic stimulation by extracellular signals in the tumour microenvironment.

STAT3 is crucial for cell development, which has been evidenced in experi-

ments using null (knock out) mice suffering from early embryonic lethality [14]. Constitutive activation of STAT3 has been found to be associated with the initiation and progression of various cancers has been found in several solid tumours and haematologic malignancies; altogether STAT3 is one of the most commonly activated transcription factors in cancer [12].

STAT3 in CLL

Although CLL is a heterogeneous disease with various genetic aberrations, there are abnormalities in CLL that occur in all patients. One of them is constitutively activated STAT3 protein [8–10]. STAT3 plays a role in promoting tumorigenesis of CLL where it represents a key pathway involved in the growth and survival of CLL cells [8,9,15,16]. In CLL, STAT3 messenger RNA (mRNA) expression is significantly higher compared to healthy B-lymphocytes [17–19]. Therefore, the regulation of STAT3 activity is highly dysregulated in CLL cells. STAT3 is involved directly or indirectly in many processes in CLL cells including proliferation, apoptosis, B-cell receptor (BCR) signalling, cytokine secretion, immune checkpoint regulation, microRNA regulation, free fatty acid metabolism and electron transport chain in the mitochondria (Fig. 2).

Constitutive activation of STAT3 in CLL cells

Constitutive Y705 phosphorylation of STAT3 occurs in several solid tumours and haematologic malignancies [12]. However, it is interesting that in CLL STAT3 is constitutively phosphorylated exclusively on S727 but not on Y705 [8,9]. Conversely, inducible Y705 phosphorylation was transient. In contrast to normal peripheral blood B-lymphocytes, constitutive S727 phosphorylation was detected in all tested samples of CLL, regardless of disease stage or treatment status. Moreover, this phosphorylation is constitutive in CLL cells. Importantly, the S727 phosphorylation also activates STAT3, which translocates to the nucleus, binds to the STAT3-specific DNA binding sites in unstimulated CLL cells and regulates the expression

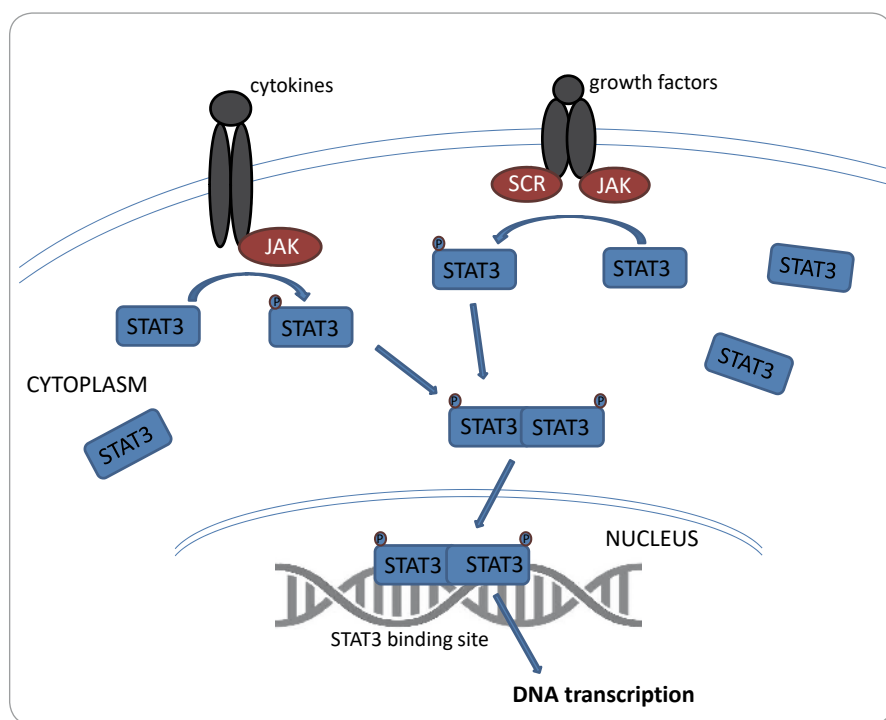


Fig. 1. General STAT3 activation. Inactive non-phosphorylated STAT3 in the cytoplasm is phosphorylated and thus activated by the JAK kinases. It enables dimerization of STAT3. The phosphorylated STAT3 dimers enter the nucleus and bind STAT3 target genes.

STAT – signal transducer and activator of transcription

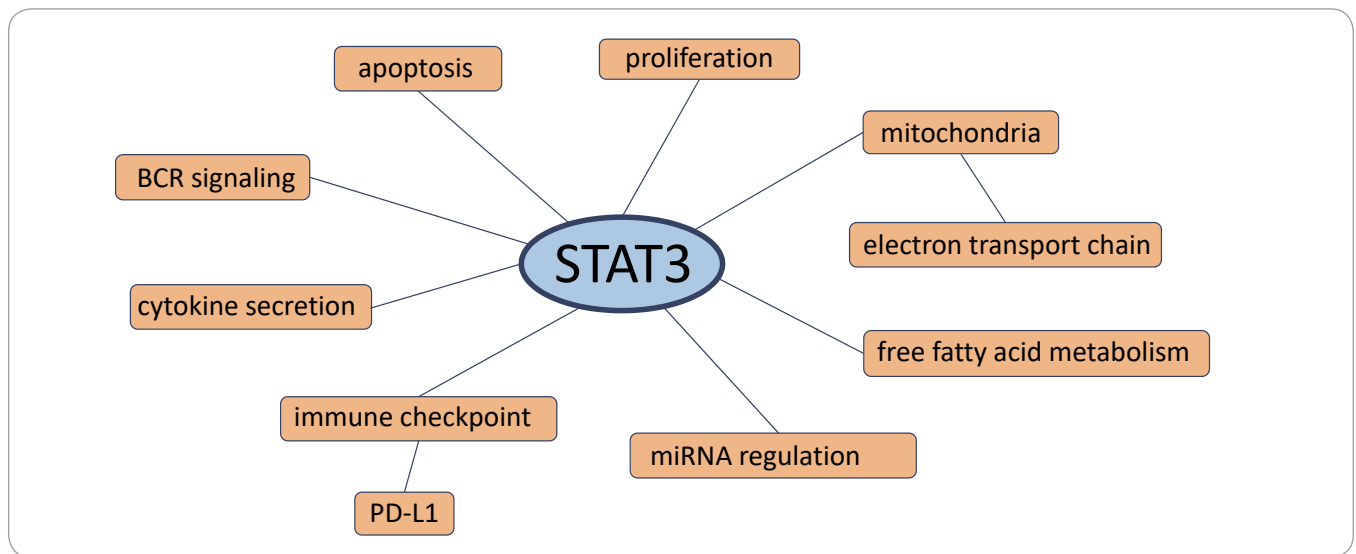


Fig. 2. Processes in which STAT3 is involved in chronic lymphocytic leukemia cells.

STAT – signal transducer and activator of transcription, BCR – B cell receptor, PD-L1 – programmed death-ligand 1, miRNA – microRNA

of STAT3-dependent genes. The elimination of STAT3 using short hairpin RNA (shRNA) reduced the mRNA levels of STAT3 and the STAT3-regulated genes such as *BCL2*, *Pim1*, *BCL-XL*, *Cyclin D1*, *p21* and *c-MYC* in a dose-dependent manner. Besides STAT3, STAT1 was also found to be constitutively activated on S727 residues in CLL cells [9]. In addition, in CLL cells, STAT3 can be activated easily through Y705 phosphorylation because CLL cells reside in the bone marrow, blood and lymph nodes and are thus exposed to various cytokines, chemokines and growth factors and therefore capable of potentiate through this phosphorylation a magnitude of STAT3 activation. Although it is not constitutive in CLL cells, increased pY705 STAT3 levels were described by Myhrvold et al. [20]. In CLL cells, the basal phosphorylation level of proteins involved in B-cell signalling was similar or lower compared to normal B cells, with the exception of pY705 STAT3, which was increased. Constitutive S727 phosphorylation of STAT3 is biologically significant and probably plays a role in the pathogenesis of CLL because it activates the transcription of pro-survival and pro-proliferative genes. The STAT3 constitutive phosphorylation on S727 residues is induced by casein kinase 2, which is required for the phosphorylation together with SD5 and BLNK; together

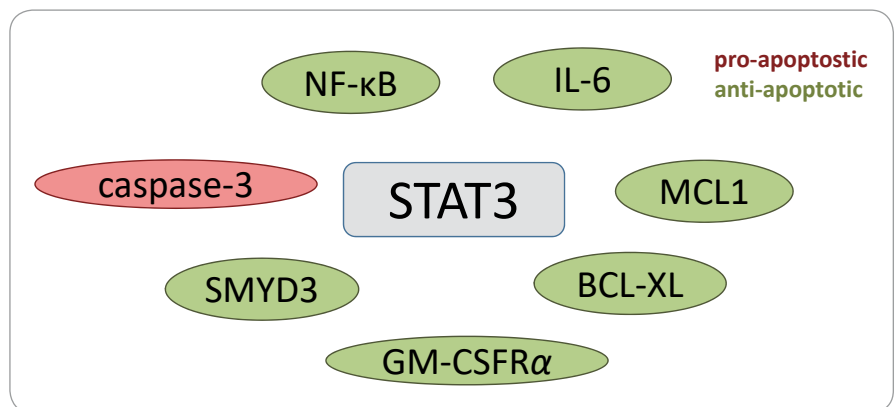


Fig. 3. Directly and indirectly regulated apoptosis by STAT3. Pro-apoptotic proteins are circled in red, anti-apoptotic proteins in green.

STAT – signal transducer and activator of transcription

they create a complex that phosphorylates STAT3 [21]. Moreover, in addition to phosphorylation, STAT3 is constitutively acetylated on K685 in CLL cells [10]. It was detected in all the analysed samples regardless of clinical characteristics, cytogenetic abnormalities or *IGHV* mutation status. Acetyltransferase p300 was detected at high levels in CLL cells and subsequently it was found that p300 induces constitutive acetylation and activation of STAT3. Acetylated STAT3 increased the transcriptional activity of STAT3 and provided CLL cells with a survival advantage. The acetylation and phosphorylation of STAT3 were found as independent events in CLL cells.

STAT3 in regulating CLL cell survival

Interestingly, it was found that the sensitivity of fresh CLL cells to spontaneous apoptosis is highly variable among different patients and inversely correlates with constitutive activation of STAT3 and NF-κB [22]. Both transcription factors maintain the levels of anti-apoptotic proteins MCL1 and BCL-XL and autocrine IL-6 secretion. The sensitivity of CLL cells to spontaneous apoptosis is thus regulated by STAT3 and NF-κB (Fig. 3).

One reason for increased proliferation in CLL cells is NF-κB activation. Yang et al. described the mechanism of NF-κB activation through interaction with

STAT3 [23]. They found that unphosphorylated STAT3 (U-STAT3) binds to NF- κ B dimers in competition with I κ B [23]. The U-STAT3/NF- κ B complex translocates to the nucleus, binds to the DNA and activates NF- κ B-regulated genes. In CLL cells, constitutively activated STAT3 induces STAT3 protein production, and thus CLL cells harbour high levels of U-STAT3. Moreover, Liu et al. confirmed that also in CLL cells U-STAT3 binds to the NF- κ B dimers, forming a complex that activates NF- κ B-regulated genes (e.g. *VEGF-C*, *CCL5* and *CXCR5*) [24]. Further, increased transcription of *SMYD3* promotes CLL survival – Lin et al. found that activated STAT3 binds directly to the *SMYD3* promoter and enhances *SMYD3* transcription [19]. Decreased phosphorylation of STAT3 or *SMYD3* knock down inhibited CLL cell growth. Another mechanism which protects CLL cells from apoptosis is STAT3-mediated GM-CSFR α (α subunit of the granulocyte-macrophage colony-stimulating factor receptor) production [25].

On the other hand, STAT3 also regulates the apoptosis of CLL cells [26]. Besides that STAT3 protects CLL cells from apoptosis, at high levels STAT3 can activate pro-apoptotic mechanisms and induce apoptosis in CLL cells. Overexpression of STAT3 upregulated caspase-3 levels and induced apoptosis. STAT3 binds with low affinity to the *caspase-3* promoter and at very high levels can activate *caspase-3* expression. This could represent a physiological negative feedback mechanism used in normal cells to counteract uncontrolled proliferation.

Microenvironment activation of STAT3 in CLL cells

Interestingly, CD5^{high}CXCR4^{dim} CLL cells, which probably recently exited the lymph node, express higher levels of pY705 STAT3 than the CD5^{dim}CXCR4^{high} CLL cells likely circulating in the peripheral blood [27]. Moreover, the expression of STAT3-target genes was significantly higher in CLL cells isolated from the lymph node than in CLL cells isolated from the blood of the same patient [27,28]. Chen et al. found that nurse-like cells could induce Wnt5a/ROR1-dependent activation of NF- κ B in CLL cells that in turn elicits au-

tochrone IL-6-induced activation of STAT3. Further, it was found that cirmtuzumab, the anti-ROR1 antibody, can inhibit the activation of both STAT3 and NF- κ B in CLL cells.

STAT3 is involved in BCR signalling in CLL cells [15,16,29]. BCR stimulation triggers survival signals in CLL cells. BCR stimulation via anti-immunoglobulin M (anti-IgM) antibodies induces transient Y705 phosphorylation and nuclear localisation of pSTAT3 [15]. In contrast, S727 phosphorylation was increased only mildly. BCR activation induces the activation of JAK2, which phosphorylates STAT3 on Y705 residues. Anti-IgM upregulates STAT3-regulated genes *CyclinD1*, *STAT3*, *p21*, *c-MYC* and *BCL2*. In addition, Rozovski et al. found that stimulation of the BCR increases the constitutive activation of NF- κ B, NF- κ B then induces the production of IL-6, which subsequently leads to phosphorylation of STAT3 on Y705 residues [29]. Furthermore, BCR signalling can also activate the second STAT3 phosphorylation; Kondo et al. found that Bruton's tyrosine kinase (BTK) is an upstream activator of pS727 STAT3 in CLL cells [16]. Ibrutinib abrogated IgM/IgG- or CD40L-induced pS727 STAT3 activation. Moreover, BTK knockout significantly reduced the constitutive level of pS727 STAT3. The effect was probably exerted indirectly through the activation of serine kinases.

Further, STAT3 regulates cytokine production in CLL cells. STAT3 was found to regulate the secretion of IL-9 and IL-10 in CLL cells [18,30]. STAT3-mediated translational upregulation of miR-155 and miR-21 promoted IL-9 secretion [18]. Concerning IL-10, the CXCL12–CXCR4–STAT3 axis regulates IL-10 production in CLL cells and their ability to suppress T-cell effector function [30].

Moreover, STAT3 directly or indirectly upregulates or downregulates microRNA gene levels in CLL cells including, for example miR-155 involved in tumourigenesis [31]. The levels of 63 microRNAs were downregulated and 9 upregulated after STAT3 shRNA transfection. Li et al. confirmed that STAT3 binds to the miR-155 gene promoter and activates miR-155 expression in CLL cells [32].

STAT3 mediates the response to ibrutinib in CLL cells

Recently, one of the important effects of ibrutinib, in addition to the inhibition of BTK, is the inhibition of STAT3 signalling, demonstrating the important role of this signalling pathway and the potential for its use in CLL therapy, either through inhibition by ibrutinib or another specific inhibitor [16]. The study showed an interesting link between the STAT3 pathway and regulation of the immune response through programmed death-ligand 1 (PD-L1) expression, which was significantly reduced following treatment with ibrutinib, enhancing the T-cell response in the CLL cells. In the peripheral blood CLL samples treated with ibrutinib, durable downregulation of PD-L1 by 3 months post-treatment was observed. This effect was mediated through the inhibition of constitutively active STAT3 on S727 in the CLL cells. In contrast, chlorambucil therapy had no effect on PD-L1 expression, suggesting a specific effect of ibrutinib treatment. PD-1/PD-L1 receptors play an important role in the anti-tumour immune response, which has recently been used for targeted anti-tumour therapy [33,34]. Moreover, the effect of inhibition of STAT3 and subsequent downregulation of PD-L1 has already been described in lymphoma-derived cell lines [35].

Effect of STAT3 on CLL cell metabolism

In addition to its classic function as the transcription factor in the nucleus, a pool of pS727 STAT3 molecules has been identified in the mitochondria, where it modulates the activity of the electron transport chain [36]. Importantly, this was also confirmed in CLL cells where the pS727 STAT3 level correlated with prolonged *in vivo* survival [37]. pS727 STAT3 was detected in the mitochondria and associated with complex I of the respiratory chain. Furthermore, STAT3 activity contributes to the CLL cell but not to healthy B cell resistance to apoptosis *in vitro*. Mitochondrial pS727 STAT3 overactivity is therefore a part of the antioxidant defence pathway of CLL cells. Thus, mitochondrial pS727 STAT3 appears to be a newly

Tab. 1. Preclinical and clinical testing of STAT3 inhibition.

Drug	STAT3 targeting	Combination	Model	Reference
JSI-124	directly	single-agent, TRAIL	cell lines, primary cells	Ishdorj et al., 2010 [40]
WP1066 and niclosamide	directly	single-agent	primary cells	Myhrvold et al., 2018 [20]
WP1066	directly	single-agent	cell lines, <i>in vivo</i>	Lin et al., 2019 [19]
C6-ceramide	indirectly	single-agent, ibrutinib	cell lines, primary cells, <i>ex vivo</i>	Doshi et al., 2017 [48]
Pyrimethamine	indirectly	single-agent	clinical trial (NCT01066663)	Brown et al., 2018 [42]
Cirmtuzumab	indirectly	single-agent	clinical trial (NCT01066663)	Chen et al., 2019 [27]
Ibrutinib	indirectly	single-agent	clinical practice	Kondo et al., 2018 [16]

STAT – signal transducer and activator of transcription, TRAIL – tumour necrosis factor-related apoptosis-inducing ligand

identified cell-protective signal involved in CLL cell survival.

Rozovski et al. found that CLL cells, similar to Burkitt's lymphoma cells but unlike normal B-lymphocytes, store lipids in the form of cytoplasmic lipid vacuoles [38]. STAT3 was found to modulate free fatty acid metabolism through aberrant lipoprotein lipase expression in CLL cells. It seems that CLL cells transformed their metabolism to oxidise free fatty acids. Activated STAT3 induces lipoprotein lipase, which catalyses the hydrolysis of triglycerides into free fatty acids. Recently, it was found that STAT3-activated CD36 facilitates fatty acid uptake in CLL cells [39].

Preclinical testing of STAT3 inhibition in CLL

The inhibition of STAT3 using JSI-124 potently induced apoptosis in B leukaemia cell lines and primary CLL cells [40]. JSI-124 reduced STAT3 S727 phosphorylation. JSI-124 also induced cell cycle arrest prior to apoptosis activation. Moreover, the knockdown of STAT3 induced apoptosis and cell cycle arrest in leukaemia cells. The blocking of STAT3 led to downregulation of an anti-apoptotic protein XIAP. STAT3 inhibitors WP1066 and niclosamide significantly reduced both pY705 STAT3 levels and cell viability [20]. STAT3 inhibition reduced cellular metabolism before cell death was induced. Moreover, WP1066 significantly inhibited tumour growth *in vivo* [19]. This suggests that STAT3 activation can be a potential target for therapy of CLL (Tab. 1).

In addition, SOCS3, a negative regulator of the JAK/STAT signalling pathway, was found to be a potentially new therapeutic target in CLL using the inhibition of Hsp90 [41]. In this study Hsp90 induced the cell death of CLL cells through upregulation of SOCS3 via the p38 pathway.

Clinical testing of STAT3 inhibition in CLL

The fact that STAT3 is a promising target for CLL therapy led to the initiation of a clinical trial investigating the efficiency of STAT3 inhibitors. STAT3 inhibitor pyrimethamine was tested in relapsed CLL/SLL (NCT01066663) [42,43]. Pyrimethamine, an anti-microbial drug that is effective in the prevention and treatment of malaria and toxoplasmosis, was found to be a potent STAT3 inhibitor in a screen of a chemical library of drugs already known to be safe in humans [44,45]. Pyrimethamine was found to inhibit STAT3 transcriptional function at concentrations known to be safe in patients. The anti-tumour effect of pyrimethamine through STAT3 inhibition was confirmed in murine models of breast cancer [46]. Pyrimethamine inhibited the expression of STAT3-regulated genes in CLL cells *ex vivo* and decreased the survival of CLL cells [42]. Importantly, peripheral blood mononuclear cells from healthy donors were not affected. A clinical trial was done in CLL patients whose disease progressed despite therapy. There were no dose-limiting toxicities and no significant drug-related

toxicities. No objective responses were observed. Half of the patients achieved stable disease for 12 months and two people for 4 and 6 months, respectively. The remaining patients had progressive disease, and all but one patient discontinued therapy for disease progression. The median overall survival was 22 months. Nevertheless, only the highest dose which was applied approached the threshold of 10 μ M of plasma concentrations, which effectively inhibited STAT3 *in vitro*. Therefore, it is necessary to increase the dose of pyrimethamine in future studies to determine its efficiency in CLL patients.

Another drug tested in clinical trial (NCT02860676) which indirectly targets STAT3 is cirmtuzumab. Cirmtuzumab, the anti-ROR1 antibody tested in a phase I clinical trial showed the mechanism of its action through downregulating the expression of STAT3 [27]. It downregulated the expression of STAT3 and NF- κ B target genes and reduced the levels of phosphorylated STAT3 and p65 detected in the CLL cells of treated patients.

Therefore, targeting STAT3 may have a high therapeutic potential because normal cells can tolerate a loss of STAT3 function [47].

Summary

CLL cells harbour constitutively activated STAT3. STAT3 is permanently phosphorylated on S727 and acetylated on K685 residues in all CLL samples [8,10]. The full activation of STAT3 occurs in the lymph nodes, where CLL cells are stimu-

lated from various stimuli which also activates Y705 of STAT3. For example, BCR signalling is able to increase both types of STAT3 phosphorylation [15,16]. Fully activated STAT3 contributes to the increased proliferation and survival of CLL cells in this compartment. It is interesting that, unlike constitutive S727 phosphorylation detected in all samples, Y705 phosphorylation status varies between samples. Although it is rare, some samples from the peripheral blood also harbour a high level of Y705 phosphorylation indicating a different stimulation of the CLL cells by the microenvironment [15; our unpublished data]. This is in line with the fact that CD5^{high}CXCR4^{dim} CLL cells (which recently exited the lymph node) express higher levels of pY705 STAT3 than the CD5^{dim}CXCR4^{high} CLL cells (circulating in the peripheral blood) [27]. It seems that after exiting the lymph node, CLL cells keep increased anti-apoptotic signalling at least for some time.

This notwithstanding, no STAT3 inhibitor has been approved at the moment for CLL therapy; the effect of STAT3 inhibition can be seen after other drugs such as ibrutinib or cirtuzumab, which indirectly block STAT3 signalling [16,27]. Therefore, the blocking of STAT3 contributes to the observed therapeutic effect of ibrutinib in the clinics.

STAT3 plays an important role in CLL biology and pathology because constitutive STAT3 activation on S727 and K685 is present in all patient samples regardless of genetic aberration presence [8,10]. Therefore, one molecular hallmark of CLL biology is the constitutive phosphorylation and acetylation, and consequently the transcriptional activation of STAT3.

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