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Imatinib Mesylate induces apoptosis in chronic myeloid leukemia cells by triggering nitric oxide production

Apoptotic effect of nitric oxide on CML cells

Aynur Karadağ Gürel[1], Tülin Özkan[2], Buket Altınok Güneş[3], Asuman Sunguroğlu[2], Fügen Aktan[4]
[1]Department of Medical Biology, School of Medicine, Usak University, Usak
[2]Department of Medical Biology, School of Medicine, Ankara University, Ankara
[3]Medical Laboratory Techniques, Vocational School of Health Services, Ankara University, Ankara
[4] Department of Biochemistry, Faculty of Pharmacy, Ankara University, Ankara, Turkey

Abstract

Aim: Chronic Myeloid Leukemia (CML) is a myeloproliferative disease characterized by abnormal clonal proliferation of hematopoietic stem cells. The over-production of nitric oxide (NO) causes reactive oxygen species and subsequent oxidative stress to cell toxicity. The production of factors such as nitric oxide (NO) and reactive oxygen species (ROS) in the cell and/or tissue environment affects the behavior of normal cells and cancer cells. In this study, we aimed to investigate the effects of nitric oxide formed as a result of Imatinib Mesylate (STI571, Gleevec®) metabolism on cell death.

Material and Methods: 32D (Control) and 32DP210 Cells were treated with 10um of Imatinib Mesylate for 24, 48 and 72 hours. Methyl tetrazolium assay (MTT) was used for cell viability. Griess assay and capillary electrophoresis were used to measure Imatinib Mesylate -mediated NO production. The number of cells leading to apoptosis was calculated by counting 1000 cells and compared with the amount of NO. Statistical analysis of the obtained data was done with SPSS for Windows statistical package program.

Results: The presence of NO is important for increased cell death in the cell culture medium. Imatinib mesylate concentrations administered to healthy and CML groups increased intra cell NO levels by a significant amount in Bcr-Abl+ CML cell lines. The production of NO per apoptotic body is decreased during the apoptosis, and analysis of NO production using CE in these cells has rapid and efficient separation ability.

Discussion: As a result, knowing the molecules and effects regulating NO activity better appears to be a target point to remove many irregularities of these molecules. Sensitive detection of these metabolic products is important as variations in NO levels in many diseases provide information about development of diseases and treatment.

Keywords

Chronic myeloid leukemia; Imatinib mesylate (STI571, Gleevec); Nitric oxide; Capillary electrophoresis; Apoptosis

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Corresponding Author: Aynur Karadağ Gürel, Usak University, Faculty of Medicine Dean's Office 4th Floor, 64200, Usak, Turkey.
E-mail: aynur.karadag@usak.edu.tr P: +90 276 221 21 21-6240 GSM: +90 5326821113 F:+90 276 221 22 34
Corresponding Author ORCID ID: https://orcid.org/0000-0002-5499-5168

Introduction

Chronic myeloid leukemia (CML) is a clonal hemopoietic stem cell disease, characterized by the presence of Bcr-Abl oncoprotein and mutual translocations between the chromosome 9 (ch9) and 22 (ch22) long arms [1]. Imatinib mesylate, known as STI571 or Gleevec, is a successful tyrosine kinase inhibitor (TKI) commonly used for CML treatment. The development of TKI significantly increased the survival of CML patients [2]. Imatinib mesylate suppresses these tyrosine kinase receptors by blocking the ATP binding site and is accepted as the first-stage treatment of CML and gastrointestinal stromal tumors [3]. Reactive oxygen species (ROS) are important for tumor cell apoptosis, mitochondrial stability and chemotherapeutic effects. Additionally, ROS may act like mitogens stimulating the proliferation and migration of tumor cells [4]. ROS can damage DNA directly or through intermediates, and plays an important role in genome integrity as increased ROS production is associated with Bcr-Abl activity. [5].

Cell toxicity may cause organ function disorder and serious health problems. Recent studies have revealed that many toxic materials, overproduction of nitric oxide, reactive oxygen species and later occurring oxidative stress may cause cell toxicity [6]. At low concentrations, intracellular oxidants act like signal transducers, inducing growth factors, hypoxia and other receptor-ligand systems [7, 8]. Additionally, these oxidants may damage lipids, proteins, RNA and DNA at concentrations above threshold levels; thus, they trigger cell death through apoptosis and/or necrosis pathways [9, 10]. ROS, like oxygen ions and peroxides, comprise natural by-products of normal oxygen metabolism and they are reactive molecules containing oxygen that play an important roles in cell signals and homeostasis [11]. The biological functions of ROS and their potential role in cancer development and disease progression have been researched in recent years. ROS mediates cancer cell apoptosis induced by a variety of anticancer agents and other stimulants. Additionally, several studies have shown that anticancer agents reduced ROS production and increased malignant cell apoptosis [12, 13] . Bcr-Abl stimulates the production of ROS. ROS induces abl kinase activation. Since ROS activates Abl kinase, the Bcr-Abl kinase inhibitor imatinib is thought to have an effect opposite to the endogenous ROS induced by Bcr-Abl [5, 14-16]. In this very complicated network of biological processes, certain molecules may have binary roles linked to activity as a part of the content and on complicated intracellular and intercellular communication paths. Some, like reactive species, play a role in protecting the regular physiological environment; however, they may become pathogenic vectors for cell injury and destruction under pathophysiological conditions and contribute to disease development [16-18]. NO is believed to play a dual role in damaging or protecting cell functions. Nitric oxide may induce both apoptosis and necrosis in a variety of cells. Additionally, the NO-dependent effect mechanism of Imatinib Mesylate has not yet been determined; however, many studies have shown that apoptosis is closely related to changes in NO levels [18]. In this study, we aimed to understand the developed cytotoxicity mechanism caused by Imatinib Mesylate derived NO in CML and Control cell lines, nitrite levels were assessed before and after imatinib administration and cell death was monitored.

Material and Methods

Cell Culture and Reagents

Murine IL-3-dependent myeloblastic cell lines 32D (normal) and 32Dp210 (CML lines) were purchased from ATCC (American Type Culture Collection). 32Dp210 were maintained in RPMI1640 supplemented with FBS (10%), 100 U/ml penicillin, and 100 mg/ml Penicillin/streptomycin at 37°C, in a humidified atmosphere of 5% CO2. The cells were plated in 96-well plates, covered with collagen (Sigma) at the density of 2x 105 /cm2. 32D cell lines were grown in RPMI supplemented with 10% fetal calf serum (FCS), glutamine, penicillin, streptomycin, and 10% conditioned medium from the WEHI-3B cell line, as a source of IL-3. WEHI-3B cells produce high levels of IL-3 and used WEHI-3B-conditioned medium as a source of IL-3. The cells were passaged every 3 days, and the supernatant was centrifuged in 2500 rpm and collected for IL3.

Imatinib Mesylate (Novartis, Basel, Switzerland) was prepared by dissolving the stock solution in Dimethyl Sulfoxide (DMSO) (Applichem, A2940) at a concentration of 2mg/ml and stored at -20°C. A dose of 10μM Imatinib Mesylate was used for this assay because we showed that 10μM was required to induce apoptosis at 24 hours.

Cell Viability

The MTT assay, reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product, was used to estimate cell viability and growth. Cells were plated in triplicate in 96-well plates at a density of 5x106 cells/well, and the next day the media was replaced with media containing Imatinib Mesylate. Cells were treated with the previously mentioned concentrations for 24, 48 and 72 hours. After 24, 48 and 72 hours, the media was replaced with MTT containing media (0.5mg/ml). The cells were incubated at 37°C for 4 hours, an equal volume of solubilization solution was added and incubated at 37°C overnight to solubilize formazan crystals. Then the absorbance at 540 nm was read using a spectrophotometric microplate reader. The viability of cells was calculated as viability percentage in comparison to non-treated cells.

Determination of Nitrite/Nitrate in Supernatans using Griess Reaction and Capillary Electrophoresis

Griess assay: Nitric oxide production was determined by measuring nitrite, the stable end product, using the Griess reagent according to the manufacturer's protocol. On a 96-well plate, 500 μl of supernatant was added, followed by 500 μl of sulfanilamide and 500 μl of N-1-naphthylethylenediamine dihydrochloride (NED). Absorbance was read at 540 nm with nitrite concentrations determined using a standard nitrite curve.

Capillary Electrophoresis: At determined times, samples taken from the cell culture were placed in 15 mL Falcon tubes, centrifuged for 10 min at 900 rpm, and then the supernatant portions were taken and samples were stored at -20 °C. Immediately before measurements, samples were thawed, diluted 1:5 with water and then samples were prepared for measurement with 0.5:1 IS and centrifuged for 5 min at 4000 rpm and measurements were performed.

Morphological Assay for Apoptotic Cells

Typical apoptotic cells, under a light microscope, show small, highly condensed chromatin bodies. Each apoptotic body has

a fragmented piece of nucleus surrounded by a viable cell membrane. In addition, for confirmation of morphological assessments of apoptosis, smears were stained with Wright-Giemsa stain. The cells were centrifuged and the pellets were resuspended in minimum volumes of PBS. The cells were fixed and stained with 1 ml per slide of Wright-Giemsa stain for 1 min. After 4 min, the slides were rinsed with water and air-dried.

Statistical Analysis

The cells were studied in triplicates; each replication three times for each assay. Statistical analysis of the groups according to the initial value of the control cells was performed according to the one-way ANOVA test. Statistical analysis of the obtained data was done with the statistical package program software SPSS for Windows version 22.0. Results were given as mean ± standard deviation and $p < 0.05$ was considered significant.

Results

Determination of Live Cell Amounts with the MTT Method in Cells with Imatinib Mesylate Administered

The number of viable cells was determined with the MTT method in the presence and absence of 10μM concentration of Imatinib Mesylate at hours 0, 4, 6, 8, 10, 12, 24, 48 and 72. According to our results, in the presence and absence of Imatinib Mesylate from the 24th hour, the number of 32D viable cells increased; however, there appeared to be no variation in the number of viable cells in the 24 hour period. However, 32D cells with Imatinib Mesylate administered were identified to have slower proliferation compared to the rates for controls. Contrary to this, for the 32Dp210 cell lines, Imatinib Mesylate caused cell death from the 24th hour, with an increase in the cell numbers for control cells in this series from the 24th hour (Figure 1).

Apoptotic Index of Cells Administered with Imatinib Mesylate

Following Imatinib Mesylate administration, preparations of 32D and 32Dp210 cells were observed under a microscope and apoptotic cell counts per 1000 cells were determined. Control and Imatinib Mesylate-administered 32D cells were observed to have a reduction in ‰ apoptotic cell counts from the 12th hour, and this reduction was statistically significant ($p<0.05$). There was no significant change observed for apoptotic cell amounts in control 32Dp210 cells. However, 32Dp210 cells with Imatinib Mesylate administered were observed to have a significant increase in apoptotic cell amounts from the 24th hour ($p<0.05$) (Figure 2). In conclusion, Imatinib Mesylate induced cell death in 32Dp210 cells, and suppressed proliferation. The microscopic appearance of apoptotic cells is shown in Figure 3.

Determination of Nitrite in Supernatans by Simple Griess Reaction

Under physiological conditions, NO is oxidized to nitrite and nitrate in a ratio of about 3:2. The conversion of nitrite to nitrate in oxygenated solutions is quite slow. According to the standard chart drawn, the amount of nitrite/nitrate taken from the cell media at different times was calculated by the Griess method. The value of nitrate concentrates per apoptotic cell ratio (‰) was evaluated according to the baseline data. According to our results, there was a significant increase in nitrate concentration per apoptotic cell at 72 hours at 32D control cells in the absence of Imatinib Mesylate.

Determination of Nitrite/Nitrate in Supernatans using Capillary Electrophoresis

Capillary electrophoresis for nitrite and nitrate estimation was performed according to a method proposed Tuncel et al. [19]. Electropherograms were drawn for samples under standardized conditions, peak areas were calculated and the nitrate concentrations for both cell lines were identified. According to the calculated concentrations, no significant increase or decrease in nitrate concentrations was observed in both cell lines.

Nitrate Concentration (with the capillary electrophoresis method)/Apoptotic Cells

Assessment of nitrate levels, identified with the capillary electrophoresis method as a marker of NO release, was assessed linked to the presence of apoptotic cells in the medium before and after Imatinib Mesylate administration. The nitrate concentrations per ‰ apoptotic cell proportion were assessed between the groups. 32D cells without Imatinib Mesylate administration had a statistically significant increase in nitrate concentration/‰ apoptotic cell ratio after 24 hours, while this ratio significantly increased at the 72nd hour for 32D cells with Imatinib Mesylate administered ($p<0.05$). For 32Dp210 cells without Imatinib Mesylate administration, no significant change was observed for 72 hours, while in the presence of Imatinib Mesylate, the ratio of nitrate concentration to ‰ apoptotic cells decreased significantly from the 24th hour.

Nitrite Concentration (with Griess method)/‰ Apoptotic Cells Results

Cell counts cannot be standardized after the 24th hour, so assessment of nitrite levels with the Griess method, as a marker of NO release, was performed linked to the presence of apoptotic cells in the medium before and after Imatinib Mesylate administration. According to our results, 32D control cells had a significant increase in nitrate concentration per ‰ apoptotic cells in the 72nd hour in the absence of Imatinib Mesylate. The ratio of nitrite concentration (μM) to ‰ apoptotic cell amount identified with the Griess method, as marker of NO release, and the ratio of nitrate (μM) to ‰ apoptotic cell proportion, identified with the capillary electrophoresis method were compared.

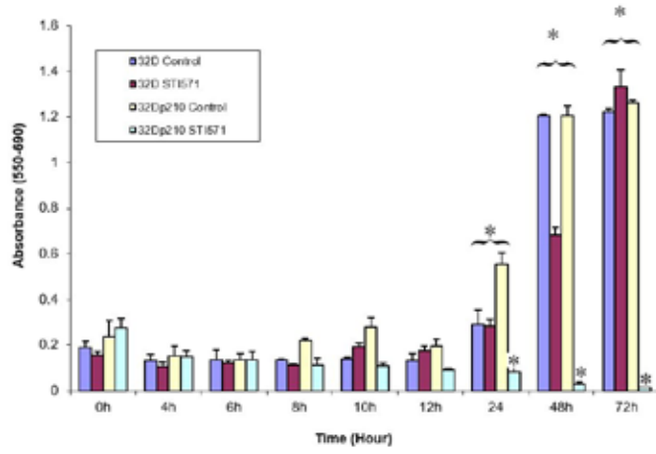

Figure 1. Cell levels identified with MTT for 32D and 32Dp210 cells as control and with 10 μM Imatinib Mesylate administered at 0, 4, 6, 8, 10, 12, 24, 48 and 72 hours. 32D C: 32D control cells, 32 D G: 32 D cell lineses with Imatinib Mesylate administered, 32Dp210 C: 32Dp210 control cells, 32Dp210 G: 32Dp210 cell lineses with Imatinib Mesylate administered. *statistical significance at $p<0.05$ compared to initial value.

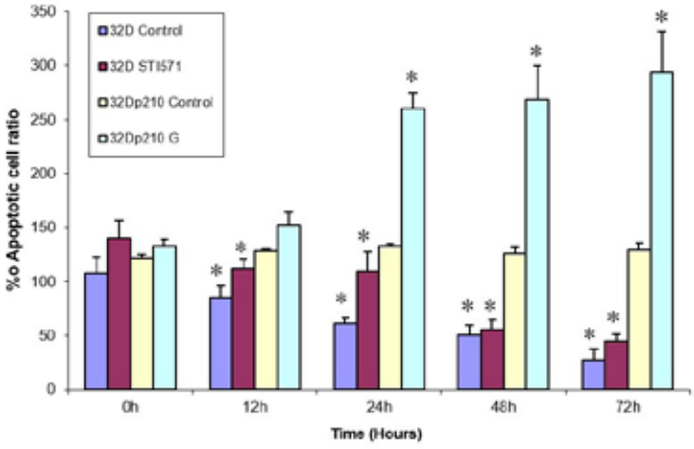

Figure 2. Apoptotic cell rates for 32D and 32Dp210 cells as control and with 10 μM Imatinib Mesylate administered at 0, 12, 24, 48 and 72 hours. 32D C: 32D control cells, 32 D G: 32 D cell lineses with Imatinib Mesylate administered, 32Dp210 C: 32Dp210 control cells, 32Dp210 G: 32Dp210 cell lineses with Imatinib Mesylate administered. *statistical significance at $p<0.05$ compared to initial value.

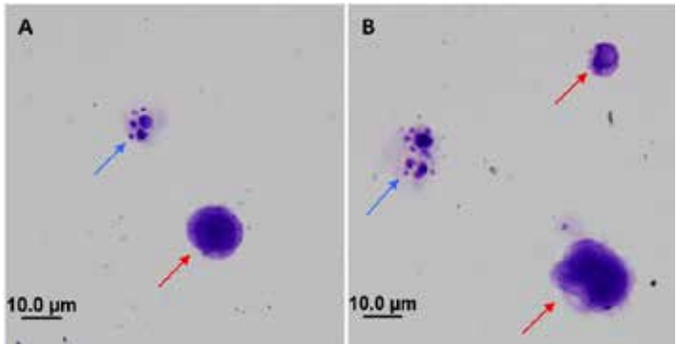

Figure 3. Photographs of normal and apoptotic cells in 32D (A) and 32Dp210 (B) cells administered Imatinib Mesylate. Blue arrows show apoptotic cells, red arrows show normal cells.

Discussion

Chronic myeloid leukemia is a hematopoietic system disease characterized by the presence of Bcr-Abl. In recent years, signal transduction inhibitors were used for leukemic cell treatment. Especially Imatinib Mesylate is a selective Bcr-Abl tyrosine kinase inhibitor affecting P210 Bcr-Abl. Some anticancer drugs display antitumor effects on cancer cells by inducing apoptosis, and targeting apoptosis has become an important point in current cancer treatments. In this study, the apoptotic effects of NO released into the cell culture medium during cell death induced by imatinib mesylate on 32D and 32Dp210 myeloid cells were investigated. In this study, Imatinib Mesylate used for CML treatment induced apoptotic cell death and nitric oxide amount, a significant parameter for apoptosis, was measured with the rapid and sensitive method of capillary electrophoresis and the Griess method.

The first member of signal transduction inhibitors and causing effect by binding to the ATP binding region of the kinase domain, Imatinib Mesylate is selectively effective on Bcr-Abl positive cells. At minimal doses, Imatinib Mesylate (0.1μM) is effective on Bcr-Abl positive cells; however, at high doses (10μM), apoptotic cell death is triggered in the majority of cells and proliferation is known to be inhibited [20]. In this study, the dose inducing maximum apoptosis of 10 μM Imatinib Mesylate was administered to 32D and 32Dp210 cells and cell death was observed for 72 hours, and a rapid increase in apoptotic cells numbers was observed in 32Dp210 cells at 24 hours and longer. Increasing apoptotic cells counts are in parallel with the reduction in the amounts of viable cells. Oetzel et al. took the duration of 48 hours in an apoptosis model induced with 10 μM Imatinib Mesylate on Bcr-Abl cells [20]. Contrary to this, our study observed that for 32D cells, there was a reduction in viable cell amounts after 24 hours and longer and a reduction in apoptotic cell levels in this period in the presence and absence of Imatinib Mesylate in the medium. 32Dp210 cells without Imatinib Mesylate administered had an increase in the number of viable cells, though there was no significant change observed in apoptotic cell levels [21]. As a result, the presence of NO is important for increased cell death in the cell culture medium. Imatinib concentrations administered to healthy and CML groups significantly increased intracell NO levels in Bcr-Abl+ CML cell lines.

Capillary electrophoresis has many superior aspects like high selectivity, rapid detection, requiring low amounts of sample and reactives, and detection levels that can be lowered to very low levels and is used for differentiation and detection of chemical compounds. It is very difficult to detect the amount of NO with very short half-life and this structure rapidly metabolizes to nitrite (NO_2^-) and nitrate (NO_3^-). As a result, the aim of the research was to measure nitrites (NO_2^-) and nitrates (NO_3^-), stable end products of NO metabolism, used as markers of NO release. In our study, samples taken at different times from 32D and 32Dp210 cell media had nitrate levels identified with capillary electrophoresis as NO release marker [19].

After administration of Imatinib Mesylate to both cell lines, there was no significant increase or reduction in nitrate levels measured with the capillary electrophoresis method. To date, no studies have been found on NO release with Imatinib Mesylate, and the information about this topic is not sufficient. For structures with anticancerogenic effect, important parameters are reducing cell proliferation and increasing apoptosis. Studies have explained that structures with anticancerogenic effect also cause iNOS inhibition. Imatinib Mesylate inhibits the JAK2 tyrosine kinase enzyme activated in 32Dp210 cells and c-Myc RNA expression [22]. In this situation, it is clear that Imatinib Mesylate with anticancerogenic and proapoptotic effect both prevents cell proliferation and causes iNOS inhibition.

In order to emphasize the relationship of NO with apoptotic cells, the proportion of NO release to apoptotic body formation was calculated. In this situation, when the nitrate concentration detected with capillary electrophoresis to ‰ apoptotic cell ratio is assessed, 32D control cells had an increase in the of amount nitrate per apoptotic cell at 24 hours and later. Linked to the increasing apoptotic bodies after Imatinib Mesylate administration to cells, the rate of this increase slowed. However, while the nitrate amounts in 32Dp210 control cells did not change, there was a rapid increase in apoptotic body levels at 24 hours and later after Imatinib Mesylate administration and linked to this, a rapid reduction in this ratio. In this situation Imatinib Mesylate slowed the proliferation of 32D cells, increased apoptosis in 32Dp210 myeloid cells, and caused a rapid reduction in nitrate levels. Results were parallel to the

values for NO release determined with capillary electrophoresis; however, due to the large standard deviation related to the method, reductions were not identified to have statistical significance. As the ‰ apoptotic cell rates were very low for 32D control cells without Imatinib Mesylate administered, the nitrite concentration to ‰ apoptotic cell rates calculated at 72 hours was found to be statistically significant compared to initial values ($p<0.005$). When we compare data obtained with the Griess method and capillary electrophoresis method, we did not identify a very good linear correlation between these values. The increase in NO synthesized from L-arginine in cancer and many pathological situations shows that NO is used as a signal molecule by apoptotic signal metabolisms. However, the response to varying signals in cells may be to stop apoptosis, just as it may inversely be to initiate apoptosis. As a result, knowing the molecules and effects regulating NO activity better appears to be a target point to remove many irregularities of these molecules. Sensitive detection of these metabolic products is important as variations in NO levels in many diseases provide information about development of diseases and treatment.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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