Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
C M B Association Publisher

Original Research

Characterization of imatinib-resistant K562 cell line displaying resistance mechanisms

Yalda Hekmatshoar[1*], Tulin Ozkan[1*], Buket Altinok Gunes[1,2], Sureyya Bozkurt[3], Aynur Karadag[1,4], Arzu Zeynep Karabay[5], Asuman Sunguroglu[1*]

[1] Department of Medical Biology, School of Medicine, Ankara University, Ankara, Turkey
[2] Vocational School of Health Services, Medical Laboratory Techniques, Ankara University, Ankara, Turkey
[3] Department of Medical Biology, School of Medicine, Istinye University, Istanbul, Turkey
[4] Department of Medical Biology, School of Medicine, Usak University, Usak, Turkey
[5] Faculty of Pharmacy, Department of Biochemistry, Ankara University, Ankara, Turkey

Correspondence to: hekmatshoar@ankara.edu.tr; tozkan@ankara.edu.tr; asungur@medicine.ankara.edu.tr

Received February 20, 2018; **Accepted** April 9, 2018; **Published** May 15, 2018

Doi: http://dx.doi.org/10.14715/cmb/2018.64.6.5

Abstract: Chronic myeloid leukemia (CML) is a hematopoietic malignancy characterized by the t(9; 22) and the related oncogene, BCR-ABL. Tyrosine kinase activity of fusion protein BCR-ABL is the main cause of CML. Even if imatinib is used as a tyrosine kinase inhibitor (TKI) for CML therapy, drug resistance may occur in patients and the clinical failure of imatinib treatment in resistant patients had resulted with the use of another alternative TKIs. BCR-ABL dependent and independent molecular mechanisms have crucial roles in drug resistance. To reveal the underlying molecular mechanisms which play significant roles in imatinib resistance in CML, we established K562 imatinib-resistant cell line (K562r5) which was continuously exposed to (5μM) imatinib to investigate molecular mechanisms which play significant roles in drug resistance. First of all, we analyzed T315I, M351T, F315L and F359C/L/V mutations with DNA sequencing as a BCR-ABL dependent mechanism in our cell lines. Moreover, we investigated BCR-ABL independent mechanisms such as apoptosis, autophagy, drug transport and DNA repair which affect drug resistance in these cell lines. *In vitro* cell viability was determined by MTT assay. DNA sequencing analysis was performed to detect BCR-ABL mutations. The apoptotic effect of imatinib on CML cell lines was tested by flow cytometric Annexin V-PE staining and caspase activation assays. Apoptotic, autophagic, drug transporter and DNA repair genes expression levels were determined by RT-PCR. The conventional cytogenetic analysis was performed on K562s and K562r cells. Our results indicate that inhibition of apoptosis, induction of autophagy, overexpression of efflux gene MDR1 and down-regulation of influx gene OCT1 play crucial roles in the progression of imatinib resistance.

Key words: Imatinib resistance; CML; Apoptosis; Autophagy; Drug transporter proteins; DNA Repair.

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disease which is characterized by Philadelphia chromosome caused by a reciprocal translocation between chromosomes 9 and 22. This translocation leads to the formation of constitutively active BCR-ABL chimeric protein which is responsible for the emergence of a leukemic clone (1). Imatinib mesylate is a tyrosine kinase inhibitor which targets BCR-ABL tyrosine kinase activity. Although treatment of CML patients with imatinib is successful, resistance against imatinib can be seen in CML patients. Imatinib resistance mechanisms can be classified as BCR-ABL dependent and BCR-ABL independent mechanisms. Mutations in the BCR–ABL tyrosine kinase domain (KD) is one of the important mechanisms in BCR-ABL dependent resistance (2).

There are several mechanisms which lead to BCR-ABL independent drug resistance. One of these mechanisms is the alteration of drug efflux protein expression. ATP binding cassette (ABC) transporter proteins such as MDR1 (Multi Drug Resistance Protein1) and MRP1 (Multidrug Resistance-Associated Protein 1) are efflux pumps which expel drugs. Increased expression levels of these efflux pumps were reported in resistant CML (3, 4). On the other hand, decreased level of influx proteins such as organic cation transporter-1 (Oct-1) seems to be responsible for inefficient drug uptake and drug resistance (5, 6). Other BCR-ABL independent imatinib resistance mechanisms are inhibition of apoptosis and induction of autophagy. Apoptosis (programmed cell death) is an important mechanism in maintaining the homeostatic balance in physiological processes and classified into extrinsic and intrinsic pathways (7,8). Intrinsic pathway can be induced by signals such as cellular stresses, DNA damage and chemo/radiotherapies which lead to activation of pro-apoptotic Bcl-2 family proteins. The pro-apoptotic Bcl-2 family members Bax, Bak, Bim and Bad induce permeabilization of the mitochondrial membrane, the release of cytochrome c and caspase activation. The extrinsic pathway is activated by binding of cell death ligands such as FAS to death receptors which are located on the cell surface. Activation of these pathways leads to cell death (9).

Autophagy as a survival mechanism is a highly conserved cellular process which occurs during starvation, nutrient deprivation, accumulation of reactive oxygen species (ROS) and chemotherapy. Autophagy is regulated by more than 30 proteins which are known as autophagy-related genes (ATGs). In addition to Atg

proteins, Beclin1 protein plays a critical role in the regulation and induction of autophagy (10). Autophagy is initiated with the formation of phagophore, autophagosome and autolysosomes respectively (11,12,13)

Genomic instability can be observed in most cancer cells. Recent studies demonstrated that BCR/ABL expression leads to small increase in DNA damage and genomic instabilities (14). Accumulation of genetic lesions is correlated with the occurrence of resistance to BCR-ABL kinase inhibitors, such as imatinib. Studies demonstrated that increased level of endogenous reactive oxygen species causes accumulation of DNA alterations such as point mutations, in BCR/ABL-positive leukemia cells (15, 16). Mismatch repair (MMR) system plays an important role in maintaining genomic stability by recognizing and removing mismatched bases from DNA and inducing apoptosis in cells with accumulated DNA lesions (17). MutS homolog 2 (MSH2) and mutL homolog 1 (MLH1) are the members of the MMR system which recognize and remove base-base mismatches and insertion-deletion mispairs. Another DNA repair protein, O6-methylguanine-DNA-methyltransferase (MGMT) reduces the mutagenic effects of alkylating agents such as temozolomide (TMZ). Recent studies showed that MGMT plays a crucial role in temozolomide resistance in Glioblastoma multiforme (GBM) patients (18). The role of MGMT is not clarified in imatinib resistance yet.

Imatinib resistance is one of the main causes of treatment failure in CML therapy, therefore, in this study, we planned to investigate the different mechanisms which involved in imatinib resistance in CML. For this purpose, we established imatinib-resistant K562 cells (K562r) which were generated from imatinib sensitive K562s cells by increasing doses of imatinib and these resistant cells were able to grow in the presence of 5 µM imatinib.

BCR-ABL mutations such as T315I play crucial roles in imatinib resistance, therefore we performed sequence analysis to detect BCR-ABL mutations in our K562 cells. In addition to BCR-ABL dependent mechanisms, there are several BCR-ABL independent mechanisms that lead to imatinib resistance. In order to investigate the BCR-ABL independent mechanisms we analysed the expression changes in apoptotic, autophagic, drug transporter and DNA repair genes in imatinib-resistant and sensitive K562 cells.

Materials and Methods

Cell lines and cell cultures

The cells were cultured with RPMI 1640 growth medium (Sigma/USA), containing 10 % fetal bovine serum (FBS)(Sigma/USA), 100 units/ml penicillin, 100g/ml streptomycin (Gibco/USA) and 1,2 ml L-Glutamine (Gibco/ USA). 0,6µM imatinib-resistant K562r (K562r0.6) cell line was a gift of Prof. Carlo Gambacorti-Passerini. Imatinib mesylate resistant cell line (K562r5) which is able to grow in the presence of 5 µM imatinib were generated by adding imatinib mesylate (Santa Cruz/USA) with stepwise increasing concentration during 18 months on imatinib mesylate-resistant K562r0.6 cell. In each step, 2-4x10^5 cells/ml were incubated with imatinib. Due to the stability of imatinib in water, imatinib stock at a final concentration of 10mM was freshly prepared every month. The medium containing fresh imatinib was added and the cell line was passaged every 3 days.

Cell viability

In order to determine the imatinib concentration in which K562r are resistant, we plated approximately 2×10^4 cells per well in a 96-well plate. K562s, K562r0.6, and K562r5 Cells were treated with different concentrations of imatinib (0.6µM and 5µM), then incubated for 48 hours at 37˚C and 5% CO2. Cell viability was evaluated with the MTT test. 10 µl (5 mg/ml) of the MTT reagent was added to each well for 2-hours at 37˚C. After 2h 100 µL detergent reagent was added to each well plate. The absorbance at 550-690nm was quantified using a spectrophotometric microplate reader (Biotek, USA) (19).

Sequence analysis

c-DNA was amplified with polymerase chain reaction (PCR) for p210 product of BCR-ABL. Semi-nested PCR was performed to amplify the complete ABL kinase domain (KD) mutations (T315I, M351T, F315L, F359C/L/V) of the BCR-ABL allele. The first PCR step amplified BCR-ABL using the forward primer 5'-AGA TGC TGA CCA ACT CGT G-3' and reverse primer 5'-GCC ATA GGT AGC AAT TTC CCA-3'. The second step amplified the ABL (exon 6) of BCR-ABL using the forward primer 5'-GAA AGA GAT CAA ACA CCC- 3' and reverse primer 5'-AAA TCA GCT ACC TTC ACC-3'. After amplification PCR products were purified with Promega A9281 Wizard SV Gel and PCR Clean-Up System (Promega, Madison, Wis., U.S.A.) according to the manufacturer's instructions and were then sequenced using Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequencing products were dissolved in 20ul formamide (Applied Biosystems) and denatured. Bidirectional sequencing was carried out in an ABI 310 Genetic Analyzer (Applied Biosystems). The sequence analyzing results were evaluated using Chromas program in terms of each mutation.

Flow cytometric analysis for apoptosis determination

K562s, K562r0.6 and K562r5 cells (2x10^5cells/well) were seeded in 6 well plates. Cells were incubated with imatinib (0.6 and 5µM) for 48 h. After the incubation period, cells were collected, washed with PBS two times and resuspended in the 1X binding buffer according to the suppliers instructions (BD Biosciences). 5 µl PE-Annexin V-PE and 5 µl 7AAD was added to 100 µl of cell suspension. After a brief vortex, cells were incubated for 15 min at room temperature. After the incubation period, cells were washed with 1X binding buffer and 200 µl 1X binding buffer was added to cells and cells were measured by flow cytometry (Accuri C6) (20).

Detection of caspase 3/7 activation

Caspase3/7 activation was determined by the Cell Event Caspase-3/7 Green Flow Cytometry Assay Kit as described by the manufacturer (Thermo Fisher Scientific) protocols. Flow cytometry tubes were prepared with

each containing 1 ml of cell suspension at $4x10^5$ cells/ml; 1 µL of Cell Event Caspase-3/7 Green Detection Reagent was then added to each sample and incubated for 30 minutes at 37 °C. During the final 5 minutes of staining, 1 µL of the 1 mM SYTOXTM AADvancedTM dead cell stain solution was added. The samples were analyzed on an Accuri C6 flow cytometer. Data plots of FL-1 and FL-4 used to show the populations of alive and apoptotic cells with the activated form of caspase 3 and 7 (21).

Quantitative Real -Time PCR

For detection of expression levels of apoptosis and autophagy-related, drug transporter and DNA repair genes, we used quantitative real-time RT-PCR. K562 cells ($5x10^5$ cells/well) were seeded in six well-plates. Cells were incubated with imatinib for 48 h. After incubation period total RNA was extracted from K562 cells by using the High Pure RNA kit (Roche, Mannheim, Germany) according to the manufacturer's protocols. cDNA was generated from RNA by reverse transcriptase (Transcriptor High Fidelity cDNA Synthesis Kit; Roche). Quantitative real-time RT-PCR was performed using SYBR Green PCR Master Mix (Roche) on the LC480 instrument. mRNA was measured relative to HPRT as an endogenous control. Experiments were performed in biological triplicates. For primer sequences see Table 1.

Determination of Pgp activity in cell lines

P-glycoprotein's ability to pump dyes such as rhodamine 123 from the cell has been used to determine the activity of Pgp (Neyfakh, 1988). For this assay, $5x10^5$ cells from each cell line were incubated with 150 ng/ml of Rh 123 (Sigma, St Louis, U.S.A) for 30 min at 37°C in RPMI medium. The cells were washed twice with PBS and resuspended in RPMI and allowed to efflux for 90 min at 37°C. The cells were then washed and analyzed on flow cytometry (Accuri C6).

Cytogenetic analysis

A conventional cytogenetic analysis was performed on metaphase cells derived from K562s and K562r5 cell lines. Briefly, the cells were synchronized using fluorodeoxyuridine, uridine, and thymidine. Colcemid was added to the culture (0.05 g/mL) for half an hour. After that cells were incubated in hypotonic solution for 30 min (0.075 M KCl) and fixed with Carnaby solution (3 parts methanol to 1 part glacial acetic acid). After G-banding, karyotypes were interpreted according to the 2009 International System for Human Cytogenetic Nomenclature (24).

Statistical analysis

Data were represented as mean ±standard deviations (SD) from triplicate experiments performed in a parallel manner. Comparisons between treated and untreated control groups were made by t-tests or ANOVA analysis where applicable. $P < 0.05$ and $P < 0.001$ was used as the cutoff for defining statistically significant differences.

Results

Effects of imatinib on cell viability of imatinib sensitive K562s and imatinib-resistant K562r cell lines

Cytotoxic effects of imatinib on K562s, K562r0.6, K562r5 cells were evaluated by MTT assay. Cell viability of all cell lines diminished significantly with increasing dose of imatinib from 0.6 µM to 5 µM in comparison with control group (without imatinib treatment) at 48h. K562s cell viability after 0.6 µM and 5 µM imatinib treatment are 52.122±2.977% and 42.42±2.473% respectively. The viability of K562r0.6 cells after 0.6 µM and 5 µM imatinib-treatment are 83.92±6.909% and 45.44±3.57% respectively. Cell viability of K562r5 cells treated with 0.6 µM and 5 µM concentrations decreased from 95.43±4.25% to 74.8±3.9%. Test results were demonstrated in Figure1.

T**able 1.** Primers were used in the Quantitative Real -Time PCR **.**

Gene	Forward (5'- 3')	Reverse(5'- 3')
BAD	GATGAGTGACGAGTTTGTGGA	CAAGTTCCGATCCCACCAG
BCL-2	CGCCCTGTGGATGACTGAGT	GGGCCGTACAGTTCCACAA
BIM	ATCTCAGTGCAATGGCTTCC	CATAGTAAGCGTTAAACTCGTCTCC
BAX	GACGGCAACTTCAACTGGG	AGGAGTCTCACCCAACCAC
BCL-XL (22)	GATCCCCATGGCAGCAGTAAAGCAAG	CCCCATCCCGGAAGAGTTCATTCACT
MCL-1	CGAACCATTAGCAGAAAGTATCAC	GATATGCCAAACCAGCTCCT
MDR-1	AAGGCATTTACTTCAAACTTGTCA	GGATTCATCAGCTGCATTTTC
MRP-1	AGTGCTTTCAGAACACGGTC	TTTCCCAGAAAGAGTAGAAGAGGT
OCT1	TCCTCTTCCTGCTCTACTACTGG	TGGTCCATTATCTTTATTGCTTCA
BCRP	CCTTGGGATACTTTGAATCAG	AGTTGACATAAATCTCCGCT
ATG5 (23)	TGGGCCATCAATCGGAAACTC	TGCAGCCACAGGACGAAACAG
ATG7 (23)	GCAAGCCCGCAGAGATGTGGA	GCAGCAATGACGGCAGGAAGC
ATG12 (23)	TCCGAGCCAGCGGCCTAACT	AAGGAGGCGCCGGAGTAGGG
BECLIN1 (23)	AGCTGCCGTTATACTGTTCTG	ACTGCCTCCTGTGTCTTCAATCTT
HPRT	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
MGMT	GAATGCCTATTTCCACCAGCC	TCTCCGAATTTCACAACCTTCAG
MSH2	GCTGGAAATAAGGCATCCAAGG	CACCAATGGAAGCTGACATATCA
MLH1	TTCGTGGCAGGGGTTATTCG	GCCTCCCTCTTTAACAATCACTT

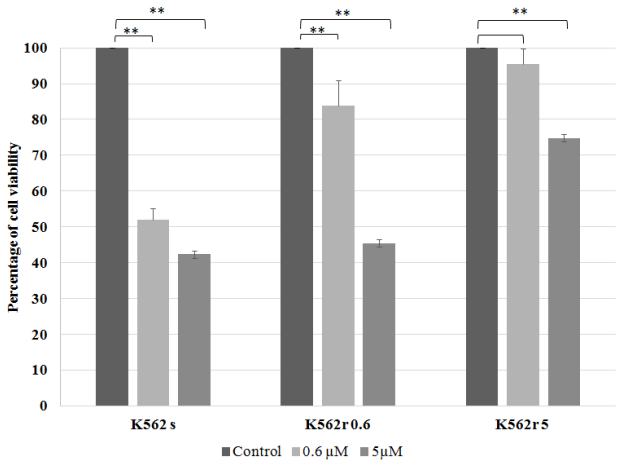

Figure 1. Effects of imatinib (0.6-5µM) on cell viability of K562s, K562r0.6, and K562r5 cells. All cells were incubated with imatinib for 48 h before analyzing cell viability with MTT. The viability of cells treated without imatinib was regarded as 100 %.(*p≤ 0.05 **p≤ 0.001).

Mutations analysis of imatinib sensitive K562s and imatinib-resistant K562r cell lines with DNA sequencing

We examined if there were any mutations (T315I, M351T, F315L, F359C/L/V) along with enhanced doses of imatinib with sequence analysis. But we did not detect any mutations in K562s, K562r0.6, and K562r5 (Fig 2).

Determination of apoptosis in imatinib sensitive K562s and imatinib-resistant K562r cell lines with flow cytometry

To determine the apoptotic effects of low (0.6 µM) and high (5 µM) imatinib doses on treated K562 cells, flow cytometric analysis with Annexin V-PE/7AAD staining was performed. Imatinib treated cell were marked with 7AAD and Annexin- V and were examined by flow cytometry (Fig. 3 A). The percentage of early apoptotic K562s, K562r0.6 and K562r5 cells (Annexin V-PE / 7AAD) incubated with 0.6 µM imatinib are higher (36.24±2.85, 21.53±2.58, 19.68±1.17% respectively) than control cells (7.07±1.47, 16.96±0.58, 17.50±0.51% respectively) at 48h (Fig. 3 B, C, D). The percentage of early apoptotic K562s, K562r0.6 and K562r5 cells treated with 5 µM imatinib are significantly higher (51.63±1.95, 31.9±2.68, 24.42±1.33% respectively) than the control group (7.07±1.47, 16.96±0.58, 17.50±0.51% respectively) at 48 h (Fig. 3 B, C, D). The percentage of late apoptotic K562s, K562r0.6 and K562r5 cells (Annexin V-PE / 7AAD) incubated with 0.6 µM imatinib (5.32±0.9, 9.05±2.85, 7.04±0.91% respectively) were higher than the control group(5.57±1.33, 7.56±1, 5.95±0,33% respectively) at 48h (Fig. 3 B, C, D). The percentage of late apoptotic K562s, K562r0.6 and K562r5 cells (Annexin V-PE / 7AAD) incubated with 5 µM imatinib higher (12.58±0.98, 13.10±2.31, 12,73±0,91% respectively) than control group (5.57±1.33, 7.56±1, 5.95±0,33% respectively) (Fig 3. B, C, D).

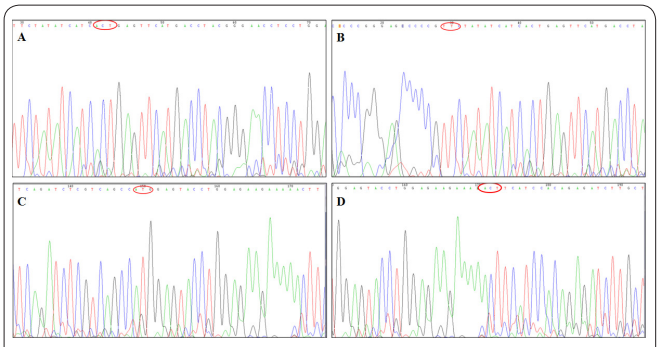

Figure 2. Sequencing results of the ABL gene (exon 6) containing previously reported mutational hotspots are represented as A) T315I mutation in which ACT→ATT; B) F311L mutation in which TTC→CTC; C) M351T mutation in which ATG→ACG; D) F359C/L/V mutation in which TTC→GTC in K562r5 cells.

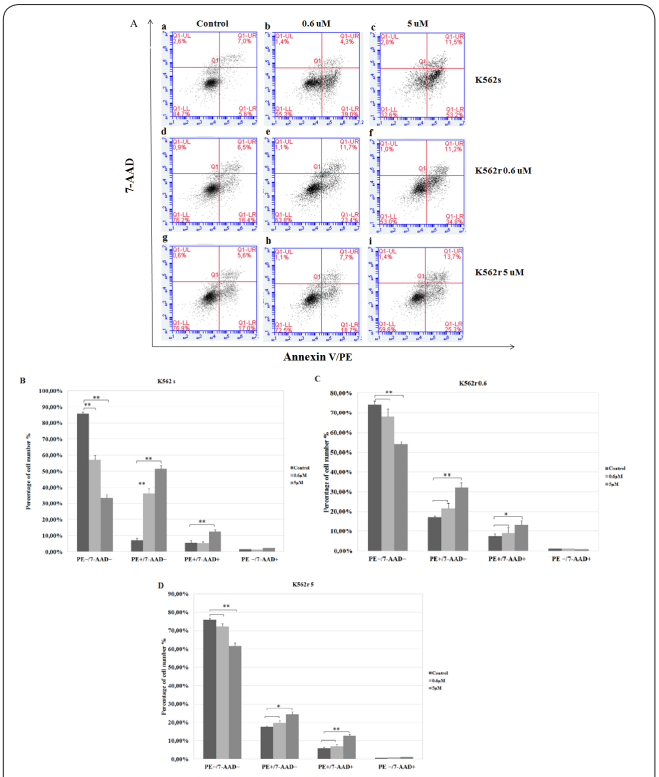

Figure 3. A) Flow cytometry results are represented as a) K562s cells without imatinib; b) K562s cells incubated with 0.6 µM imatinib; c) K562s cells incubated with 5 µM imatinib; d) K562r0.6 cells without imatinib; e) K562r0.6 cells incubated with 0.6 µM imatinib; f) K562r0.6 cells incubated with 5 µM imatinib; g) K562r5 cells without imatinib; h) K562r5 cells incubated with 0.6 µM imatinib; i) K562r5 cells incubated with 5µM imatinib; B) Flow cytometry results of K562s cells with/without imatinib were shown as bar graphs; C) Flow cytometry results of K562r0.6 cells with/without imatinib were shown as bar graphs; D) Flow cytometry results of K562r5 cells with/without imatinib were shown as bar graphs. Percentage of viable cells (Annexin V-PE $^{-}$/7-AAD^{-}), early apoptotic cells (Annexin V-PE $^{+}$/7-AAD^{-}), late apoptotic cells (Annexin V-PE^{+}/7-AAD^{+}), and necrotic cells (Annexin V-PE $^{-}$/7-AAD^{+}) are shown as means ± SD. (*p≤ 0.05 ** p≤0.001).

Detection of caspase 3/7 activation in imatinib sensitive K562s and imatinib-resistant K562r cell lines with flow cytometry

To confirm our annexin V assay, we examined caspase 3/7 activation after 48h incubation with imatinib. According to our data caspase 3/7 were activated in K562s cells in a dose dependent manner. The highest activation was detected in 5µM imatinib treated K562s cells (157,753.35±10422.3667) in comparison with 0.6µM imatinib treated K562s cells (129,314.93±7941.905853) and K562s control cells (15,296.75±361.0338877)

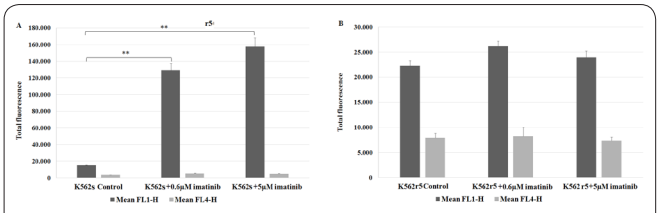

Figure 4. A) Caspase 3/7 activation after 48h of incubation with 0.6 μM and 5 μM imatinib in K562S cells, B) Caspase 3/7 activation after 48h of incubation with 0.6 μM and 5 μM in K562r5 cells, ** p≤0.001.

(Fig.4A). Whereas, caspase activation in the 0.6 and 5 μM imatinib treated K562r5 cells was not statistically significant (Fig.4B).

Expression levels of apoptotic genes in imatinib sensitive K562s and imatinib-resistant K562r cell lines

To evaluate the expression level of pro-apoptotic and anti-apoptotic genes, we compared K562r0.6 and K562r5 cells with K562s (treated with 0.6μM imatinib) and K562s (treated with 5μM imatinib) respectively as a control group. Antiapoptotic gene *BCL-2* expression increased significantly as a 3.83 fold in K562r0.6 cells when compared to 0.6 μM imatinib treated K562s cells, and when the dosage of imatinib was increased to 5 μM, its expression level increased as a 3.38 fold in comparison with 5 μM treated K562s cells. *MCL-1* expression level increased dramatically from 1.1 fold to 24.83 fold when imatinib doses increased from 0.6 μM to 5 μM in comparison with their imatinib treated control groups (p≤ 0.05). *BCL-XL* gene expression was increased from 0.75 fold to 1.48 fold in K562r0.6 and K562r5 when compared with K562s imatinib-treated cells in a dose-dependent manner. Expression fold change of pro-apoptotic gene BIM in K562r0.6 and K562r5 imatinib-resistant cells are 0.26 and 0.46 in comparison with K562s 0.6 μM and 5 μM imatinib-treated cells respectively (p≤ 0.001). The expression level of *BAD* gene decreased dramatically from 12.14 fold to 0.2 fold in K562r0.6 and K562r5 imatinib- resistant cells respectively. *BAX* gene expression fold changes were 0.31 and 0.84 in K562r0.6 and K562r5 imatinib-resistant when compared to K562s 0.6 μM a 5 μM imatinib incubated cells (Fig 5A).

Expression levels of drug transporter genes in imatinib sensitive K562s and imatinib-resistant K562r cell lines

We examined expression levels of *MDR-1, MRP-1, OCT-1* genes in K562s and K562r cells by quantitative real-time PCR. The expression level of the *MDR-1* gene was found as increased 350.97 fold in K562r0.6 cell line compared to 0.6 μM imatinib-treated K562s, moreover, MDR-1 expression level increased 135.70 fold in imatinib-resistant K562r5 cell lines in comparison with K562s cells treated with 5 μM imatinib. These results were found as statistically significant.

MRP-1 gene expression decreased 0.694 and 0.652 fold in imatinib K562r0.6 and K562r5 cells compared with K562s incubated with 0.6 μM and 5 μM respectively(p ≤0.05).

The fold change in *OCT-1* gene expression which encodes an influx protein decreased in both imatinib-resistant K562r0.6 and K562r5 cell lines 0.26 and 0.23

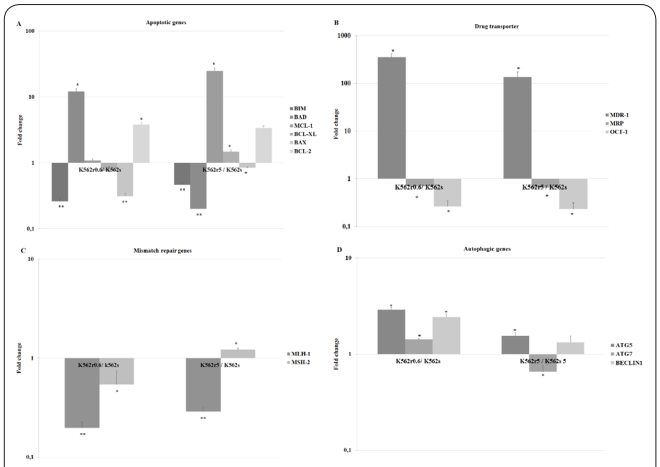

Figure 5. A) Expression of apoptosis-related genes *BAX, BCL-2, BAD, BIM, BCL-XL* and *MCL1* after treatment with imatinib; B) Expression of drug transporter genes *MDR1*, *MRP1* and *OCT1* after treatment with imatinib; C) Expression of DNA repair genes *MLH1* and *MSH2* after treatment with imatinib; D) Expression of autophagic genes *ATG5, ATG7*, and *BECLIN1* after treatment with imatinib. Levels of expression are compared with 0.6 μM and 5 μM imatinib treated K562s, and *HPRT* mRNA was used as an internal control. Experiments were conducted in triplicate. Error bars indicate ± s.d. significantly different from control (*p≤ 0.05 ** p≤0.001).

fold in comparison with control group K562s treated with 0.6 μM and 5 μM imatinib respectively (p <0.05) (Fig 5B).

Expression levels of DNA repair genes in imatinib sensitive K562s and imatinib-resistant K562r cell lines

We evaluated expression levels of *MLH1* and *MSH2* genes which play a role in DNA mismatch repair. We found that expression level of *MSH2* gene decreased (0.54 fold) in K562r0.6 cells whereas gene expression is increased significantly to 1.22 fold in K562r5 cells in comparison with control group K562s treated with 0.6 μM and 5 μM imatinib respectively. Another mismatch repair gene *MLH1* gene expression decreased in both K562r 0.6 and K562r5 (0.19 and 0.28 fold respectively) in comparison with 0.6 μM and 5 μM imatinib treated K562s cells (Fig 5C).

MGMT is another gene which plays an important role in DNA repair system. According to our results, we did not detect any expression of this gene in all K562 cells.

Expression levels of genes which play roles in autophagy in imatinib sensitive K562s and imatinib-resistant K562r cell lines

ATG5, *ATG7*, and *BECLIN1* are genes playing roles in autophagy. *ATG5* expression level increased 2.9 and 1.5 fold in K562r0.6 and K562r5 cells respectively compared with 0.6 μM and 5 μM imatinib treated K562s cells respectively (p≤0.05). *ATG7* gene expression increased 1.42 fold in K562r0.6 cell lines whereas it decreased 0.65 fold in K562r5 cells in comparison with K562s cells incubated with 0.6 μM and 5 μM imatinib respectively (p≤0.05). *BECLIN1* gene expression increased significantly in K562r0.6 2.42 fold, on the other hand, *BECLIN1* expression level increased in

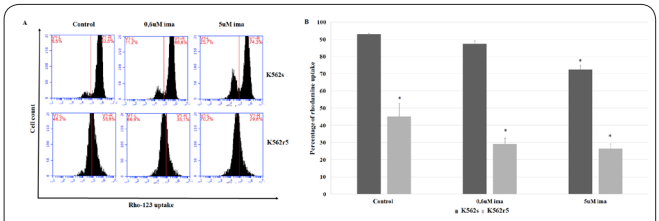

Figure 6. A) Accumulation of rhodamine 123 in K562s and K562r5 incubated with 0.6 µM and 5 µMimatinib; B) Percentage of rhodamine uptake in K562s and K562r5 incubated with 0.6 µM and 5 µM imatinib. (*p≤ 0.05).

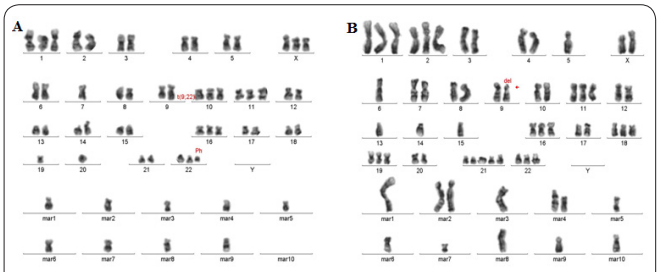

Figure 7. A) Karyotypic image of K562s cells (57, XX, +X, +1, +7, t(9;22)(q34;q11.2), +10, +11, +16, -19, -20, +22, +mar1, +mar2, +mar3, +mar4, +mar5, +mar6, +mar7, +mar8, +mar9)
B) Karyotypic image of K562r5 cells (63, XX, +1, +2, -5, -6, del(9)(p21), +11, -13, -14, -15, +16, +18, +19, +21, +21, +22, +mar1, +mar2, +mar3, +mar4, +mar5, +mar6, +mar7, +mar8, +mar9, +mar10, +mar11, +mar12).

K562r5 1.33 fold but this increased value was not found statistically significant (p>0.05) (Fig 5D).

Determination of Pgp activity with flow cytometry

In this study, we performed the Rhodamine-123 efflux assay to investigate the Pgp activity in our cell lines in the presence of 0.6 and 5 µM imatinib.

Mean total fluorescence of rhodamine uptake in K562s control, 0.6 and 5 µM imatinib treated cells are 614809.58± 20841.05, 458413.92±42053.98 and 393303.23±7152.83 respectively. Mean total fluorescence of rhodamine uptake in K562r5 control, 0.6 and 5 µM are 140314.87±20044.21257, 97982.66±6256.11and 97883.99±8322.98, respectively. Our data showed that MDR activity in K562r5 cells is more than K562s. K562r5 cells pump rhodamine from the cell and data was represented as percentage in Fig 6.

Cytogenetic analysis of imatinib-sensitive K562s and imatinib-resistant K562r cell lines

We analyzed the chromosomes of imatinib-sensitive and resistant K562 cells and we found that K562r5 cells have more complex karyotype than K562s cells. The images of karyotype are shown in figures 7 A and B.

Discussion

Imatinib is used as a first line TKI in the treatment of CML, however emergence of imatinib resistance is a major problem which results with the clinical failure of imatinib therapy that leads to the use of other alternative TKIs. BCR-ABL dependent and independent molecular mechanisms have crucial roles in drug resistance in CML. Even if BCR-ABL dependent mechanisms, for instance, BCR-ABL mutations, over-expression, and gene amplification are associated with resistance, there are several BCR-ABL independent mechanisms, such as changed expressions of drug transporters, apoptotic and autophagic genes which are responsible for resistance. In this study, we established K562 imatinib-resistant cell line (K562r5) which was continuously exposed to increasing doses of imatinib. We analysed molecular mechanisms which play a role in drug resistance phenotype. First of all, we focused on the BCR-ABL dependent mechanisms. For this purpose, we analysed mutations with DNA sequencing and we did not observe BCR-ABL point mutations (T315I, M351T, F315L, F359C/L/V) in our cell lines. These results indicated that BCR-ABL independent mechanisms, like apoptosis, autophagy, drug transporters and DNA repair might affect drug resistance in these cells.

BCR-ABL independent mechanisms of imatinib resistance involve apoptosis inhibition and autophagy induction. The Bcl-2 family anti-apoptotic proteins, such as Bcl-2, Bcl-Xl, and Mcl-1 are important for cell survival, whereas, the Bcl-2 family pro-apoptotic proteins, such as Bid, Bim, Bad, Bax, and Bak are required to initiate cell death apoptosis (9).

In this study, we analysed the mRNA expression levels of pro-apoptotic (*BIM, BAD*, and *BAX*) and anti-apoptotic genes (*MCL-1, BCL-XL*, and *BCL-2*) which can be responsible in imatinib resistance of K562 cells. It has been reported that down-regulation of pro-apoptotic genes play a critical role in drug resistance of various cancers (25, 26, 27). According to our data, *BIM, BAX* and *BAD* gene expression levels decreased, dose-dependently, in comparison with imatinib treated K562s cells.

Upregulation of anti-apoptotic genes such as *BCL-2, BCL-XL*, and *MCL1* correlated with chemoresistance in acute myeloid leukemia (AML) (26, 28). In our study, *BCL-XL* and *MCL-1* gene expression level increased dramatically in K562r5 cells compared with K562s and K562r0.6 cells, moreover, the Bcl-2 gene was up-regulated in both K562r imatinib-resistant cells in comparison with their imatinib-treated K562s cells.

Recent studies indicated that anti-apoptotic proteins such as Bcl-2, Bcl-Xl, and Mcl-1 play important role in mediating apoptosis or autophagy. Interaction of Beclin-1 as an essential protein in induction of autophagy with Bcl2 in the endoplasmic reticulum (ER) regulates autophagy and apoptosis. Formation of Beclin-1 and Bcl2 complex suppressed Beclin1 from inducing autophagy. Moreover, an interaction of Beclin-1 with Mcl-1 in mitochondria inhibits induction of autophagy by Beclin1 (29, 30, 31). It is also reported that dissociation of Beclin-1 from Bcl-Xl caused initiation of autophagy (32).

Our results demonstrated that anti-apoptotic and *BECLIN1* genes expression increased in imatinib-resistant K562 cells. Several articles showed that imatinib-induced autophagy in BCR-ABL expressing hematopoietic cells (33, 34). It was reported that 5 µM imatinib treatment induced apoptosis in K562 cells (34) and our data represented the same results with our imatinib-sensitive cells. According to our data, 0.6 and 5 µM imatinib-induced apoptosis and autophagy in K562s cells and autophagic gene expression increased dose-dependently in these cells. Moreover, in 0.6 and 5µM imatinib treated K562r cells autophagy was induced whereas apop-

tosis was inhibited in comparison with 0.6 and 5 μM imatinib treated K562s cells respectively.

In addition to *BECLIN-1, ATG5* and *ATG7* genes play a significant role in activating autophagy by forming pre-autophagosome. Our results indicated that *ATG5* gene was overexpressed in both K562r0.6 and K562r5 cells whereas, ATG7 gene expression decreased with increasing dosage of imatinib from 0.6 μM to 5 μM. It can be concluded that inhibition of apoptosis and induction of autophagy lead to imatinib resistance in K562r cells.

Other molecular mechanisms are involved in drug resistance of hematopoietic malignancies such as drug transporter proteins including efflux and influx proteins which may also influence cell response to imatinib. The multidrug resistance (MDR) phenotype is related to increased expression of efflux pumps, such as Mdr-1, Mrp-1 and decreased expressions of influx transporters, such as Oct-1 (3, 4, 5, 6). According to our study, the *MDR-1* gene is overexpressed significantly in K562r0.6 and K562r5 cells. *MRP-1* gene expression decreased significantly in both concentrations of imatinib in K562r cells. In this study, we suggested that imatinib increased gene expression level of *MDR-1* and decreased *MRP-1* gene expression in imatinib-resistant cells. Although imatinib is the substrate of both Mdr-1 and Mrp1 proteins, in this study, we found that *MDR1* gene plays a major role in imatinib resistance rather than *MRP1* gene. In addition to efflux proteins, we investigated gene expression level of influx protein Oct-1. Our results demonstrated that with increasing concentrations of imatinib (0.6 μM to 5 μM), the expression level of *OCT-1* gene was decreased. Several studies indicated that *OCT-1* gene expression was reduced after imatinib treatment (5,6).

Recent studies showed that BCR-ABL expressing cells accumulate genomic lesions so there are several repair mechanisms that repair these genomic abnormalities and repair faults in their genes (35). Mismatch repair system (MMR) is responsible for repairing point mutations or inducing apoptosis in the case of increasing DNA lesions in the cell. Our results showed that *MLH-1* gene expression was down-regulated in both K562r0.6 and K562r5 cells compared with their control group K562s cell, Moreover, the expression level of *MSH2* was decreased in K562r0.6 but *MSH2* gene expression was increased in K562r5 cells. It is reported that BCR-ABL abolishes MMR activity in order to inhibit induction of apoptosis (36). In our study, with increasing the concentration of imatinib from 0.6 to 5μM, the expression of mismatch repair genes, especially *MSH2* was increased in a dose-dependent manner, whereas *MLH1* was down-regulated in all cells. In addition to MMR repair system, we analyzed *MGMT* gene expression in K562s and K562r cells, but according to our result, we could not detect any *MGMT* expression in all cells. It can be concluded that *MGMT* gene is methylated in K562s and K562r cells.

In addition to determining gene expressions in all cell lines, we performed cytogenetic analysis to determine chromosomal alterations in K562r5 resistant cells. Although we could not speculate about a direct effect of the chromosomal anomalies on the imatinib resistance in these cell lines, we showed that the resistant cells have more complex karyotype than sensitive cells.

Imatinib and other TKIs are used for the treatment of CML patients, but the occurrence of resistance to TKIs leads to treatment failure in CML. Although BCR-ABL mutations are identified as the main causes of TKIs resistance there are mechanisms which are classified as BCR-ABL independent mechanisms. BCR-ABL independent mechanisms such as inhibition of apoptosis, induction of autophagy, altered expression levels of drug transporter proteins and inactivation of DNA repair system could contribute to the progression of drug resistance. As it was shown in our study, several mechanisms may contribute to imatinib resistance at the same time. In this case, inhibition of only one of these mechanisms could not be enough to increase the effect of TKIs in CML therapy. According to these data, it can be suggested that using inhibitors to target different molecules at the same time could increase the effect of imatinib in CML therapy.

Acknowledgments

We thank Zeynep Biyikli Gencturk for assistance in statistical analysis. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Jain P, Kantarjian H, Patel K P, Gonzalez GN, Luthra R, Kanagal Shamanna R, Sasaki K, Jabbour E, Romo CG, Kadia TM, Pemmaraju N, Daver N, Borthakur G, Estrov Z, Ravandi F, O'Brien S, Cortes J. Impact of BCR-ABL transcript type on outcome in patients with chronic-phase CML treated with tyrosine kinase inhibitors. Blood. 2015;10-674242.

2. Diamond JM , Melo JV. Mechanisms of resistance to BCR-ABL kinase inhibitors. Leuk Lymphoma. 2011; 1:12-22.

3. Jordanides NE, Jorgensen HG, Holyoake TL, Mountford JC. Functional ABCG2 is overexpressed on primary CML CD34+ cells and is inhibited by imatinib mesylate. Blood. 2006;108:1370–3.

4. Dharmapuri G, Doneti R, Philip GH, Kalle AZ. Celecoxib sensitizes imatinib-resistant K562 cells to imatinib by inhibiting MRP1-5, ABCA2 and ABCG2 transporters via Wnt and Ras signaling pathways. Leuk Res. 2015; 696-701.

5. Kim YK, Lee SS, Jeong SH, Ahn JS, Yang DH, Lee JJ, Shin MG, Kim HJ. OCT-1, ABCB1, and ABCG2 Expression in Imatinib-Resistant Chronic Myeloid Leukemia Treated with Dasatinib or Nilotinib. Chonnam Med J. 2014; 50(3) 102-11.

6. Alves R, Fonseca AR, Gonçalves AC, Ferreira-Teixeira M, Lima J, Abrantes AM, Alves V, Rodrigues-Santos P, Jorge L, Matoso E, Carreira IM, Botelho MF, Sarmento-Ribeiro AB. Drug transporters play a key role in the complex process of Imatinib resistance in vitro. Leuk Res. 2015; 39(3): 355-60.

7. Jacobson MD, Weil M, Raff MC. Programmed cell death in animal development. Cell. 1997; 88 347–354.

8. Danial NN, Korsmeyer SJ. Cell death: critical control points. Cell. 2004;116 205–219.

9. Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, Dawson TM, Dawson VL, El-Deiry WS, Fulda S, Gottlieb E, Green DR, Hengartner MO, Kepp O, Knight RA, Kumar S, Lipton SA, Lu X, Madeo F, Malorni W, Mehlen P, Nuñez G, Peter ME, Piacentini M, Rubinsztein DC, Shi Y, Simon HU, Vandenabeele

P, White E, Yuan J, Zhivotovsky B, Melino G, Kroemer G. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death, Cell death and differentiation. 2012; 19(1) 107-20.
10. Oberstein A, Jeffrey PD, Shi Y. Crystal structure of the Bcl-XL-Beclin 1 peptide complex: Beclin 1 is a novel BH3-only protein. The Journal of biological chemistry. 2007; 282(17) 13123-32.
11. Klionsky DJ. Look people, "Atg" is an abbreviation for "autophagy-related." That's it. Autophagy. 2012; 8(9) 1281–2.
12. Ganley IG, Wong PM, Gammoh N, Jiang XJ. Distinct autophagosomal-lysosomal fusion mechanism revealed by thapsigargin-induced autophagy arrest. Mol Cell. 2011; 42(6): 731–43.
13. Suzuki K, Kirisako T, Kamada Y, Mizushima N, Noda T, Ohsumi Y. The pre autophagosomal structure organized by concerted functions of APG genes is essential for.2001; 10.1093.20.21.5971.
14. Penserga ET, Skorski T. Fusion tyrosine kinases: a result and cause of genomic instability. Oncogene. 2007; 26:11–20.
15. Nowicki MO, Falinski R, Koptyra M, Slupianek A, Stoklosa T, Gloc E, Nieborowska-Skorska M , Blasiak J, T. Skorski, BCR/ABL oncogenic kinase promotes unfaithful repair of the reactive oxygen species-dependent DNA double-strand breaks. Blood. 2004; 104:3746–53.
16. Koptyra M, Falinski R, Nowicki MO, Stoklosa T, Majsterek I, Nieborowska-Skorska M, Blasiak J, korski TS. BCR/ABL kinase induces self-mutagenesis via reactive oxygen species to encode imatinib resistance. Blood. 2006; 108:319–27.
17. Stojic L, Brun R, Jiricny J. Mismatch repair and DNA damage signaling. DNA Repair. 2004; 3:1091–101.
18. Perazzoli G, Prados J, Ortiz R, Caba O, Cabeza L, Berdasco M, Gónzalez B, Melguizo C. Temozolomide Resistance in Glioblastoma Cell Lines: Implication of MGMT, MMR, P-Glycoprotein and CD133 Expression. PLoS One. 2015; 8;10(10).
19. Koc A, Ozkan T, Hekmatshoar Y, Gurkan-Alp AS, Aktan F, Buyukbingol Z, Sunguroglu A, Buyukbingol E, Karabay AZ. Apoptotic Effects of Some Tetrahydronaphthalene Derivatives on K562 Human Chronic Myelogenous Leukemia Cell Line. Anticancer Agents Med Chem. 2018; 7;17(14):1924-1930.
20. Yilmaz B, Karabay AZ. Food Additive Sodium Benzoate (NaB) Activates NFκB and Induces Apoptosis in HCT116 Cells. Molecules. 2018; 22;23(4).
21. Ozkan T, Hekmatshoar Y, Ertan-Bolelli T, Hidayat AN, Beksac M, Aki-Yalcin E, Yalcin I, Sunguroglu A. Determination of the apoptotic effect and molecular docking of benzamide derivative XT5 in K562 cells. Anticancer Agents Med Chem. 2017; doi: 10.2174/1871520618666171229222534.
22. Gwak HS, Shingu T, Chumbalkar V, Hwang YH, DeJournett R, Latha K, Koul D, Alfred Yung WK, Powis G, Farrell NP, Bögler O. Combined action of the dinuclear platinum compound BBR3610 with the PI3-K inhibitor PX-866 in glioblastoma.Int J Cancer. 2011; 15;128(4):787-96.
23. Ding ZB, Shi YH, Zhou J, Qiu SJ, Xu Y, Dai Z, Shi GM, Wang XY, Ke AW, Wu B, Fan J. Association of autophagy defect with a malignant phenotype and poor prognosis of hepatocellular carcinoma. Cancer Res. 2008; 15, 68(22), 9167-75.
24. Shaffer LG, Slovak ML, Campbell LJ. An international system for human cytogenetic nomenclature. Basel: S. Karger.2009.
25. Kuribara R, Honda H, Matsui H, Shinjyo T, Inukai T, Sugita K, Nakazawa S, Hirai H, Ozawa K, Inaba T. Roles of Bim in apoptosis of normal and Bcr-Abl-expressing hematopoietic progenitors. Mol Cell Biol. 2004; 24(14) 6172-83.
26. Ferreira A F, de Oliveira GL, Tognon R, Collassanti MD, Zanichelli MA, Hamerschlak N, de Souza AM, Covas DT, Kashima S, de Castro FA. Apoptosis-Related Gene Expression Profile in Chronic Myeloid Leukemia Patients after Imatinib Mesylate and Dasatinib Therapy. Acta Haematol. 2015; 133(4) 354-64.
27. Kuroda J, Puthalakath H, Cragg MS, Kelly PN, Bouillet P, Huang DC, Kimura S, Ottmann OG, Druker BJ, Villunger A, Roberts AW, Strasser A. Bim and Bad mediate imatinib-induced killing of Bcr/Abl+ leukemic cells, and resistance due to their loss is overcome by a BH3 mimetic. Proc Natl Acad Sci U S A. 2006; 3;103(40) 14907-12.
28. Pan R, Ruvolo VR, Marina Konopleva JW, Reed JC, Pellecchia M, Andreeff M, Ruvolo PP. Inhibition of Mcl-1 with the pan–Bcl-2 family inhibitor (–)BI97D6 overcomes ABT-737 resistance in acute myeloid leukemia. Blood . 2015; 126:363-372.
29. Germain M, Nguyen AP, Le Grand JN, Arbour N, Vanderluit JL, Park DS, Opferman JT, Slack RS. MCL-1 is a stress sensor that regulates autophagy in a developmentally regulated manner. EMBO J. 2011; 19;30(2) 395-407.
30. Marquez RT, Xu L. Bcl-2:Beclin 1 complex: multiple, mechanisms regulating autophagy/apoptosis toggle switch. Am J Cancer Res. 2012; 2(2) 214-21.
31. Elgendy M, Minucci S. A novel autophagy-independent, oncosuppressive function of BECN1: Degradation of MCL1. Autophagy. 2015; 11(3) 581-2.
32. Kim SY, Song X, Zhang L, Bartlett DL, Lee YJ. Role of Bcl-xL/Beclin-1 in interplay between apoptosis and autophagy in oxaliplatin and bortezomib-induced cell death. Biochem Pharmacol. 2014; 15;88(2) 178-88.
33. Bellodi C, Lidonnici MR, Hamilton A, Helgason GV, Soliera AR, Ronchetti M, Galavotti S, Young KW, Selmi T, Yacobi R, Van Etten RA, Donato N, Hunter A, Dinsdale D, Tirrò E, Vigneri P, Nicotera P, Dyer MJ, Holyoake T, Salomoni P, Calabretta B. Targeting autophagy potentiates tyrosine kinase inhibitor-induced cell death in Philadelphia chromosome-positive cells, including primary CML stem cells. J Clin Invest. 2009; 119:1109–1123.
34. Crowley LC, Elzinga BM, O'Sullivan GC, McKenna SL. Autophagy induction by Bcr- Abl-expressing cells facilitates their recovery from a targeted or nontargeted treatment. Am J Hematol. 2011; 86:38–47.
35. Burke BA, Carroll M. BCR-ABL: a multi-faceted promoter of DNA mutation in chronic myelogeneous leukemia. Leukemia. 2010; 24(6): 1105–1112.
36. Stoklosa T, Poplawski T, Koptyra M, Nieborowska-Skorska M, Basak G, Slupianek A, Rayevskaya M, Seferynska I, Herrera L, Blasiak J, Skorski T. BCR/ABL inhibits mismatch repair to protect from apoptosis and induce point mutations. Cancer Res. 2008; 68:2576–2580.