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Docetaxel enhances the cytotoxic effects of imatinib on Philadelphia positive human chronic myeloid leukemia cells

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Chronic myelogenous leukemia (CML) results from a translocation between chromosomes 9 and 22 which generates BCR/ABL fusion protein and characterized by uncontrolled proliferation of immature white blood cells. Imatinib, a molecularly targeting anticancer agent, is used widely for the treatment of CML and showed significant activity in chronic and accelerated phases but much less in blast crisis phase. The resistance to imatinib especially in blast crisis phase is recognized as a major problem in the treatment of CML patients. Docetaxel is shown to arrest cells in G2/M phase of the cell cycle which makes cells more sensitive to chemo- and radiotherapy. In this study, we aimed to increase chemosensitivity of human K562 CML cells to imatinib in combination with docetaxel. Taken together, our results showed that the combination of imatinib and docetaxel decreased cellular proliferation and increased apoptosis in human K562 chronic myeloid leukemia cells as compared to any agent alone. Imatinib and docetaxel induced apoptosis through caspase-3 enzyme activity and mitochondrial membrane potential.

Keywords: Chronic myeloid leukemia, imatinib, docetaxel, BCR/ABL, apoptosis

Introduction

Imatinib (imatinib mesylate, STI571, Gleevec) is a widely used anticancer agent for the treatment of chronic myeloid leukemia (CML).¹⁻³ Chronic myeloid leukemia is a hematopoietic stem cell disorder which is the result of the reciprocal translocation between chromosomes 9 and 22. This translocation forms a fusion protein gathering two genes, the BCR gene from chromosome 22 and the ABL gene from chromosome 9. BCR/ABL fusion protein is encoded by this hybrid gene with a constitutive tyrosine kinase activity inducing cellular proliferation, preventing apoptosis and decreasing cellular adhesion.^{2,4-6} Development of imatinib is an important avenue for the treatment of CML. Imatinib is able to

recognize and bind to 21 amino acids of the BCR/ABL ATP-binding site. Binding of imatinib to that site will inhibit the autophosphorylation of BCR/ABL and thus its substrates.^{2,6,7} In the beginning, imatinib presented very high hematological and cytogenetic responses in chronic and accelerated phases of CML patients. But, in blast crisis phase, the emergence of hematologic and cytogenetic resistance to imatinib is recognized as a major problem in the treatment of patients with CML.^{2,3,6,8}

Docetaxel is an anticancer agent which induces apoptosis by suppressing the microtubule dynamics of the mitotic apparatus. Docetaxel binds to taxel side of the β -tubulin stabilizing the microtubules and promotes the polymerization of microtubules. Thus, depolymerization of microtubules into free tubulin monomers is prevented causing inhibition of the metaphase anaphase transition, blocking mitosis and inducing apoptosis. Besides inducing cell death, docetaxel arrests cells in G2/M phase altering the

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centromere organization as a microtubule-stabilizing agent.^{9,10} By this G2/M arrest ability, docetaxel has been shown to increase the sensitivity of cancer cells to chemotherapy and radiotherapy besides inhibiting cell proliferation.¹¹

In this study, we tried to increase sensitivity of K562 cells to imatinib by combination with docetaxel and showed the responsible signaling pathways that induce apoptosis.

Methods

Cell lines and culture conditions

K562 human CML cells were obtained from the German Collection of Microorganisms and Cell Cultures. These cells were cultured in RPMI 1640 growth medium containing 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen) at 37°C in 5% CO₂.

Measurement of cell growth by XTT

The IC₅₀ values (the drug dose in which the 50% of cells can survive) of imatinib and docetaxel were determined by XTT as described previously.⁹ Briefly, 96-well plates were seeded with 2×10^4 cells/well containing 100 µl of the growth medium in the absence or presence of increasing concentrations of imatinib and docetaxel. They were incubated at 37°C in 5% CO₂ for 72 h. Then, they were treated with 40 µl XTT for 4 h. After that, plates were read under 490 nm wavelengths by Elisa reader (Thermo Electron Corporation Multiskan Spectrum, Finland). Finally, IC₅₀ values were calculated according to cell survival plots.

Measurement of caspase-3 enzyme activity

Caspase-3 enzyme activity of the cells was assessed using the caspase-3 colorimetric assay kit (R&D Systems, USA) as described previously.⁴ Firstly, the cells that had been induced to apoptosis were collected by centrifugation at 1000 rpm for 10 min. Then, the collected cells were lysed by adding 100 µl of cold lysis buffer (1 ×). After incubation of the cells on ice for 10 min, they were centrifuged at 14000 rpm for 1 min. Supernatants were removed to new Eppendorf tubes and the reaction mixture was prepared in 96-well plates adding 20 µl assay buffer (5 ×), 25 µl of sample, 50 µl of sterilized water and 5 µl of caspase-3 colorimetric substrate. After 2 h incubation at 37°C, the plate was read under 405 nm wavelength light (Thermo Electron Corporation Multiskan Spectrum, Finland)

Detection of the loss of mitochondrial membrane potential (MMP)

APO LOGIX JC-1 Assay Kit (Cell Technology, USA) was used to measure the mitochondrial membrane potential in K562 cells as described previously.⁵ JC-1, a unique cationic dye, was used to determine the loss of the MMP. In the beginning, the cells that had been induced to apoptosis were collected by centrifugation at 1000 rpm for 10 min. Supernatants were removed and 500 µl of JC-1 dye was added onto the pellets. After incubation at 37°C in 5% CO₂ for 15 min, they were centrifuged at 1000 rpm for 5 min. Then, 2 ml of assay buffer was added onto the pellets which were centrifuged for 5 min at 1000 rpm. All pellets were resuspended with 500 µl assay buffer and 150 µl from each of them was added into the 96-well plate. The aggregate red form has absorption/emission maxima of 585/590 nm and the green monomeric form has absorption/emission maxima of 510/527 nm. The plate was read in these wavelengths by fluorescence Elisa reader (Thermo Varioskan Spectrum, Finland).

Phosphatidylserine exposure

Apoptotic cell death was evaluated by using annexin V/propidium iodide double staining method based on apoptosis-related cell membrane modifications. Firstly, cells that had been induced to apoptosis were washed with cold PBS and then resuspended in 1 × binding buffer. 100 µl from each solution was transferred to Eppendorf tubes. Five microliters Annexin V-FITC and 5 µl PI was added on the cells. After gentle vortexing, they were incubated for 15 min at room temperature in the dark. Four hundred microliters 1 × binding buffer was then added into each tube and they were analyzed by flow cytometry (BD Facsanto flowcytometry, Belgium) within 1 h.

Results

Docetaxel or imatinib showed a dose-dependent cytotoxicity on human K562 chronic myeloid leukemia cells

We have shown antiproliferative effects of docetaxel or imatinib on K562 cells exposed to increased concentrations of the drugs by XTT cell proliferation assay. The results showed that there were dose dependent decreases in cell proliferation as compared to untreated controls. There were 12, 16, and 26% decreases in cell proliferation in 1, 10, and 100 nM imatinib applied K562 cells, respectively, as compared to untreated controls (Fig. 1). On the other hand, in K562 cells exposed to 1, 10, and 100 nM docetaxel, there were 10, 67 and 75% decreases in cell

proliferation, respectively, as compared to untreated controls. IC₅₀ values of imatinib (Fig. 1) and docetaxel (Fig. 2) in K562 cells were calculated from cell proliferation plots and were found to be 280 and 7.3 nM, respectively.

Synergistic/additive effects of docetaxel and imatinib on human K562 chronic myeloid leukemia cells

To examine the possible synergistic/additive cytotoxic effects of docetaxel and imatinib, K562 cells were exposed to combination of IC₅₀ levels of docetaxel (7.3 nM) and increasing concentrations of imatinib from 0.1 to 1000 nM and proliferation rates were determined by XTT cell proliferation assay. There were 6, 12, 16, 26, and 80% decreases in cell proliferation in 0.1, 1, 10, 100, and 500 nM imatinib treated K562 cells, respectively, while the combination of these doses with 7.3 nM docetaxel (IC₅₀ value) resulted in 53, 57, 62, 81, and 93% decreases cell proliferation, respectively, as compared to untreated controls (Fig. 3). These results strongly suggest that the combination of imatinib and docetaxel has shown significant cytotoxic effect, comparing any agent alone, in human K562 chronic myeloid leukemia cells.

Significant increase in caspase-3 enzyme activity determined in response to combination of imatinib and docetaxel as compared to any agent alone

Caspase-3 enzyme activity was measured to show the synergistic effects of imatinib and docetaxel on K562 cells. The K562 cells exposed to 7.3 nM docetaxel, 10 and 280 nM imatinib alone presented 1.7-, 1.3- and 1.6-fold increases in caspase-3 enzyme activity, respectively, as compared to untreated controls. On the other hand combination of 7.3 nM docetaxel with 10 or 280 nM imatinib resulted in 2 and 2.4-fold increase in caspase-3 activity in K562 cells, respectively, compared to untreated controls (Fig. 4).

Synergistic effects of combination of imatinib and docetaxel on mitochondrial membrane potential in K562 cells

To confirm the synergistic effect of the combination of imatinib and docetaxel, mitochondrial membrane potential (MMP) was measured by JC-1 MMP Assay Kit. Cells were treated with 7.3 nM docetaxel, 10 nM, 280 nM imatinib alone and the combination of 7 nM docetaxel and 10 or 280 nM imatinib. There were 1.3-, 1.3- and 1.5-fold increases in cytoplasmic/monomeric JC-1 in 7.3 nM docetaxel, 10 and 280 nM imatinib alone treated K562 cells, respectively, compared to control cells (Fig. 5). Combination of 7.3 nM docetaxel with the same doses of imatinib increased the cytoplasmic/monomeric JC-1, 1.6- and

17-fold, respectively, as compared to untreated controls (Fig. 5).

Combination of imatinib and docetaxel induced human K562 CML cells to apoptosis

Apoptosis and cell viability in K562 cells were measured by AnnexinV-FITC staining by flow cytometry. As shown in Fig. 6, there were 15.5, 16.4 and 25.1% increases in apoptosis in response to 7.3 nM docetaxel, 10 and 280 nM imatinib in K562 cells, respectively. Exposure of K562 cells to the combination of 7.3 nM docetaxel and 10 or 280 nM imatinib increased the apoptosis percentage 26.1 and 37.2% respectively.

Discussion

Treatment of cancer with more than one agent is called combination therapy. In combination therapy, to increase the effect of the one agent, another agent which has different effective mechanism can be used. Recent studies have shown that because treating different targets gives more effective results, combination therapy is a good alternative way for the treatment of cancer.¹²⁻¹⁴

Chronic myeloid leukemia is the result of the reciprocal translocation between chromosomes 9 and 22. This translocation between these two chromosomes causes the formation of BCR/ABL fusion protein.^{5,9} BCR/ABL fusion protein contributes many signaling pathways in cell-cycle showing high tyrosine kinase activity resulting in CML. Imatinib which is the first generation of the tyrosine kinase inhibitors, is used in the treatment of CML targeting the tyrosine kinase site of the BCR/ABL fusion protein. It binds to the ATP-binding side and inhibits the tyrosine kinase activity of BCR/ABL.^{5,9,15} Recent studies have shown that CML patients have well responses to imatinib at chronic and accelerated phases. But, the emergence of resistance to imatinib at blast crisis phase complicates the CML treatment with imatinib. Combination therapy is an alternative way to treat the diseases with more than one agent. The object of this application is to enhance the effect of the one agent with some other agents. In this study, we aimed to increase the efficiency of imatinib on Philadelphia chromosome positive K562 cells with a combination of docetaxel.

Imatinib/docetaxel combination has been used recently for the treatment of different types of cancers by different groups. In one study, it was shown that the imatinib/docetaxel combination showed significant cytotoxic effect for the treatment of androgen-independent prostate cancer (AIPC).¹⁰

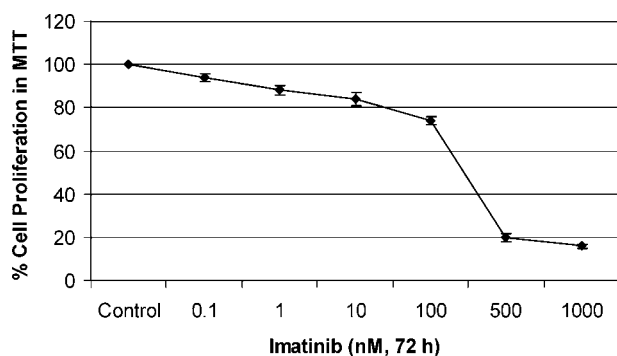


Figure 1 Effects of imatinib on the growth of K562 cells. The IC50 concentration of imatinib was calculated from cell proliferation plots. The XTT assays were performed using triplicate samples in at least two independent experiments. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance, and $p < 0.05$ was considered significant

Imatinib/docetaxel combination was also applied for the patients with advanced, platinum-resistant ovarian cancer and primary peritoneal carcinomatosis together and the results indicated that there was little induction of responses for heavily pretreated patients.¹⁶

There are some other studies used combinations of imatinib with some other agents to increase the susceptibility of BCR/ABL positive CML cells. Kancha and his coworkers emphasized the effectiveness of combination of imatinib and a nuclear

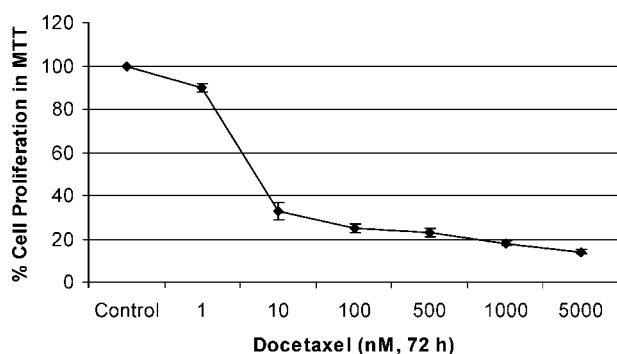


Figure 2 Effects of docetaxel on the growth of K562 cells. The IC50 concentration of zoledronic acid was determined by XTT assay for each cell line as described. The XTT assays were performed using triplicate samples in at least two independent experiments. The error bars represent the standard deviations, 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 $p < 0.05$ was considered significant

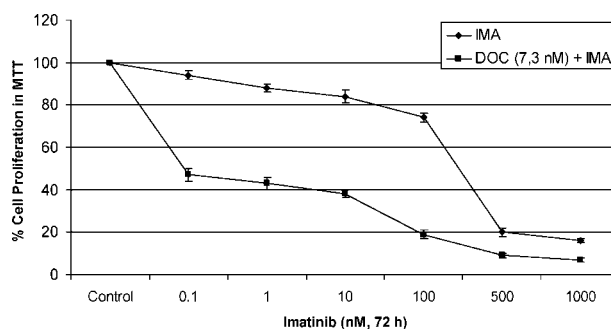


Figure 3 Effects of docetaxel and imatinib combination on the growth of K562 cells. Cytotoxicity was determined by the XTT cell proliferation test in a 72 h culture. The XTT assays were performed using triplicate samples in at least two independent experiments. The error bars represent the standard deviations, 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 $p < 0.05$ was considered significant

transport blocker, leptomyacin B, to imatinib-resistant Ba/F3 cells.¹⁷ In another study, imatinib applied to neuroblastoma cells combining with retinoic acid and γ -irradiation. The results showed that there were synergistic cytotoxic effects of imatinib and retinoic acid application as compared to any agent alone.¹⁸

Docetaxel has also been used as a chemosensitising agent for the treatment of different types of cancer with different anticancer agents. Docetaxel with a combination of zoledronic acid showed significant cytotoxicity as compared to only zoledronic acid applied hormone-sensitive prostate carcinoma cells. The combination of these agents presented high

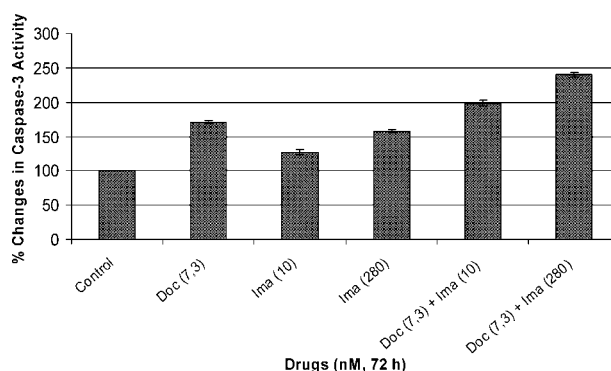


Figure 4 Percent changes in caspase-3 enzyme activity in docetaxel and imatinib combination or any agent alone exposed K562 cells. The results are the means of two independent experiments. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. $p < 0.05$ was considered significant

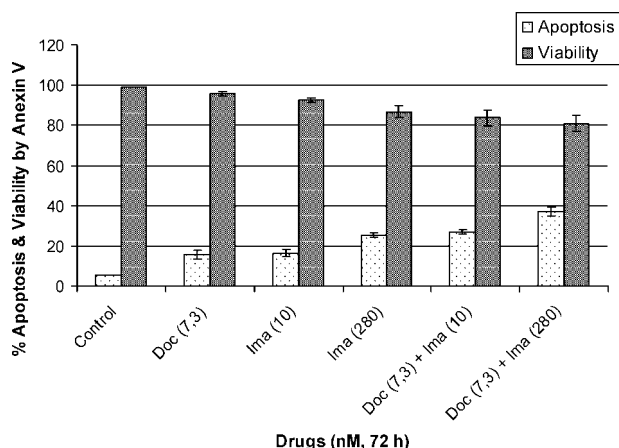


Figure 5 Percent changes in cytoplasmic/monomeric JC-1 in docetaxel and imatinib combination or any agent alone exposed K562 cells. The results are the means of two independent experiments. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. $p < 0.05$ was considered significant

synergistic effect on the induction of the apoptosis on LnCap cells.¹⁹ In one another study, docetaxel was applied to hormone-refractory prostate cancer (HRPC) with a combination of bevacizumab and showed synergistic apoptotic effect on HRPC cells. Results showed that the combination of docetaxel and bevacizumab is tolerable and active for HRPC patients.²⁰ It was also shown by different studies that the combination of docetaxel and gemcitabine had

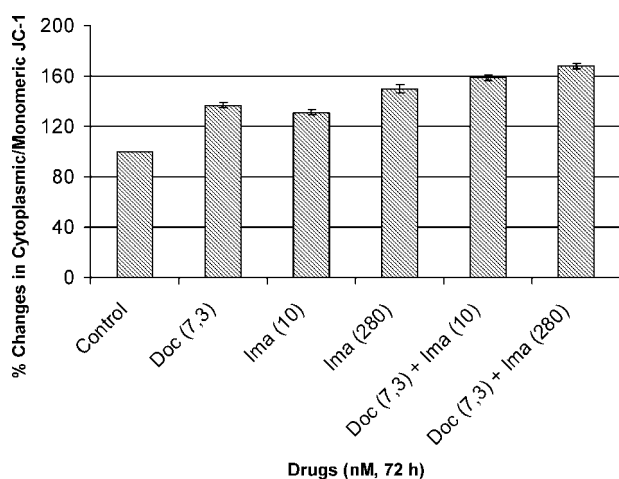


Figure 6 Percent changes in apoptotic cells by anexin V-FITC in docetaxel and imatinib combination or any agent alone exposed K562 cells. The results are the means of two independent experiments. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. $p < 0.05$ was considered significant

synergistic cytotoxic effect for the treatment of the pediatric sarcomas and for elderly patients with lung adenocarcinomas. The results of this study indicated that the combination of docetaxel with gemcitabine had effective and tolerable antitumor activity in both sarcomas and elderly patients with lung adenocarcinomas.^{21,22}

In parallel with the literature, our results strongly indicated that docetaxel increased the chemosensitivity of BCR/ABL positive human K562 chronic myeloid leukemia cells to imatinib. Imatinib/docetaxel combination induces apoptosis through decrease in mitochondrial membrane potential and increase in caspase-3 enzyme activity.

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