Vol.1, No.4, 320-324 (2009)
doi:10.4236/health.2009.14052

Inhibition of proteasome by bortezomib increase chemosensitivity of bcr/abl positive human k562 chronic myeloid leukemia cells to imatinib

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Received 18 July 2009; revised 28 July 2009; accepted 14 August 2009.

ABSTRACT

Chronic myeloid leukaemia (CML) results from a translocation between chromosomes 9 and 22 which generates the BCR/ABL fusion oncoprotein. BCR/ABL has constitutively tyrosine kinase activity resulting in leukemogenesis. Imatinib, a competitive inhibitor of the BCR/ABL tyrosine kinase, is the common treatment of CML. Despite the outstanding results of imatinib in the chronic phase of CML, cases of treatment failure have been reported, resulting in heterogeneous molecular response. Bortezomib is a reversible inhibitor of the 26S proteasome inducing cell cycle arrest in G2/M phase, apoptosis by inhibition of NF-kB. In this study, we examined the possible synergistic apoptotic effects of the imatinib/bortezomib combination and the responsible apoptotic mechanisms induced by this combination in K562 cells. The results of this study showed increased cytotoxicity by XTT assay in combination of imatinib and bortezomib as compared to any agent alone. On the other hand, synergistic apoptotic affects of combination of these agents were also confirmed by changes in caspase-3 enzyme activity and mitochondrial membrane potential. Taking together, all the results, confirming each other, showed that the combination of the imatinib and bortezomib has considerable synergistic effects on the apoptosis through increase in caspase-3 enzyme activity and decrease in mitochondrial membrane potential in human K562 CML cells.

Keywords: Imatinib; Bortezomib; Combination Therapy; CML, BCR/ABL

1. INTRODUCTION

Chronic myeloid leukemia (CML) is characterized by a reciprocal translocation between chromosomes 9 and 22 bringing together BCR and ABL genes to form BCR/ABL fusion protein. BCR/ABL protein, having constitutive tyrosine kinase activity, is responsible for the pathogenesis of CML. Imatinib (imatinib mesylate, STI571, Gleevec) is a tyrosine kinase inhibitor with good efficacy and recently recommended as first line therapy for the chronic phase CML. BCR/ABL has a special ATP binding site close to the substrate proteins binding region. Imatinib works by binding for blocking function of BCR/ABL ATP binding site. When the ATP binding site is filled by imatinib, ATP cannot donate the phosphate and BCR/ABL can no longer activate downstream signaling proteins that promote chronic myeloid leukemia [1,2,3,4,5]. Although imatinib has an outstanding outcomes in the chronic phase of CML, cases of treatment failure has been reported. Resistance to imatinib is a major problem in CML patients in blast crisis phase and has been principally associated with BCR/ABL tyrosine kinase domain point mutation decreasing the affinity of imatinib to BCR/ABL protein. In addition, amplification of the BCR/ABL oncogene, aberrant ceramide metabolism, inhibition of apoptotic pathways, and reduction in effective intracellular concentrations of the drug by altered drug efflux or influx are responsible for imatinib resistance [6,7]. As a result of disease persistence and resistance imatinib monotherapy cannot be referred to a cure for the majority of patients with CML [8,9,10,11,12]. The primary and acquired resistance to imatinib is the developing problem in current CML treatment and has various underlying mechanisms [1]. On the other hand, the combination of imatinib with anti-CML agents, may be able to kill drug resistant cells as highly as drug-sensitive parental BCR/ABL$^+$ leukaemic cells, unless the completely refractory to the partner drugs is developed by leukaemic cells.

The main pathway of protein degradation in eukaryotes is through ubiquitin-proteosome pathway [13,14]. Bortezomib has been common knowledge as a dipeptide boronic acid, potent and reversible inhibitor of the 26S proteasome [15,16]. Antitumor activity of bortezomib

has been proved in myeloma, myeloid leukemia, lymphoma, prostate, breast, colon, and lung cancers. For patients with myeloma and/or relapsed and refractory diseases bortezomib shows promising results [17]. Bortezomib induces cell cycle arrest in G2M phase and apoptosis by inhibition of NF-kB [14,18,19,20]. Studies strongly implicate the activity of the transcription factor NF-κB in promoting chemoresistance, cytokine-mediated proliferation, tumor metastasis, and angiogenesis. Bortezomib, as a proteosome inhibitor, also enhances the sensitivity of tumor cells to chemotherapy and radiation and reverses chemoresistance. Induction of apoptosis involves increased Bcl-2 phosphorylation and cleavage, accumulation of cyclins A and B1 and increased stability of p53 and up-regulated p53-induced gene expression [21].

In this study we describe synergistic cytotoxicity when cells are treated with the imatinib in combination with bortezomib and responsible signaling pathways that induce apoptosis in human K562 CML cells.

2. METHODS

Cell lines, Growth Conditions, and Drugs. Human K562 CML cells were obtained from German Collection of Microorganisms and Cell Cultures. Human K562 CML cells were maintained in RPMI 1640 growth medium containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37℃ in 5% CO_2. Imatinib was a generous gift from Novartis (USA) and bortezomib was from Gulhane Medical School, Department of Hematology. Imatinib and bortezomib were dissolved in distilled water and 10 mM stock solutions were prepared.

Measurement of growth by XTT. The IC50 values of imatinib and bortezomib that inhibited cell growth by 50% were determined from cell proliferation plots obtained by XTT as described previously [7]. Briefly, $2x10^4$ cells were seeded into the 96-well plates that contain 200 µl growth medium and the absence or presence of increasing concentrations of bortezomib and imatinib, and incubated at 37˚C in 5% CO_2 for 72 hours. Afterwards, they were treated with 40 µl XTT reagent for 4 hours. Then, the absorbances of the samples under 490 nm wavelength were measured by Elisa reader (Thermo Electron Corporation Multiskan Spectrum, Finland). At the end, IC50 values were calculated to cell survival plots.

Measurement of caspase-3 activity. Caspase-3 activity was determined using the caspase-3 colorimetric assay (R&D Systems, USA). To start with, the cells that treated with bortezomib and imatinib for 72 hours were collected by centrifugation at 1000 rpm for 10 minutes. Afterwards, pellets were treated with 100 µl of cold lysis buffer (1X) in order to obtain cell lysate. Then cell lysates were incubated on ice for 10 minutes and they were centrifuged at 14000 rpm for 1 minute. After that, supernatants were transferred to new eppendorf tubes. For measuring caspase activity, reaction mixture that include 20 µl assay buffer (5X), 25 µl of sample, 50 µl of sterilized water and 5 µl of caspase-3 colorimetric substrate was prepared in 96-well plates adding and incubated for 2 hours incubation at 37˚C. samples was read under 405 nm wavelength by Elisa reader (Thermo Electron Corporation Multiskan Spectrum, Finland). Absorbances were normalized to protein levels as determined by Bradford Assay.

Detection of the loss of mitochondrial membrane potential (MMP). The loss of mitochondrial membrane potential was detected by JC-1 mitochondrial membrane potential (MMP) kit (Cell Technology, USA). The loss of mitochondrial membrane potential is a hallmark for apoptosis. Firstly, the cells treated with imatinib and bortezomib were collected by centrifugation at 1000 rpm for 10 minutes. Supernatants were discarded and 500 µl of JC-1 dye was added onto pellets and incubated at 37˚C in 5% CO_2 for 15 minutes. Then samples were centrifuged at 1000 rpm for 5 minutes and 2 ml of assay buffer was added onto the pellets and they were centrifuged for 5 minutes 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).

3. RESULTS

The cytotoxic effects of imatinib or bortezomib alone or in combination on K562 cells. IC50 values of imatinib and bortezomib were found to be 267 nM (**Figure 1**) and 65 nM (**Figure 2**) in K562 cells exposed to increasing concentrations of imatinib and bortezomib for 72 hours, respectively. There were decreases (from 8 to 82%) in proliferation of K562 cells exposed to increasing nanomolar concentrations of imatinib (from 0,2 nM to 600 nM) for 72 hours as compared to untreated controls (Figure 1). On the other hand 10-, 50-, and 100 nM bortezomib resulted in 5-, 36-, and 70% decreases in cell proliferation (**Figure 2**), respectively. The same concentrations of imatinib with a combination of 65 nM bortezomib decreased cellular proliferation significantly (from 82 to 90%), as compared to untreated controls (**Figure 3a**). There were 50% decrease in proliferation of K562 cells in response to 65 nM bortezomib (**Figure 3b**).

The effects of Imatinib/Bortezomib combination on caspase-3 enzyme activity in K562 cells. Caspase-3 enzyme activity analyses showed that there were 1.21-

and 1.29-fold increase in 0.5- and 5 nM imatinib alone applied K562 cells, respectively while 65 nM bortezomib increased the caspase-3 enzyme activity 1.24-fold increase by itself (**Figure 4**). On the other hand, the same concentrations of imatinib in combination with 65 nM bortezomib increased the enzyme activity 2.4- and 2.85-fold, respectively (**Figure 4**).

The effects of combination of Imatinib/Bortezomib on mitochondrial membrane potential. Treatment with imatinib/Bortezomib caused a significant loss of MMP, as measured by increased accumulation of of cytoplasmic/mitochondrial form of JC-1, in K562 cells as compared any agent alone. There were 1.19- and 1.31-fold increase in cytoplasmic/mitochondrial JC-1 in 0.5- and 5 nM imatinib applied K562 cells, respectively, while 65 nM bortezomib decreased MMP 1.16 fold by itself. On the other hand, the same concentrations of imatinib with 65 nM bortezomib decreased MMP 1.88- and 2.79-fold, respectively (**Figure 5**).

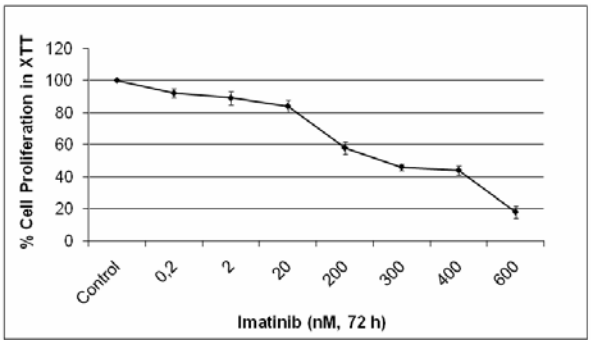

Figure 1. Effects of imatinib on the growth of K562 cells. The IC50 concentration of imatinib was calculated from cell proliferation plots. The XTT assays were performed using triplicate samples in at least two independent experiments. Statistical significance was determined using two-way analysis of variance, and ($P < 0,05$) was considered significant.

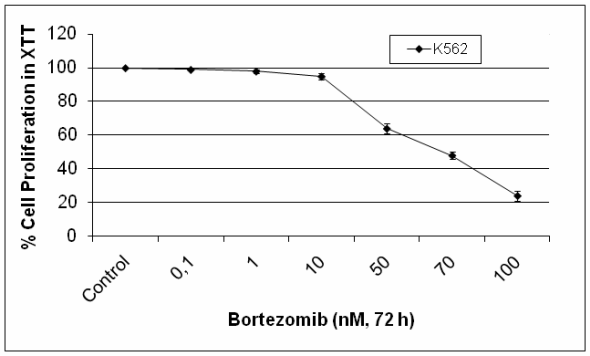

Figure 2. Effects of bortezomib on the growth of K562 cells. The IC50 concentration of bortezomib was determined by XTT assay for each cell line as described. The XTT assays were performed using triplicate samples in at least two independent experiments. Statistical significance was determined using two-way analysis of variance, and ($P < 0,05$) was considered significant.

4. DISCUSSION

The way for treatment of cancer more than one agent is

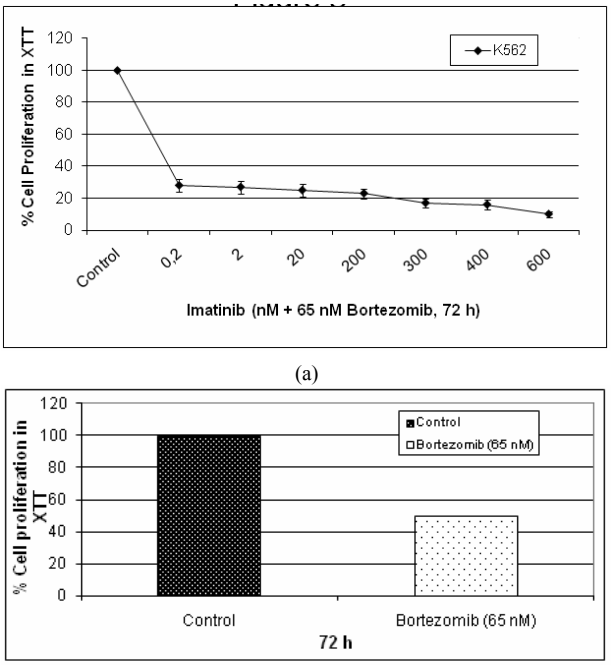

Figure 3. Effects of bortezomib and imatinib combination on the growth of K562 cells (a). Effects of 65 nM bortezomib by itself on K562 cells as compared to untreated controls (b). Cytotoxicity was determined by the XTT cell proliferation test in a 72 hours culture. The XTT assays were performed using triplicate samples in at least two independent experiments. Statistical significance was determined using two-way analysis of variance, and ($P < 0,05$) was considered significant.

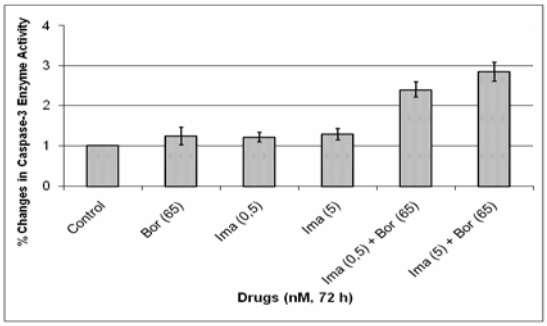

Figure 4. Percent Changes in Caspase-3 Enzyme Activity in bortezomib and imatinib combination or any agent alone exposed K562 Cells. The results are the means of two independent experiments. $P < 0,05$ was considered significant.

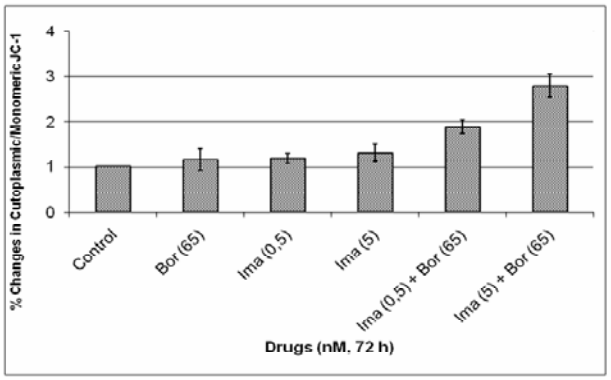

Figure 5. Percent changes in cytoplasmic/mitochondrial JC-1 in bortezomib and imatinib combination or any agent alone exposed K562 Cells. The results are the means of two independent experiments. P < 0,05 was considered significant.

known as combination therapy. The main aim of the combination therapy is to get more effective result with at least two agents than any agent alone. For this purpose, the agents targeting the different mechanisms and having effect on different ways are preferred. Several groups demonstrated that targeting the combination therapy increases the effect of one agent and it appears a very good alternative way for the treatment of cancer [22, 23, 24]. Thus the complete destruction of BCR/ABL$^+$ leukemic cells may require BCR/ABL inhibitors in combination with other therapeutic agents that modify the molecular pathways that control cell survival. The treatment of Philadelphia chromosome-positive (Ph$^+$) leukemias has improved markedly owing to the development of inhibitors of BCR/ABL tyrosine kinase, as a first member tyrosine kinase inhibitors imatinib. However, resistance to imatinib resulting from various molecular mechanisms, such as BCR/ABL overexpression, aberrant ceramide metabolism, mutations within the ima- tinib-binding site of BCR/ABL, expression of a drug efflux pump or compensatory overexpression of related Src family kinases [4,7].

Bortezomib reduces NF-kb activation and inhibits its translocation to the nucleus. On the other hand having the ability to arrest the cells in G2/M, it can increase the sensitivity of cancer cells to chemotherapy and radiotherapy. The BCL-2 family plays significant roles as a key activators of mitochondrial apoptosis in mediating bortezomib toxicity [14,25].

There were some recent studies showing increased synergistic apoptotic effects of imatinib and/or bortezomib with a combination of other anticancer agents in hematological malignancies. In isolated chronic lymphoblastic cells, bortezomib has been shown to induce considerably higher level of apoptosis than either methylprednisolone or fludarabine and the combination of fludarabine plus bortezomib increased apoptosis more as compared to any agent alone, even in fludarabine-resistant cells [15]. In 2008, Wiberg and his coworkers explored 115 samples of tumour cells that bortezomib is more active in haematological malignancies than in solid tumour samples. Their results showed a strong synergy with arsenic trioxide or irinotecan and bortezomib [13]. In an interesting study by Yong et al., in CD34$^+$ cells of fourteen CML patients from different phases and in K562 cells, as a positive control, bortezomib treatment improved sensitization, upregulated the expression of TRAIL receptors on quiescent leukemic CD34$^+$ cells and increased their susceptibility to expanded donor NK cells [27]. Likewise, another data support a model in which the combination of sorafenib, multikinase inhibitor, and bortezomib showed synergistic cytotoxicity by modulating Akt and JNK signaling to activate apoptosis [28]. On the other hand, bortezomib in combination of histone deacetylase inhibitor, SAHA, was subjected to another study demonstrating that bortezomib and SAHA combination resulted in imatinib-sensitive and -resistant BCR/ABL positive cells to apoptosis through induction of JNK and p21CIP1 [29].

To overcome resistance problems in CML, combinations of drugs with imatinib provided an emerging therapeutic concept. The recent study by Cortes and his coworkers showed effectiveness of imatinib with lonafarmib in CML patients who were initially resistant to imatinib. Combination of imatinib with cytarabine also showed synergistic cytotoxic effects in CML patients [30]. It was also shown by our group that imatinib in combination with docetaxel and fludarabine presented synergistic apoptotic effects in K562 cells (Unpublished data). All examinations showed that combination of bortezomib with imatinib induces apoptosis synergistically.

In this study we evaluated the possible synergy of combination of imatinib and bortezomib. Our data provide that bortezomib enhanced the chemosensitivity of BCR/ABL positive human K562 CML cells to imatinib. Combination therapy can be revealed promising results for new approaches to underlying mechanism of drug resistance. The combination of these agents induced apoptosis through decrease in mitochondrial membrane potential and increase in caspase-3 enzyme activity.

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