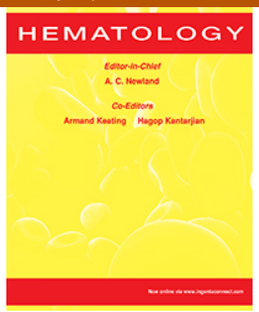

Hematology

ISSN: 1024-5332 (Print) 1607-8454 (Online) Journal homepage: http://www.tandfonline.com/loi/yhem20

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To cite this article: Huseyin Atakan Ekiz, Geylani Can, Ufuk Gunduz & Yusuf Baran (2010) Nilotinib significantly induces apoptosis in imatinib resistant K562 cells with wild-type BCR–ABL, as effectively as in parental sensitive counterparts, Hematology, 15:1, 33-38, DOI: 10.1179/102453310X12583347009775

To link to this article: http://dx.doi.org/10.1179/102453310X12583347009775

Published online: 18 Jul 2013.

Nilotinib significantly induces apoptosis in imatinib resistant K562 cells with wild-type BCR–ABL, as effectively as in parental sensitive counterparts

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Chronic myeloid leukemia (CML) is a hematological malignancy characterized by high levels of immature white blood cells. CML is caused by the translocation between chromosomes 9 and 22 (which results in the formation of the Philadelphia chromosome) creating BCR–ABL fusion protein. Imatinib and nilotinib are chemotherapeutic drugs which specifically bind to the BCR–ABL and inhibit cancer cells. Nilotinib is more effective in this respect than imatinib. We have shown that nilotinib induces apoptosis in imatinib-resistant K562 CML cells which have the wild-type BCR–ABL fusion gene almost to the same extent as it does in the parental sensitive cells by the increase in caspase-3 enzyme activity and the decrease in mitochondrial membrane potential. This effect of nilotinib, even in low concentrations, may indicate the efficacy of the usage of nilotinib in imatinib-resistant CML with less risk of undesired cytotoxic effects in the remaining cells of the body.

Keywords: CML, imatinib, nilotinib, drug resistance, BCR–ABL

Introduction

Chronic myeloid leukemia (CML) is a hematological malignancy in which healthy cells in the bone marrow are replaced by malignant leukemic cells. The disease is characterized by elevated numbers of immature white blood cells. The main cause of CML is a reciprocal translocation between the long arms of chromosomes 9 and 22 (creating Philadelphia chromosome, Ph), which results in production of the BCR–ABL fusion protein.[1–3] This fusion protein shows constitutive tyrosine kinase activity, resulting in strong oncogenic properties. BCR–ABL protein is also thought to have significant roles in cell growth, proliferation, differentiation, migration, adhesion and prevention of apoptosis.[3,4] Thus, targeting this protein by tyrosine kinase inhibitors is an important approach for prevention of the leukemogenesis in CML.[5]

Imatinib (STI-571, Gleevec) is the first target-specific drug for the treatment of cancers including Ph-positive CML and gastrointestinal tumors.[6] This drug specifically binds to the ATP-binding domain of BCR–ABL fusion protein and prevents its activation and subsequent phosphorylation of the downstream proteins.[7] Although this drug was a breakthrough for the treatment of CML, there is increasing number of cases where patients have developed resistance to the imatinib treatment. This resistance might be conferred by five known mechanisms.[8,9] Most cases of resistance to imatinib result from mutations in the drug-binding pocket of the fusion protein.[10] Two of

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Received 15 June 2009; accepted 7 September 2009
DOI 10.1179/102453310X12583347009775

the mechanisms involve decreased availability of the drug either by binding to plasma proteins, or by functioning of the efflux proteins such as P-glycoprotein on the plasma membrane.[11] BCR–ABL gene amplification is another resistance mechanism which causes increased levels of fusion protein reducing the efficacy of the drug *in vivo*. Besides the mechanisms stated above, resistance might be developed independently of BCR–ABL. Activation of other kinases are implicated in this type of resistance. We have also shown in our previous studies for the first time that ceramide metabolizing genes and their end products are also involved in imatinib resistance in CML cells. In addition to the known mechanisms of resistance, clinical cases highlight that there are still uncharacterized resistance mechanisms in CML patients.

Nilotinib (AMN107, Tasigna) is a second-generation tyrosine kinase inhibitor targeting the BCR–ABL fusion protein. Nilotinib has more favorable binding energetics and higher selectivity than the imatinib.[12] Thus, it becomes particularly useful for the treatment of Ph-positive CML which has developed resistance to imatinib.[13] Usage of increasing concentrations of chemotherapeutic drugs in the clinic to treat cancers might possibly cause undesired cytotoxic effects on the healthy cells. Thus, using the least drug concentrations possible is the main aim in the treatment of various cancers. Current study shows the effects of low concentrations of nilotinib on both parental sensitive and imatinib-resistant K562 CML cells which do not have any mutations in the drug-binding pocket of the BCR–ABL fusion product.

Methods

Cell lines and growth conditions

Human K562 CML cells were obtained from German Collection of Microorganisms and Cell Cultures. K562 cells were maintained in RPMI 1640 growth medium containing 10% fetal bovine serum and 1% penicillin–streptomycin at 37°C in 5% CO_2. The Ph^+ human K562 cells were exposed to increasing concentrations of imatinib stepwise for 2 year time period starting from 50 nM. Subpopulations of cells those were able to grow in the presence of 3 μM imatinib, were then selected and referred to as K562/IMA-3.

Assessment of cellular growth by XTT cell proliferation assay

The IC_{50} values of imatinib and nilotinib that inhibit cell growth by 50% were determined from cell proliferation plots obtained by XTT cell proliferation assay (Biological Industries, Israel) as described previously.[1] Briefly, 2×10^4 cells were seeded into the wells of 96-well plates that contain 200 μl growth medium in the absence or presence of increasing concentrations either imatinib or nilotinib, and incubated at 37°C in 5% CO_2 for 72 h. After this, cell suspensions were treated with 40 μl XTT reagent for 4 more hours. Then, the absorbances of the samples under 490 nm wavelength of light were measured by ELISA reader (Multiskan Spectrum, Thermo Electron Corporation, Vantaa, Finland). At the end, IC_{50} values were calculated from the cell survival plots.

Measurement of caspase-3 enzyme activity

Caspase-3 enzyme activity was determined using caspase-3 colorimetric assay (R&D Systems, Minneapolis, MN, USA). The cells that had been treated with different concentrations of nilotinib for 72 h were collected by centrifugation at 1000 *g* for 10 min. Pelleted cells were treated with 100 μl of cold lysis buffer ($\times 1$) in order to obtain the cell lysate. Then, the cell lysates were incubated on ice for 10 min and were centrifuged at 14,000 *g* for 1 min. Following the centrifugation, supernatants were transferred to new microcentrifuge tubes. For measuring caspase-3 enzyme activity, reaction mixture including 20 μl of assay buffer ($\times 5$), 25 μl of the samples, 50 μl of distilled water and 5 μl of caspase-3 colorimetric substrate were applied to the cell suspensions in 96-well plate and incubated for 2 h at 37°C in CO_2 incubator. Absorbances of the samples were read under 405 nm wavelength of light via the ELISA reader (Multiskan Spectrum, Thermo Electron Corporation).

Detection of the mitochondrial membrane potential

The loss of mitochondrial membrane potential as a hallmark of apoptosis was detected by JC-1 mitochondrial membrane potential (MMP) detection kit (Cell Technology, Mountain View, CA, USA). First, the cells treated with different concentrations of nilotinib were collected by centrifugation at 1000 *g* for 10 min. Supernatants were discarded and the pellets were mixed with 500 μl of JC-1 dye and incubated at 37°C in 5% CO_2 for 15 min. Then the mixtures were centrifuged at 1000 *g* for 5 min. Pellets were resuspended in 2 ml of blank media in order to remove the excess dye and centrifuged for 5 min at 1000 *g*. All pellets were resuspended with 500 μl of blank media and 150 μl from each of them was added into the black 96-well plate. The aggregate red form

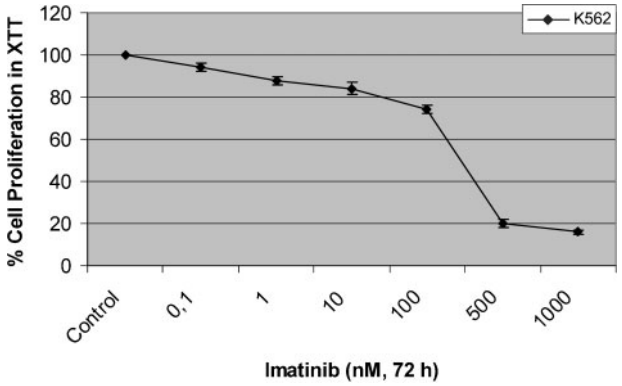

Figure 1 Effects of imatinib on the growth of K562 cells. The IC_{50} concentration of imatinib was calculated from cell proliferation plots. The XTT assays were performed using triplicate samples in at least two independent experiments. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance, and $P<0{\cdot}05$ was considered significant

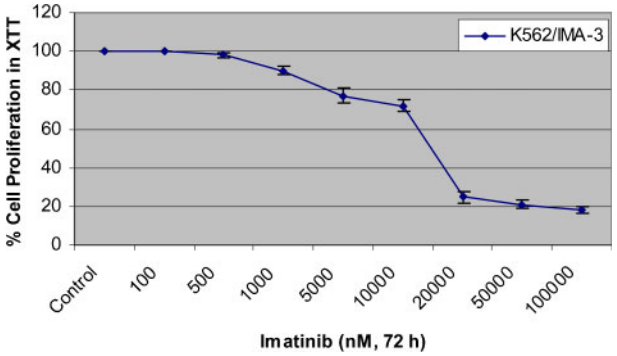

Figure 2 Effects of imatinib on the growth of K562/IMA-3 cells. The IC_{50} concentration of imatinib was determined by XTT assay for K562/IMA-3 cells as described. The XTT assays were performed using triplicate samples in at least two independent experiments. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance, and $P<0{\cdot}05$ was considered significant

of the dye which remains within the intact mitochondria has absorption/emission maxima of 585/590 nm and the monomeric green form of the dye which is released to the cytoplasm due to the loss of the integrity of the mitochondrial membrane has absorption/emission maxima of 510/527 nm. The plate was read at these wavelengths by fluorescence ELISA reader (Multiskan Spectrum, Thermo Electron Corporation).

Sequence analysis of K562/IMA-3 cell line

Total RNA was isolated from three million K562/IMA-3 cells via NucleoSpin RNA II Kit (MACHEREY NAGEL GmbH & Co. KG, Düren, Germany). Then total RNA was converted to cDNA using M-MuLV Reverse Transcriptase (Fermentas Life Science, Burlington, Ont., Canada). After that, polymerase chain reaction (PCR) was performed with B2B and CA3 primers and PCR product was run in agarose gel. BCR–ABL band was isolated from gel by DNA Gel Extraction Kit (Fermentas Life Science). Afterwards, sequencing PCR was carried out to the purified product with CA3 primer using BigDye Terminator V3.1 Cycle Sequencing Kit. Finally, sequence analysis was carried out (Applied Biosystems 3130xl).

Results

Cellular growth assessment via XTT cell proliferation assay

IC_{50} value of imatinib was calculated as 280 nM in sensitive K562 cells (Fig. 1) and 14,680 nM in resistant K562/IMA-3 cells (Fig. 2). These results showed that K562/IMA-3 cells are 52-fold more resistant as compared to parental sensitive counterparts. Proliferation assay in the presence of nilotinib gave the IC_{50} values of 42 and 84 nM for sensitive parental K562 (Fig. 3) and K562/IMA-3 cells (Fig. 4), respectively.

Measurement of caspase-3 enzyme activity

In order to examine cytotoxic effects of nilotinib on parental and imatinib-resistant K562 cells, both cells

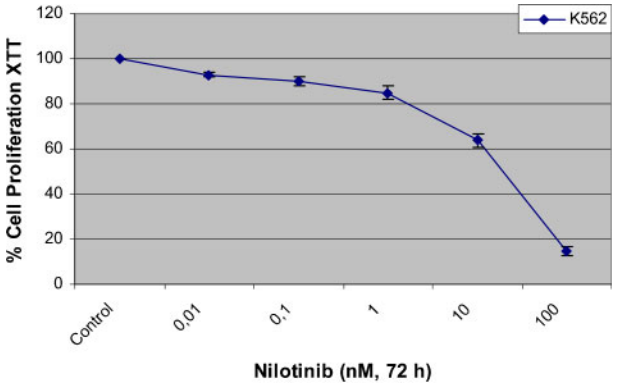

Figure 3 Effects of nilotinib on the growth of K562 cells. Cytotoxicity was determined by the XTT cell proliferation test in a 72 h culture. The XTT assays were performed using triplicate samples in at least two independent experiments. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance, and $P<0{\cdot}05$ was considered significant

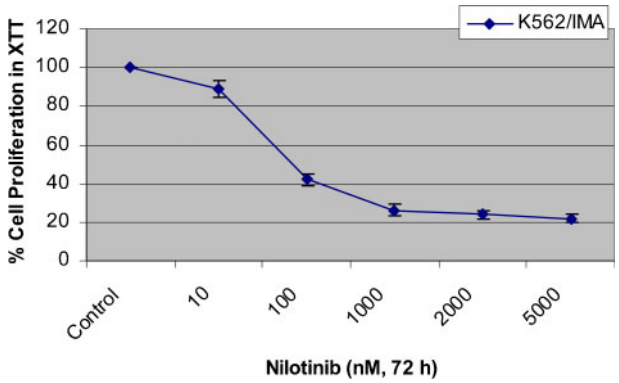

Figure 4 **Effects of nilotinib on the growth of K562/IMA-3 cells. Cytotoxicity was determined by the XTT cell proliferation test in a 72 h culture. The XTT assays were performed using triplicate samples in at least two independent experiments. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance, and $P<0{\cdot}05$ was considered significant**

were exposed to low concentrations of nilotinib for 72 h and the changes in caspase-3 enzyme activity were determined. The results revealed that there were 1·29 and 1·25-fold increases in caspase-3 enzyme activity in 1 nM nilotinib applied K562 and K562/IMA-3 cells, respectively (Fig. 5). These comparisons were carried out with the control sample which was composed of sensitive K562 cells cultured in the absence of the drug. This result showed the induction of apoptosis upon drug treatment. The closeness of those values indicates that apoptosis is induced in both sensitive and resistant K562 cells at similar rates. On the other hand, steady state levels of

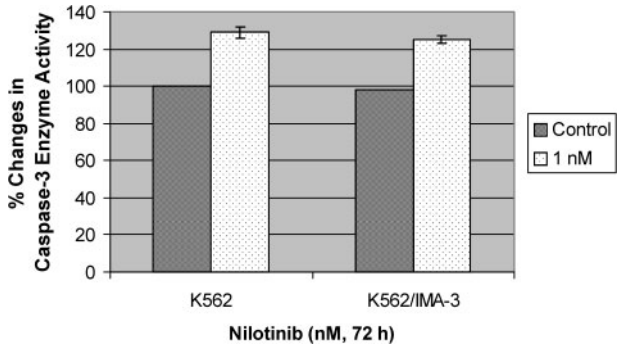

Figure 5 **Percent changes in caspase-3 enzyme activity in nilotinib exposed K562 and K562/IMA-3 cells. The results are the means of two independent experiments. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. $P<0{\cdot}05$ was considered significant**

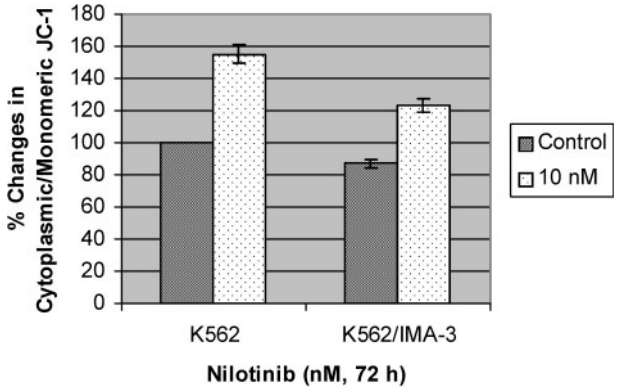

Figure 6 **Percentage changes in cytoplasmic/aggregated JC-1 in nilotinib-treated K562 and K562/IMA-3 cells. The results are the means of two independent experiments. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. $P<0{\cdot}05$ was considered significant**

caspase-3 enzyme activity results showed that there was 2% decrease in caspase-3 enzyme activity in K562/IMA-3 cells comparing to K562 cells. When the K562/IMA-3 cells in the absence and in the presence of 1 nM nilotinib are compared with each other, there is 1·27-fold increase in caspase-3 enzyme activity, indicating that the rate of apoptotic induction is almost the same with the sensitive cells (Fig. 5).

Detection of mitochondrial membrane potential

We examined the changes in MMP in response to nilotinib in K562 and K562/IMA-3 cells exposed to 10 nM nilotinib for 72 h. There were 1·55- and 1·23-fold increases in cytoplasmic/aggregated JC-1 ratios in K562 and K562/IMA-3 cells, respectively, as compared to untreated K562 control (Fig. 6). Steady state levels of cytoplasmic/aggregated JC-1 were decreased 13% in K562/IMA-3 cells compared to parental sensitive cells. When we compare changes in cytoplasmic/aggregated JC-1 in 10 nM nilotinib applied K562/IMA-3 cells with untreated counterparts, there were 1·48-fold increase in cytoplasmic/aggregated JC-1 ratio (Fig. 6). Again, the close values indicate the similar amounts of changes in MMP meaning similar induction of apoptosis. The findings from MMP detection correspond to the previous results in which caspase-3 activations were assessed.

Sequence analysis of BCR–ABL gene

Sequence of the BCR–ABL fusion gene was found as seen in Fig. 7. The sequence was compared with the known wild-type BCR–ABL and there were not any mutations in the BCR–ABL of the K562/IMA-3 cells (Fig. 7)

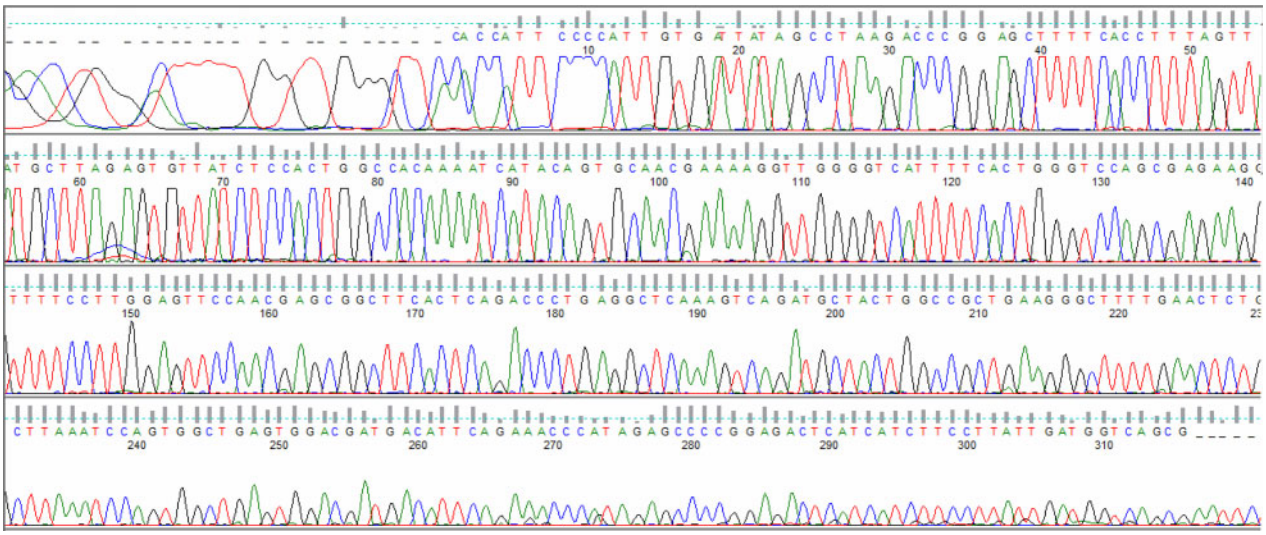

Figure 7 Sequence of the BCR–ABL fusion gene in K562/IMA-3 cells

Discussion

This study revealed the cytotoxic and apoptotic effects of nilotinib on imatinib-resistant CML cells especially in low concentrations. Results indicate that the growth of the imatinib-resistant cells is inhibited and they undergo apoptosis almost as effectively as the imatinib-sensitive cells.

Imatinib was developed for selective inhibition of tyrosine kinases expressed in malignant cells.[14] Being selective for cancerous cells, the drug has high success rates in the treatment of Ph^+ CML.[15] With the increasing number of patients using this drug, increasing number of cases are being reported in which imatinib resistance is gained. In such cases, the dosage of the drug should be increased to have a therapeutic response against the cancer. Although imatinib is a target-specific drug, it has dose-dependent hematological side effects on all hematopoietic lineages such as inhibition of platelet derived growth factor receptor, human stem cell factor receptor c-kit,[16] ABL-related gene[17] and ABL tyrosine kinases.[6,18–20] Non-hematological side effects of imatinib are not generally important to cause cessation of the treatment, but the cytotoxic effects on hematopoietic cells prevent the usage of the drug in high concentrations to treat resistant cancers.[21]

Nilotinib was developed after the discovery of imatinib and it has more effective suppression ability with the increased specificity to the CML cells.[12,22] The mechanism of action is similar to that of imatinib, but 32 of the known 33 mutations in the BCR–ABL fusion protein responsible for imatinib resistance remain ineffective to develop resistance against nilotinib.[23] Enhanced specificity and efficacy of nilotinib make it possible to obtain similar amounts of therapeutic effect in lower concentrations, which could only be provided by considerably high concentrations of imatinib. Imatinib treatment might not be tolerated well in aged patients, which brings along the necessity of using a more selective drug.[21,24] Thus, nilotinib became particularly useful for the treatment of CML, especially in old patients, and the in the patients with imatinib-resistant CML.[25] Similarly, resistant cells which are unresponsive to imatinib treatment necessitate the usage of a more powerful drug, which is the nilotinib for these particular cases.

Although nilotinib is highly specific for malignantly transformed cells, there is still a possibility of developing adverse effects.[23,26] In the clinic, these adverse effects might limit the dosage of the drug. Lower drug concentrations are less likely to have undesired cytotoxicity on the healthy cells. The results of the present study indicated the efficacy of little amounts of nilotinib on both imatinib-resistant and -sensitive cells. Inhibition of cellular growth and induction of apoptosis by such low concentrations of nilotinib in CML cells are thus promising for the treatment with minimum side effects which are mainly dose-dependent. Obtaining cellular responses, as in this study, indicates the highly selective inhibitory effect of nilotinib on CML cells, in spite of the developed resistance mechanisms against chemotherapeutic drugs.

The efficacy of nilotinib in the treatment of imatinib-resistant CML was extensively studied before.[22,27] In those studies, it was shown that mutations in the ATP binding cassette of BCR–ABL reduces the binding affinity of imatinib, while nilotinib binding is not affected by the majority

of those mutations. Current study indicates that nilotinib is not only effective for the treatment of imatinib-resistant CML with mutations in BCR–ABL fusion product, but also for the inhibition of imatinib-resistant CML cells which have the wild-type BCR–ABL. This effect of nilotinib might indicate that the other mechanisms of drug resistance in CML are somehow inadequate for nilotinib. BCR–ABL activity is known to be important for the development of CML, but there are also other targets for inhibition of CML as known.[28] In this case nilotinib might have a role in suppressing alternative signaling pathways which contribute to the leukemogenesis and cancer cell survival in a BCR–ABL independent manner. Besides those signaling pathways, nilotinib might have low efflux rates from the cell due to its molecular structure and physicochemical properties. Thus, the bioavailability of nilotinib inside the cytoplasm remains higher, making the drug possible to exert its function for suppression of the CML. Future studies could be concentrated on revealing the possible mechanisms in which nilotinib overcomes the drug resistance in CML. Taken together, all these data suggest that nilotinib is effective for inhibition of Ph^+ CML even in low concentrations and thus, it may provide a treatment with the least risk of developing undesired cytotoxic effects on the remaining cells of the body. The extents of the changes in both sensitive and resistant cells are almost the same, indicating that efficacy of the nilotinib still holds for the CML cases in which resistance was developed to imatinib.

Acknowledgement

We thank Izmir Institute of Technology, and Biotechnology and Bioengineering Research facility staff for their technical help.

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