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Nilotinib exerts a therapeutic approach via JAK/STAT pathway and cytokine network in chronic myeloid leukemia cells

Nilotinib, kronik miyeloid lösemi hücrelerinde JAK/STAT yolu ve sitokin ağı üzerinden terapötik bir yaklaşım uygular

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ABSTRACT

Aim: Chronic myeloid leukemia (CML) displays a constitutive tyrosine kinase (TK) activity which in turn leads to the activation of various signaling pathways and the outcome of leukemic phenotype. Activated STAT5A and STAT5B from JAK/STAT pathway induce cell growth, proliferation, differentiation, and survival of leukemic cells which are promoted by a cytokine network. Since the second-generation tyrosine kinase inhibitor nilotinib has the advantage of inhibiting this oncogenic TK activity; we aimed to investigate the underlying mechanism of its therapeutic approach and how it induced apoptosis via analyzing the forthcoming molecular targets of the pathway.

Materials and Methods: By Nilotinib treatments, cell viability and proliferation assays, apoptotic analysis, expressional regulations of STAT5A&5B mRNA transcripts, protein expression levels, and also cytokines' expressional assessments were determined in CML model K562 cells, *in vitro.*

Results: Nilotinib treatment in a time and dose-dependent manner assessed a therapeutic approach by decreasing leukemic cell proliferation and survival; inducing leukemic cell apoptosis, down-regulating STAT5A&5B mRNA, and protein expression levels, and regulating cytokine expressional network.

Conclusion: Nilotinib-mediated therapeutics could be dependent on targeting JAK/STAT pathway members STAT5A and STAT5B, besides; regulating the cytokine network might be another underlying mechanism for sensitization and response of K562 cells to nilotinib in leukemia pathogenesis.

Keywords: Nilotinib, chronic myeloid leukemia, JAK/STAT pathway, cytokines, apoptosis.

ÖZ

Amaç: *Kronik miyeloid lösemi (KML), çeşitli sinyal yollarının aktivasyonuna ve lösemik fenotipin sonucuna yol açan yapıcı bir tirozin kinaz (TK) aktivitesi gösterir. JAK/STAT yolundan aktive edilmiş STAT5A ve STAT5B, bir sitokin ağı tarafından teşvik edilen lösemik hücrelerin büyümesini, çoğalmasını, farklılaşmasını ve hayatta kalmasını indükler. İkinci nesil tirozin kinaz inhibitörü nilotinib, bu onkojenik TK aktivitesini inhibe etme avantajına sahip olduğundan; terapötik yaklaşımının altında yatan mekanizmayı ve yolun gelecekteki moleküler hedeflerini analiz ederek apoptozu nasıl indüklediğini araştırmayı amaçladık.*

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Yöntem: *Nilotinib tedavileri ile hücre canlılığı ve proliferasyon deneyleri, apoptotik analiz, STAT5A&5B mRNA transkriptlerinin ekspresyonel düzenlemeleri, protein ekspresyon seviyeleri ve ayrıca sitokinlerin ekspresyonel değerlendirmeleri, in vitro olarak CML model K562 hücrelerinde belirlendi.*

Sonuç: *Nilotinib tedavisi, zamana ve doza bağlı bir şekilde lösemik hücre proliferasyonunu ve sağkalımını azaltarak terapötik bir yaklaşımı değerlendirdi; lösemik hücre apoptozunu indüklemek, STAT5A&5B mRNA'yı aşağı regüle etmek ve protein ekspresyon seviyelerini düzenlemek ve sitokin ekspresyon ağını düzenlemek.*

Çözüm: *Nilotinib aracılı terapötikler, JAK/STAT yolu üyeleri STAT5A ve STAT5B'nin hedeflenmesine bağlı olabilir, ayrıca; sitokin ağının düzenlenmesi, lösemi patogenezinde K562 hücrelerinin nilotinibe duyarlılığı ve tepkisi için başka bir altta yatan mekanizma olabilir.*

Anahtar Sözcükler: *Nilotinib, kronik miyeloid lösemi, JAK/STAT yolu, sitokinler, apoptoz.*

INTRODUCTION

Chronic myeloid leukemia (CML) is a clonal, myeloproliferative disease that develops the Philadelphia chromosome (Ph). The *Abelson (Abl1)* gene on chromosome 9 is translocated to the *Breakpoint cluster region (Bcr)* on chromosome 22, forming a fused oncogene Bcr-Abl on the truncated chromosome 22; referred to as t(9;22) (q34;q11) reciprocal translocation (1). This oncogene encodes the *BCR-ABL* oncoprotein, which promotes the constitutive stimulation of *ABL*-related tyrosine kinase activity.

Tyrosine kinases have an extracellular ligand-binding domain; thus, it induces dimerization and phosphorylation of the cytoplasmic domain of tyrosine kinases, resulting in further regulation of several cytoplasmic signaling pathways (2). Dysregulation of these pathways causes physiological changes such as cell proliferation and differentiation, which in turn develops cancer, and *ABL*-related tyrosine kinase activity specifically plays a crucial role as the sole driver in the pathogenesis of CML (3, 4). *ABL*-dependent tyrosine kinase activity is responsible for malignant transformation by activating downstream signaling pathways; specifically, JAK/STAT (Janus kinase/signal transducer and activator of transcription); which causes abnormal cell survival and proliferation of leukemic cells.

The JAK/STAT signaling pathway plays a key role in various cytokines, immune system regulators, hormones, and hematopoiesis factors (5). There is a receptor on the surface of the target cell that binds to specific cytokines. Cytokines are secreted by diverse types of cells in response to different stimuli including infection or tissue damage, thus modulating the immune response and other biological activities. The proliferation of leukemic cells is promoted by a cytokine network which is constructed by the leukemic cells and their microenvironment (6). Among the cytokines, interleukins (ILs) and interferons (IFNs) are crucial for microenvironment-modulated myeloid leukemia progression (7). Due to STAT5A/B signaling, activated STAT5 proteins are known to be crucial to the development of myeloproliferative diseases and hematopoietic cancers by playing a critical role in *Bcr-Abl*-mediated anti-apoptotic response and leukemic cell survival (8). Thus, the disruption of cytokine signaling through STAT5 results in a variety of cellular effects, ranging from an impaired immune system and damaged erythropoiesis to hematological malignancies such as CML (9).

The STAT family consists of seven members, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6, and each participates in the signalization; depending on which cytokine binds to its receptor on the cell surface (10). Activation of JAK2 via many hematopoietic and other cytokines leads to phosphorylation, thus activation of STATs (11). Each STAT member is characterized by certain domains that perform specific functions in the activation and transcription process. The N-terminal and SH2 domains are responsible for binding and interactions between dimers and proteins. Also, the N-terminal domain is involved in STAT phosphorylation. In this process, STATs bind to the promoter region of target genes via the DNA binding domain, forming a protein-DNA complex to regulate transcription (12). Since the suppression of *STAT* gene expression resulted in inhibition of oncogenesis and leukemogenesis, as well as induction of tumor cell apoptosis; STATs become the primary target of emerging anticancer agents (13).

In the last decade, specific inhibitors against tyrosine kinases have been developed to control tumor progression and have proven to be highly effective compared to other treatments (2). Tyrosine kinase inhibitors (TKIs) are potentially competitive inhibitors for ATP binding sites and inhibit substrate binding at the kinase domain. TKIs can inhibit the phosphorylation of *BCR-ABL* oncoprotein (14). Nilotinib is a second-generation tyrosine kinase inhibitor (TKI), which is highly effective in the treatment of CML patients after the failure of imatinib therapy and has increased selectivity for *BCR-ABL* and is highly effective in treating CML patients after failure of imatinib therapy (15); which makes it worthy to further clinical research (16). Since JAK2/STAT5 signaling is a vital therapeutic target in CML and its inhibition by the TKI nilotinib accelerates the contribution of obtaining disease eradication (17), we aimed to clarify the possible underlying mechanism for the action of nilotinib; whether it exerted this therapeutic effect upon expressional regulation of STAT5A, STAT5B, and the cytokines.

MATERIALS and METHODS

Culturing Conditions for K-562 Cells and Nilotinib Treatment

Human chronic myelogenous leukemia cell line K–562 was purchased from the ECACC (European Collection of Cell Cultures) company. They were cultured in RPMI 1640 medium containing 100 U/ml of penicillin-streptomycin, 1% L-glutamine, and 10% heat-inactivated fetal calf serum, at 37°C in humidified air containing 5% CO_2. Cells exhibiting a 95% survival rate and 85% confluency with the logarithmic growth phase were undertaken for further experimental studies.

The powder form of Nilotinib (Tasigna) was kindly provided by Novartis Oncology (Basel, Switzerland) and was dissolved in Dimethylsulfoxide (DMSO) to achieve the stock solution. Serial concentrated dilutions of nilotinib were prepared from this stock solution in a serum-free medium and an experimental setup was generated for the assays.

Cytotoxicity assay for Nilotinib

Cell viability and proliferation of untreated control and nilotinib-treated cells were assessed by

Cell Proliferation Reagent WST-1 (Cat. No. 11644807001, Roche Applied Science, Mannheim, Germany) to obtain the IC_{50} value with a duration of 72 hours. K562 cells at a density of 30 $x10^3$ cells/ml were seeded in 96 well plates in 0.1 ml medium per well. Then, cells were treated with increasing doses of nilotinib in a 0.1 ml serum-free medium to maximally 100 nM. Following the cell proliferation assay, the absorbance of each sample was measured spectrophotometrically at 450-620 nm with a microplate reader (Multiscan FC Thermo Fisher Scientific Thermo, Vantaa, Finland) in triplicates. All experimental setup was carried out in triplicates with x5 repeats in each assay. The obtained data was assessed with the GraphPad Prism 5.01; cell proliferation curves were generated and thus IC_{50} value was calculated for nilotinib in K562 cells.

Apoptotic Analyses Assays

The amount of apoptotic and alive cells for untreated control and IC_{50} dose of nilotinib treated cells were analyzed via two different methods: The first apoptosis assay was a photometric enzyme immunoassay as “Cell Death Detection ELISA Kit” (Cat No: 11774425001, Roche Applied Science, Mannheim, Germany) which allowed the detection of mono- and oligo nucleosomes and thus gave rise to measuring apoptotic cell death. The absorbance of each sample was measured spectrophotometrically at 405 nm with a microplate reader (Thermo, Vantaa, Finland). Secondarily, Caspase-3 activity was measured in both nilotinib-treated and untreated cells by using the Caspase-3 Colorimetric Assay Kit (Cat. No: K106, abID: ab39401, BioVision Research Products, Mountain View, CA, USA) and measured the absorbance spectrophotometrically at 405 nm. In conclusion, apoptotic cell rates were calculated after taking the average values of triplicated absorbance measurements for each sample.

Determining STAT and Pro-inflammatory Cytokine Gene Expression Profiles by real-time qRT-PCR

In the mRNA expression analyses assay, leukemic cells were reverse transfected with the determined 48th h IC_{50} dose of nilotinib for a 24-96 h time course; also, another set of cells was incubated for the same time interval without treatment that served as untreated control cells. At the end of 96 hours of incubation, all cells were collected, and total RNA extraction was performed from both nilotinib-treated and

untreated control cells using the guidelines" MagnaPure LC RNA Isolation Kit" (Cat. No: 04802993001, Roche Applied Science, Mannheim, Germany) manual in MagNA Pure Compact Instrument (Cat. No: 03731146001, Roche Diagnostic, Mannheim, Germany). The amount and quality of isolated RNAs were measured by NanoDrop™ 2000/2000c spectrophotometer (Cat. No: ND2000CLAPTOP, Thermo Fisher Scientific™, Waltham, Massachusetts, U.S.), and 100 ng RNA was reverse transcribed into cDNA via "Transcriptor High Fidelity cDNA Synthesis Kit" (Cat. No:5081955001, Roche Applied Science, Mannheim, Germany) following the instructions. STAT5A (NM_003152) and STAT5B (NM_012447) mRNA expression levels were evaluated by using the "LightCycler – h-G6PDH Housekeeping Gene Set" (Cat No: 3261883, Roche Applied Science, Mannheim, Germany) and "LightCycler® FastStart DNA Master HybProbe" (Cat No: 03003248001, Roche Applied Science, Mannheim, Germany) in LightCycler® 2.0 Instrument (Product No: 03531414001, Roche LifeScience, Mannheim, Germany) as relative quantification, in triplicates. In brief, the gained mRNA copy number of target genes either STAT5A or -5B was divided by the reference housekeeping G6PDH; referred to as the relative ratio. The copy numbers were obtained from the standard curve which was derived from standards ranging between 5×10^2 and 5×10^6 copy numbers of G6PDH. The primers and hybridization probes used for the amplification of STAT5A and STAT5B are given in Table-1.

Table-1. The primers and hybridization probes used for the amplification of STAT5A and STAT5B.

NO	Genes	Fold Change	Fold Regulation (Log2 Transformation)	p-value
1	**IFNA16**	**0.03**	**-5.06**	***P< 0.05***
2	**IFNA2**	**0.07**	**-3.84**	***P< 0.05***
3	**IFNA7**	**0.08**	**-3.64**	***P< 0.05***
4	**IL6**	**0.14**	**-2.84**	***P< 0.05***
5	**IL16**	**0.16**	**-2.64**	***P< 0.05***
6	**IL8**	**0.21**	**-2.25**	***P< 0.05***
7	**IL4**	**0.25**	**-2.00**	***P< 0.05***
8	**IFNG**	**0.25**	-2.00	-
9	**IL13**	0.26	-1.94	**-**
10	**IL17A**	0.26	-1.94	**-**
11	**TNF**	0.30	-1.74	**-**
12	**IL15**	0.32	-1.64	**-**
13	**LTA**	0.34	-1.56	**-**
14	**IL1B**	0.37	-1.43	**-**
15	**IL18**	0.40	-1.32	**-**
16	**Hs18s**	0.49	-1.03	**-**
17	**IL5**	0.49	-1.03	**-**
18	**IL3**	0.50	-1.00	**-**
19	**GUSB**	0.55	-0.86	**-**
20	**IL2**	0.57	-0.81	**-**
21	**IL1A**	0.63	-0.67	**-**
22	**IL12A**	0.87	-0.20	**-**
23	**IL12B**	0.91	-0.14	**-**
24	**IL10**	1.04	0.06	**-**
25	**IFNA6**	1.13	0.18	**-**
26	**IL9**	1.22	0.29	**-**
27	**IFNA1**	1.89	0.92	**-**
28	**IFNA17**	**5.21**	**2.38**	***P< 0.05***
29	**IFNA8**	**160.90**	**7.33**	***P< 0.05***
30	**IFNB1**	**216.77**	**7.76**	***P< 0.05***

Table-2. Effect of Nilotinib on Expression of Cytokine and Proinflammatory Genes in K-562 Cells.

STAT5A (NM_003152)	***STAT5B (NM_012447)***
F:5'-GAAGCTGAACGTGCACATGAATC-3'	F:5'-AGTTTGATTCTCAGGAAAGAATGT-3'
R:5'-GTAGGGACAGAGTCTTCACCTGG-3'	R:5'-TCCATCAACAGCTTTAGCAGT-3'
FL: ACAGGACTGTGAACTTCTCCTCTGTCACGG-FL	FL: TTGGGAGACTTGAATTACCTTATCTACGT–FL
LC: CTCTGCACCCCGCCGGTCAG–p	LC: TTCCTGATCGGCCAAAAGATGAA-p

As for the cytokines' expressional evaluation, a pre-designed plate Custom 96 StellARrayTM qPCR Array (Cat no: 00201486, Lot no: 3PH290001; BAR HARBOR BioTechnology, Trenton, ME, USA) consisting of 30 genes from the cytokine network and 2 housekeeping genes [GAPDH and HPRT1; cytokines in Table-2) was used with GENECOPOEIA-BlazeTaq SYBR Green qPCR Mix (Genecopoeia, Cat. No: QP032) kit on LightCycler® 480 Instrument II System (Material No: 05015278001, Roche Diagnostics, Mannheim, Germany) as real-time qRT-PCR. After qRT-PCR analyses, mRNA expression values were determined using the database Qiagen Gene Globe Data Analysis System (https://dataanalysis2.qiagen.com/pcr). Gene expression levels were normalized using housekeeping genes, and the fold changes were calculated using the $2^{-\Delta\Delta CT}$ method. The Student's t-test of the replicated CT values was used to calculate the *p* values.

Western blot analyses

For determining STAT5A and STAT5B protein expression levels, a similar reverse transfection of the experimental setup was organized as given in the qRT-PCR session. At the end of 96 hours, all cells were collected and lysed in the "Complete lysis-M buffer" (Cat No: 04719956001, Roche Applied Science, Mannheim, Germany) with the addition of "cOmplete™ Protease Inhibitor Cocktail Tablets" (Cat No:11697498001, Roche Applied Science, Mannheim, Germany) kit following the instructions. The isolated protein amounts were calculated by the Bradford method by using bovine serum albumin (BSA) standards ranging between 0.25 and 2 mg/ml concentrations. Finally, 40 µg of each protein extract was resolved at 8% SDS-PAGE gel and transferred to PVDF membranes using a dry transfer system iBlot™ 2 Gel Transfer Device (Catalog number: IB21001, Thermo Fisher Scientific Thermo, Vantaa, Finland). The used primer antibody concentrations were; both 1:1,000 diluted STAT5A (06–968, Upstate Biotechnology, USA), *STAT5B* (06-969, Upstate Biotechnology), and β-Actin (#4967, Cell Signaling Technology, USA). Primary antibody incubation, blotting, and secondary antibody incubation were done via "iBlot® Western Detection Kit" (Invitrogen Corporation, Carlsbad, CA, USA) for chromogenic detection of proteins in the iBlot system. The results were evaluated a with gel imaging system (Chemi Smart 2000, Vilber Lourmat, Marne-la-Vallée, Cedex, France).

Statistical Analyses

The Nilotinib IC_{50} value was calculated with the GraphPad Prism 5.01. STAT5A, STAT5B, and Pro-inflammatory cytokine gene expression levels detected by qRT-PCR and apoptotic analyses assay results were evaluated with Student T-test referring to the significance of $p < 0.05$ in SPSS 15.0 software.

RESULTS

Nilotinib Displays Cytotoxic Effects upon Leukemia Cells in a time and dose-dependent manner

Cell proliferation assay WST-1 indicated that Nilotinib decreased leukemic cell proliferation significantly in a time and dose-dependent manner in K-562 cells. The IC_{50} value of Nilotinib in K-562 cells was calculated as 34.5 nM for 48th hours (Figure-1).

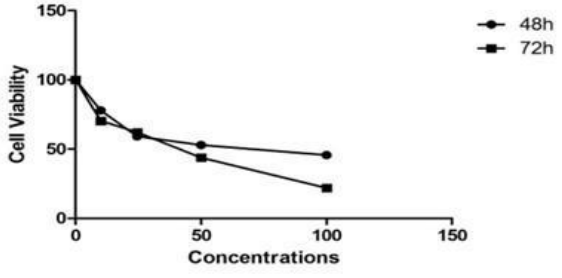


Figure-1. Cell proliferation curve for nilotinib: IC_{50} was calculated as 34.5 nM for 48th

Nilotinib Efficiently Triggers Apoptotic Cell Death of Leukemic Cells

Apoptotic changes of both nilotinib-treated and untreated control cells were evaluated via cell death detection and caspase-3 activity measurement assays in the scope of the determined IC_{50} dose for K562 cells. From both of the assays, the obtained absorbance levels indicated that nilotinib significantly induced leukemic cell apoptosis compared to untreated control cells. In detail, the apoptosis rate was increased by 47.36% (1.89-fold, *p*=0.0012) following the cell death detection assay in the nilotinib-treated group, compared to control cells (Figure-2). These results indicated the number of apoptotic nucleosomes was increased following nilotinib treatment in leukemic cells. As for the caspase 3 activity measurement assay results, the number of apoptotic cells was increased by 52.8% (2.1-fold, *p*=0.045) in nilotinib-treated cells (Figure-3). So, both of the assays pointed out that nilotinib triggered leukemic cell apoptosis approximately x2-fold significantly.

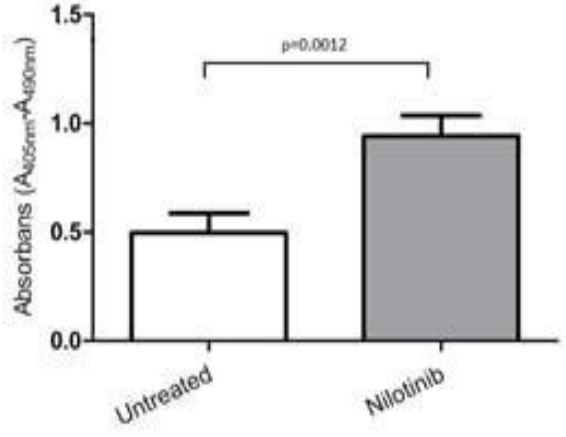


Figure-2. Apoptotic cell death detection assay result: Nilotinib triggered leukemic cell apoptosis significantly as 1.89 fold upregulation.

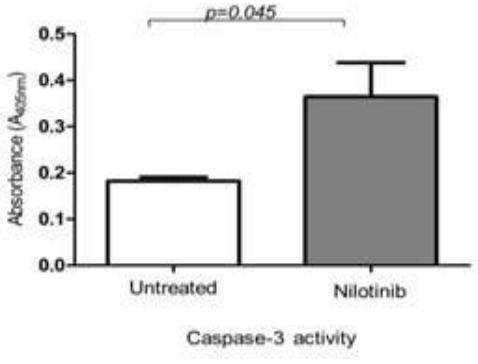


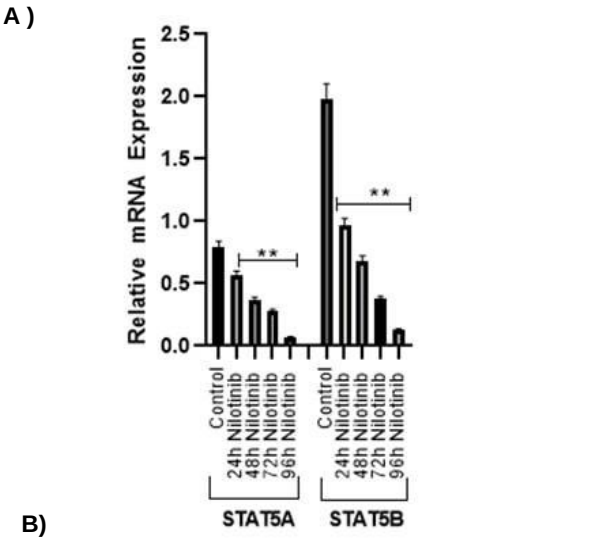
Figure-3. Measurement of caspase-3 activity assay: apoptosis was significantly induced due to nilotinib treatment as 2.1-fold increase.

Nilotinib Downregulated STAT5A & STAT5B mRNA and Protein Expression Levels and Regulated the Cytokine Expressional Network

**mRNA Transcript Levels:* The expression profiles of STAT5A and STAT5B mRNA transcripts were highly expressed in untreated cells with superiority of STAT5B with ~ twice higher than STAT5A ($p < 0.005$) (Figure-4A). Due to the IC_{50} dose of nilotinib treatment, STATs relative expressions were both significantly downregulated. STAT5A mRNA expression was downregulated by 1.41-fold and 2.15-fold respectively for the 24th and 48th hours of nilotinib treatment compared to untreated control cells (both $p < 0.05$). More significant STAT5A mRNA expressional reductions were detected as 2.87-fold (*p= 0.0033*) and 10.99-fold (*p<0.0001*) at 72nd and 96th hours, respectively in nilotinib-treated K562 cells (Figure-4B). As for STAT5B, mRNA expression levels were also dramatically decreased by 2.05-fold and 2.9-fold (both $p<0.05$) at the 24th and 48th hours, respectively. The suppression rates were higher by 5.25-fold (*p*=0.0032) and 15.08-fold ($p<0.0001$) at 72th and 96th hours respectively, following nilotinib treatment compared to untreated control cells (Figure-4C).

**Protein Expression Levels:* Western Blot results indicated that both STAT5A and 5B expressions were downregulated following nilotinib treatment. While STAT5A expression was specifically highly downregulated at the 96th hour, STAT5B was decreased starting from the 24th hour to the 96th hour with increased levels of downregulation. Protein expression assay exhibited similar mRNA expression results as STAT5B being more downregulated at 96th hours following nilotinib treatment (Figure-5).

* *Cytokine Expressional Network:* The cytokines' expressional profiles pointed out that nilotinib triggered expressional regulation of the network, compared to untreated control cells. Nilotinib significantly caused expressional decreases of several genes involved in pro-inflammatory cytokines such as IFNA16, IFNA2, IFNA7, IL6, IL16, IL8, IL4, and IFNG. Besides, IFNA17, IFNA8, and IFNB1 expressions were significantly upregulated in K562 cells due to nilotinib treatment. The fold changes and fold regulations of the evaluated genes are presented in Table-2. Also, a graphical presentation of the expressional regulations is given in Figure-6.



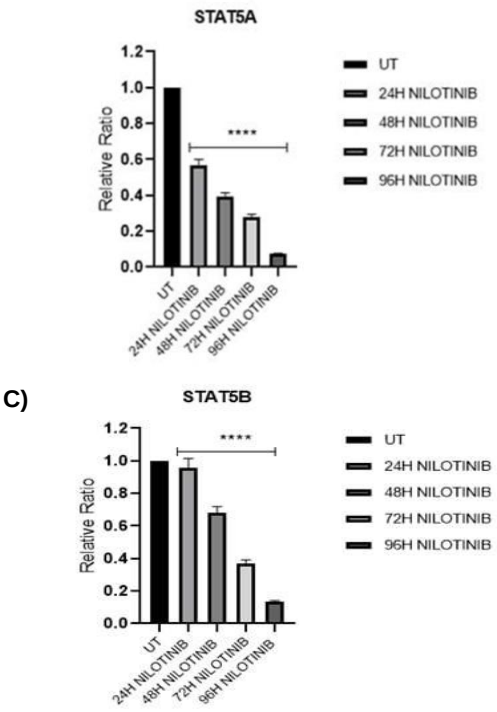


Figure-4. The mRNA expression patterns of STAT5A and STAT5B due to nilotinib treatment: A) Comparison of STAT5A and STAT5B expression profiles pointed out higher expression levels of STAT5B compared to STAT5A. B, C) Changes in STAT5A and STAT5B expression for a duration of 96 hours due to nilotinib treatment. both STAT5A and STAT5B are significantly downregulated for each hour.

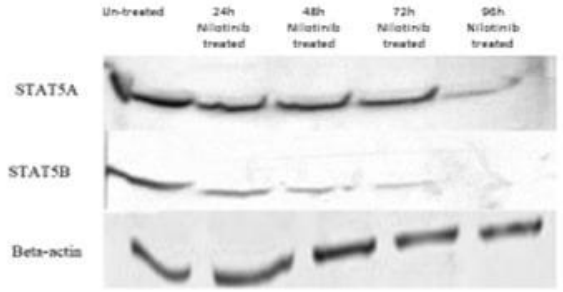


Figure-5. The protein expression patterns of STAT5A and STAT5B due to nilotinib treatment: Both STAT5A and STAT5B protein expression levels were downregulated following the IC_{50} dose of nilotinib treatment, especially at the 96th hour.

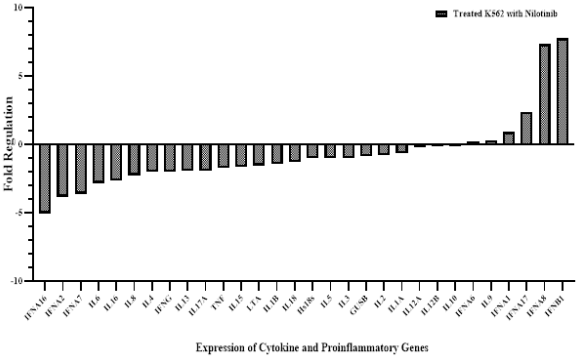


Figure-6. Graphical presentation of the expressional regulations of the genes involved in cytokine and pro-inflammatory network due to nilotinib treatment

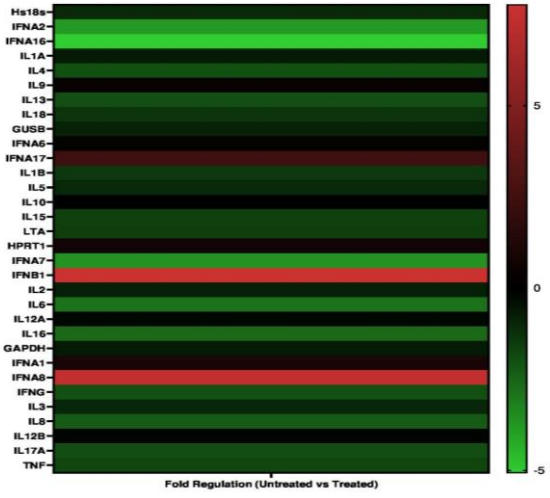


Figure-7. Representative heat maps for upregulated and downregulated genes in untreated and treated cells

DISCUSSION

STAT proteins play significant roles in the JAK-STAT signaling pathway and are activated by phosphorylation in the JAK2-STAT5 axis; which in turn accelerates the signaling network of *BCR-ABL1*, therefore triggering CML pathogenesis (17-19). Besides the usage of tyrosine kinase inhibitors, resistance to TKIs remains one of the major causes of treatment failure and patient death in CML (20). Thus, alternative strategies in the case of targeting the activation of the pathway; especially the major elements such as STAT5 trigger a potential therapeutic approach whether a TKI resistance exists or not.

This study focuses on determining the effects of second-generation TKI nilotinib upon CML cells, specialized in terms of STAT5 and cytokine network expressional regulation. Initially, we detected that STAT5B expression was higher in CML cells compared o STAT5A and, following nilotinib treatment, STAT5B exhibited higher rates of expressional reduction. The main reason for this may be related to their different functional roles in BCR-ABL signaling as being phosphorylated and activated. Both STAT5A/5B act as the critical network downstream of BCR–ABL signaling and are essential for the initiation and maintenance of CML. BCR-ABL signaling directly activates STAT5B at a higher rate than STAT5A because STAT5A remains partially in the cytoplasm, whereas STAT5B is fully activated in the nucleus (21, 22).

Then, we have shown that both STAT5A and STAT5B mRNA and protein levels expressional upregulation was decreased due to nilotinib treatment, and leukemic cell apoptosis was induced with inhibited leukemic cell proliferation in a time and dose-dependent manner. Several studies have also shown that the JAK-STAT signaling pathway is a potential survival mechanism of CML leukemia stem cells (18, 19); thus, playing roles in the regulation of cell proliferation and survival (23) via the JAK2/STAT5 axis as protecting cells from TKI-induced apoptosis (24). Similarly, Baśkiewicz-Masiuk et al. have also focused on determining the effect of suppression of STAT5A and STAT5B expression upon clonogenicity and apoptosis of CML and acute myeloid leukemia (AML) cells. They have reported that STAT5 was a potent target for a therapeutic approach, so we do, as an inducer of leukemic cell apoptosis. In another study, it was also reported that targeting and downregulating STAT5A and STAT5B mRNA levels via oligodeoxynucleotides (ODNs) reduced the proliferative potential of CML and AML blasts and induced apoptosis (25). Some other studies have also reported an association between nilotinib treatment and induced apoptosis. Ekiz et al. showed that nilotinib-induced apoptosis in imatinib-resistant K562 CML cells with increased caspase-3 enzyme activity (26) similar to our induced apoptosis data. Since our results put forward an interaction between nilotinib therapy and STAT5 expressional regulation in CML cells; Kantarjian et al. have also reported the correlation between oral nilotinib dose and phosphorylation of AKT, CRKL, STAT1, and STAT5 proteins in 119 either imatinib-resistant CML or acute lymphoblastic leukemia (ALL) patients. In all four signaling molecules, phosphorylation was reported to be significantly reduced at day 15 of nilotinib treatment compared to baseline after adjusting for multiple testing (27).

Since STAT5A and STAT5B are also activated by many cytokines which result in expressional increases leading to a leukemic phenotype and escape from apoptosis; we finally analyzed the differing expressional profiles of the cytokine network members following nilotinib treatment. We investigated significant genes like IFNA2, IFNA7, IFNA8, IFNA16, IFNA17, ILs, and IFN that are relevant to immune cytokine signaling pathways (28). The genes that have roles in the immune response are overexpressed in leukemia patients such as IFNA2, IFNA7, IFNA8, IFNA10, IFNA14, and IFNA16 (29, 30). We evaluated that IFNA16 showed the highest significant reduction as 5.06-fold downregulation, then IFNA2 with 3.84-fold and IFNA7 at 3.64-fold decreases following nilotinib treatment as pointing out the therapeutic reflection of the drug. Especially IFNA7 is upregulated in leukemia as regulating interferon signaling and also JAK/STAT signaling thus should be downregulated (31). Among those that we analyzed, only IFNA8 expression was increased, whereas its immunogenomic structure was reported to have an association with survival (32). This controversy might be related to the molecular pathogenesis of CML in case of gain of resistance to TKI or nilotinib not perfectly targeting all leukemic cells. As another possibility, in a current study by Ge Q, IFNA8 has been reported to be aberrantly activated in the AKT pathway (32); thus an AKT inhibitor cotreatment might be an alternative approach in nilotinib-

based CML treatment since IFNA8 is a positive regulator of peptidyl-serine phosphorylation of STAT proteins (33). The expression of genes associated with immune system regulation, especially IFNA17 was significantly related to prognostic markers for disease-specific survival, and IFNA17 was down-regulated in many types of cancer (34). In this study, we demonstrated that IFNA17 was significantly 2.38-fold up-regulated due to nilotinib treatment in CML cells, indicating a view of its therapeutic mechanism.

As for interleukins, IL-6 is one of the anti-inflammatory cytokines that is thoroughly studied and is regarded as a significant target for clinical interventions. IL-6 has been proposed to be a worthy prognostic biomarker in CML since accelerating JAK/STAT signaling (35,36). We detected that IL-6 expression was significantly decreased by -2.84-fold due to treatment, indicating the therapeutic power of nilotinib. In a current study, IL6 serum expression levels were found to be extremely high in various phases of CML patients (37). Similarly, many other studies have focused on IL-6 as a significant immunomodulatory factor that has been closely related to the development of several cancers and should be targeted for cure (38-40).

IL-8 (or CXCL8) is a chemokine and expressional upregulated in AML and correlated with poor prognosis (41). IL-8 is also regarded to play an important role in the chronic lymphocytic leukemia (CLL) biological process. High IL-8 serum levels have been determined in most patients compared to healthy donors and it was reported that IL-8 extended leukemic cell survival (42). In the present study, IL-8 expression was significantly 2.25-fold down-regulated following nilotinib treatment, giving rise to a therapeutic benefit. Similarly, Hantschel t al has reported that IL8 expression was upregulated due to *Bcr-Abl* expression and was inhibited by nilotinib in K562 cells, giving rise to a conclusion that IL-8 played a potential pathophysiological role and could be a useful marker for monitoring CML inhibitor efficacy (43). Also, IL16 expression was downregulated by 2.84-fold in nilotinib-treated cells. IL16 is a chemotactic cytokine that is expressional increased in various cancers and apoptosis is regulated (44, 45). Downregulation in IL16 insights the inhibition of leukemic cell proliferation and induced apoptosis due to caspase-3 activity in our nilotinib-treated K562 cells. Rather than CML, the interaction of IL-16 plasma expression levels and tumor progression has been reported for many types of cancer; especially breast cancer, multiple myeloma, and cutaneous T-cell lymphoma (46) whereas our study contributes to IL16 as a potential target in CML pathogenesis.

Finally, the *interferon-β (IFNB1)* expression was found to be upregulated in nilotinib-treated K562 cells. *IFNB1* belongs to the type I IFNs and exhibits antiproliferative, antiangiogenic, proapoptotic, and immune-modulatory functions. *IFNB1* is located on chromosome 9 and has been demonstrated to be rearranged or deleted in leukemia (47); thus acting via the JAK-STAT pathway to modulate the transcription of target genes (48). Due to nilotinib treatment, its expressional increase might lead to the gain of function in CML therapeutics.

CONCLUSION

STAT5A and *STAT5B* are significant molecular targets in the research of CML therapy. Nilotinib may exert its therapeutic effect by downregulating leukemic cell proliferation and inducing apoptosis by regulating the JAK/STAT pathway and the cytokine network.

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