Imatinib Induces Down-Regulation of Bcl-2
an Anti-Apoptotic Protein in Prostate Cancer
PC-3 Cell Line

Seyed Ataollah Sadat Shandiz¹, Mehdi Shafiee Ardestani², Shiva Irani¹ and Delavar Shahbazzadeh³*

¹Department of Biology, Science and Research Branch
Islamic Azad University, Tehran, Iran

²Department of Radiopharmacy
Faculty of Pharmacy, Tehran university of Medical Sciences
Tehran, Iran

³ Biotechnology Research Center
Pasteur Institute of Iran, Medical Biotechnology Group
Venom and Toxin Lab, Tehran, Iran
*Corresponding author

Copyright © 2014 Seyed Ataollah Sadat Shandiz, Mehdi Shafiee Ardestani, Shiva Irani and Delavar Shahbazzadeh. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Over expression of Bcl-2, an anti-apoptotic protein, has been associated with several malignancies. The Bcl-2 protein is a highly conserved member of the Bcl-2 family and constitutes a prominent regulator of apoptosis. In the present study, we investigated the effect of imatinib on cell viability, anti cancer effect through modulation of Bcl-2 gene expression in prostate cancer (PC-3) human cell lines. PC-3 cell lines were treated with various concentrations of imatinib (3, 5, 12.5, 25, 50, 100μM) for 24h. Cell viability was assessed using MTT assay and IC₅₀ value was determined. Then, real time PCR method was also applied for the investigation of Bcl-2 gene expression in cancer cells. The quantity of Bcl-2 gene
expression compared to GAPDH gene (reference gene) was analyzed. The real time PCR results showed that imatinib can inhibitory effect on viability via down regulation of anti apoptotic Bcl-2 gene expression in Prostate PC-3 cancer cells. The results presented here warrant further investigation on other cell lines as well as animal tumor models and subsequent clinical studies.

Keywords: Imatinib, Bcl-2, Apoptosis, Prostate Cancer

Introduction

Prostate cancer is the most common solid neoplasm diagnosed in men and the third leading cause of death due to cancer in males [1, 2]. The failure of conventional radio- and chemo-therapy and increasing incidence and mortality caused by advanced invasive prostate cancer, there is an urgently needed to explore novel agents and new targets in the search for more productive treatment [3]. Bone metastases are a common complication of cancer patients, with up to 70% of patients with advanced breast or prostate cancer [4]. Understanding the gene expression during tumor progression has been the topic of intense method for inhibition of tumor progression. Several proteins gene expression and signaling cascades are found to be essential in the regulation of apoptosis. Anti-apoptotic Bcl-2 family members (for example Bcl-2 and Bcl-XL) are demonstrated to be trail of important proteins functioning in the apoptotic process or clinical cancer therapy [5]. The Bcl-2 protein is a highly conserved member of the Bcl-2 family and constitutes an important regulator of apoptosis. Over expression of the anti-apoptotic protein Bcl-2 has been associated with several malignancies [6]. Nowadays, Molecular targeted therapy is designed to inhibit key signaling pathways involved in apoptosis and metastasis. Different clinical and preclinical studies on molecular targeted therapy have demonstrated that it has great promise in the treatment of several malignant tumors [7]. Imatinib (also known as Gleevec; Novartis Pharma, Basel, Switzerland), is the first member of a new class of agents that act by inhibiting specific tyrosine kinases. Gleevec is a 2-phenylaminopyrimidine derivative that its competes with ATP and inhibit specific tyrosine kineses like Bcr-Abl fusion oncprotein in chronic myeloid leukaemia (CML), as well as inhibits the activation of platelet-derived growth factor PDGF (αβ) and c-kit which regulate major cellular events and is currently used in research and treatment of several solid tumors [8-10]. Also, it was shown that imatinib can decrease the progressive growth of experimental bone metastases in human prostate cancer and its activity has been implicated in both epithelial and stromal mechanisms of prostate cancer progression [11]. The aim of the current study was to investigate the effect of imatinib on cell viability, anti cancer effect through modulation of Bcl-2 gene expression in prostate cancer (PC-3) human cell lines.
Imatinib induces down-regulation of Bcl-2

Materials and Methods

Cell culture and Drug treatment

The human cell line prostate cancer (PC-3) was purchased from the Pasteur institute cell bank in Tehran (Iran). The cell line were grown in DMEM medium supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 10% heat inactivated fetal calf serum (FCS) (All from Gibco, Scotland) at 37 °C in a 5% CO2 atmosphere. After 24-48 hours incubation period the attached cells were harvested with 0.25% trypsin (Sigma, USA) the cells were counted and then were distributed into 96-well plates with 10000 cells in each well. The plates were incubated 24-48 h under a humid atmosphere (37°C, 5% CO2) to permit the cells attaches to the bottom of the well. Different concentrations of imatinib (3, 5, 12.5, 25, 50 and 100μM) were treated into grown cell (1×10⁴ cells/well) and the plates were incubated for 24 hours at 37°C in order to further application.

Cell viability assay

The cell viability was evaluated by using methyl thiazolyl tetrazolium bromide (MTT, Sigma, USA) assay [12]. Briefly, 10μl of the MTT [3-(4, 5-dimethylethiazol-2, 5 diphenyl tetrazolium bromide] solution (5 mg/ml in PBS) was added to each well. The plates were incubated for 4 h at 37 °C. Then, for solubilization of the MTT crystals, 100μL of isopropanol (Merck, Germany) was added to the wells. Finally, the optical density of each well was determined against blank reagent with a multi-well scanning spectrophotometer (ELISA reader, Organon Teknika, Netherlands) at a wavelength of 570nm. The Percentage of cell viability related to control (untreated) was calculated by [A]test/[A]control×100. Where [A]test is the absorbance of the test sample and [A]control is the absorbance of control sample [13]. In addition, optical density (OD) value was determined to percentage of viability by using equation 1.

Viability (%) = (OD value of test samples / OD value of test controls) × 100 (Equation 1).

RNA isolation and cDNA Synthesis:

The PC-3 cell line was seeded into 6 well plates (5×10⁴ cells/well) and incubated for 24 hours, and afterwards, the cells were treated with imatinib for another 24 hours. Total cellular RNA was extracted from the untreated and treated cells using the RNA-isolation kit (Qiagen, RNeasy Plus Mini Kit 50) according to the manufacturer’s instructions. The concentration and purity of the isolated RNA were performed by using a photonanometer (IMPLEN, Germany) RNA isolated with 260/280nm absorption ratio>1.8 was subjected to study. Complementary DNA synthesis was performed using PrimeScript™ 1st strand cDNA Synthesis
Kit (Takara, China) in 20 μl reaction mixture containing 3 μl of total RNA (1 μg), 4 μl PrimeScript™ Buffer 5x, 1μl of dNTP mix (10mM), 1 μl oligo dT Primer (50 μM), 1μl Random 6 mers (50 μ M), 1 μl RNase inhibitor (40 units), 1 μl PrimeScript™ RTase (200 units) and 8 μl nuclease-free water. Thermal cycling was incubated in the ABI 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) for 10 minutes at 30 °C, for 50 minutes at 42 °C (cDNA synthesis) and 5 minutes at 95 °C (reverse transcriptase inactivation) followed by cooling on ice for 5 min.

Agarose gel electrophoresis

In order to performed electrophoresis, quantity of 5μl of 100 bp ladder (Fermentase- Germany) as well as each one of real time PCR products were transferred into the well installed in 1% Agarose Gel. After staining the gel with ethidium bromide, fragments were visualized by trans-laminator system (UV dock, England) so that the formed bands are to be observed.

Real-time-PCR

A SYBR Green real-time PCR was carrying out on cDNA extracted from cell after treatment with imatinib. The expression of target gene was quantified with SYBR Green PCR Master Mix using an ABI 7300 real-time PCR system (Applied Biosystems, USA). After denaturation at 95 °C for 10min, 40 cycles were followed by 95 °C for 15s and 60°C for 1 min. Each PCR amplification reaction was performed in 20 μl reaction mixture containing 10 μl Power SYBR Green PCR Master Mix (2x), 2 μl cDNA sample (100 ng), 1 μl of each primer (0.4μM), and 6 μl double-distilled water. In this study, two pairs of primers were applied: one pair to amplify Bcl-2 gene as target, the other for the reference gene, GAPDH (Table1). Primers were designed using primer express software, v.3.0 (Applied Biosystems,USA). Primer specificity was tested utilizing BLAST program (http://www.ncbi.nlm.nih.gov/blast). The gene expression was calculated using comparative threshold cycle (ΔΔCt). Thereafter, mean threshold cycle (mCt) value of reference gene (GAPDH) was subtracted from mCt value of the target genes (Bcl-2) to Obtain ΔCt and ΔΔCt values of each sample were calculated from corresponding Ct values; where ΔΔCt = [mCt target – mCt GAPDH] (treated sample) - [mCt target – mCt GAPDH] (untreated sample). Finally, Bcl-2/GAPDH gene expression ratio was calculated using the ratio formula (Ratio = 2 ^-ΔΔCt).

Statistical Analysis

All experiments were done in duplicate, and the results were presented as mean ± standard deviation. The experimental data were analyzed by using SPSS. The P-value of <0.05 was considered statistically significant for one way ANOVA analysis.
Results

**MTT assay results**

Different concentrations of imatinib have cytotoxicity effect on cancer cell line at 24h. At concentration of 3, 5, 12.5, 25, 50, 100μM of imatinib, the viability of PC-3 was reduced to 93.73±0.02, 65.65±0.07,(statistically insignificant, \( p>0.05 \)), 33.13 ± 0.03 (\( p<0.05 \)), 31.51± 0.01(\( p<0.05 \)), 24.84±0.005(\( p<0.05 \)) and 9.6 ± 0.002% (\( p<0.001 \)), respectively (Figure1). The mean viability percentage after treatment with imatinib was significantly decrease in prostate cancer cells at a concentration of 5μM and higher. The IC\(_{50}\) value was considered as the concentration of the imatinib that caused a 50% decrease in cell viability relative to the control which was constituted by cell media and isopropanol without imatinib. The IC\(_{50}\) of imatinib on prostate cancer PC-3 was calculated 29.53μM for 24h.

**Amplification and melting curve analysis**

The melting curve analysis was performed based on the temperature (x axis) and dF/dT derivation (y axis). The reproducibility of a melting curve is high with a standard deviation of only 0.1°C between runs. It was generated to screen for primer dimmers and to document single product formation for each gene. Each valid amplification reaction revealed a single peak at expected Tm. The melting peaks have been drawn at 86.01 and 86.4°C for Bcl-2 and GAPDH genes. The amplification curves and melting curves of Bcl-2 and GAPDH gene expression acquired from real time PCR analysis was shown in Figure 2. Furthermore, Gel electrophoresis analysis of PCR products showed specific amplification sequence of interest (Figure 3).

**Relative quantification analysis using amplification plots**

The relative gene expression between two samples (treated and untreated) can be determined by the difference in their Ct values of exponential phase. The mCt, mΔCt and ΔΔCt value for GAPDH and Bcl-2 genes were calculated in IC\(_{50}\) concentration of imatinib. The Bcl-2/GAPDH gene expression ratio equals to \( 2^{-\Delta\Delta Ct} \). The calculated \( 2^{-\Delta\Delta Ct} \) values were 0.27±0.01 (\( p<0.05 \)) for Bcl-2 in 29.53μM of imatinib (Figure.4).

Discussion

Tyrosine kinases Inhibitors (TKIs) are promising anticancer agents that often induce apoptosis and slow progression growth of experimental bone metastases in neoplastic cells. Imatinib is the first successful member of new class of TKIs that act by inhibiting specific tyrosine kinases like Bcr-Abl fusion oncoprotein in CML, c-Kit in gastrointestinal stromal tumors (GIST) and is
currently under evaluation in clinical trials for prostate cancer, malignant gliomas, ovarian cancer and carcinoid tumor [14,23]. Imatinib interferes with a specific molecular target involved in tumor growth and progression. These targets include growth factor receptors, cell-cycle proteins, signaling molecules, modulators of apoptosis, and molecules involved in invasion and angiogenesis, which are essential for development and homeostasis in normal tissues. In other investigations, the \textit{in-vitro} cytotoxic effects of imatinib on various cancer cell lines have been reported [15]. In the present work, we examined the cytotoxic effect of imatinib on the prostate cancer (PC-3) by MTT assay. Treatment of PC-3 with Imatinib induced the morphological changes that verify the MTT assay by changing in morphological cell appearance which reveal the increase in apoptotic cell population. Apoptosis is controlled by pro-apoptotic and anti-apoptotic effectors that involve a wide variety of cellular proteins. The product of the Bcl-2 family gene serves as critical regulators of pathways involved in apoptosis, acting to either promoting cell survival (for example BCL2, BCL-XL, BCL-W, A1 and Mcl-1) or promote cell death (for example BAD, BAK, BAX, BCL-XS and BIK), and go through homo- and hetero dimerization, rely upon their expression levels [16]. Bcl-2 protein inhibit the release of cytochrome c from mitochondria elicited by Bax proteins, resulting in inhibition of apoptotic death [17]. Many researchers exhibited that imatinib induce apoptosis in several human solid tumors, including thyroid cancer[18], small cell lung cancer [19] ovarian, breast cancer, glioblastoma, dermatofibrosarcoma protruberans [20] and demonstrated that BCL2, BCL-XL, BAD and BAX gene expression were differentially regulated by imatinib [21]. Similar study was demonstrated that imatinib can be down regulated expression of antiapoptotic proteins, including Bcl-xl, Bcl-2, and Mcl-1 in human K562, c-KIT-dependent Gastrointestinal stromal tumor and cholangiocarcinoma cancer cell lines [22]. To evaluate if imatinib treatment also results in apoptosis induction, our results showed that imatinib induces a programmed cell death mediated by down regulation of anti apoptotic Bcl2 gene expression on PC3 Cell line. Therefore, imatinib remains a promising candidate for the treatment of prostate cancer in the future.

\textbf{Conclusion}

Based on the result obtained from our study, imatinib induced the down regulation of \textit{Bcl}-2 expression, an anti-apoptotic protein, in prostate cancer PC-3 cells. Thus, it may be a good candidate for utilize as an inhibitor of the growth of cancer cells \textit{in-vivo} and the treatment of prostate cancer. However, for a definitive conclusion, the results presented here warrant further investigation on other cell lines as well as animal tumor models and subsequent clinical studies.
Imatinib induces down-regulation of Bcl-2

References


Imatinib induces down-regulation of Bcl-2


Table 1. Primers of Bcl-2 and GAPDH genes used for real time PCR analysis.

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bcl-2</strong></td>
<td>Forward: 5'-TGTGGATGACTGAGTACCTGAACC-3'</td>
</tr>
<tr>
<td></td>
<td>Revers: 5'-CAGCCAGGGAGAAATCAAACAGAG-3'</td>
</tr>
<tr>
<td><strong>GAPDH</strong></td>
<td>Forward: 5'-CGTCTGCGGCTATCAACTTTTG-3'</td>
</tr>
<tr>
<td></td>
<td>Revers: 5'-CGTCTGCGGCTATCAACTTTTG-3'</td>
</tr>
</tbody>
</table>

Figures:

**Figure 1.** Cell viability assay of prostate cancer PC-3 cells after treatment with Imatinib within 24 h. The data was expressed as the mean±SD from 3 independent experiments.
Figure 2. (A) Amplification (B) and Melting curve analysis for PCR product obtained with the specific primer pairs for Bcl-2 and GAPDH genes. NTC: non-template control.
Imatinib induces down-regulation of Bcl-2

Figure 3. Gel electrophoresis analysis of the PCR products. Lane 1: NTC for Bcl-2 gene. Lane 2: 122 bp PCR product of Bcl-2 gene. Lane 3: 102 bp PCR product of GAPDH gene. Lane 4: NTC for GAPDH gene. NTC: non-template control. M: Molecular Size marker -100bp ladder

Figure 4. The impacts of imatinib to expression of Bcl-2 mRNA levels in PC-3 cells. The expression of mRNAs was analyzed by Real-time PCR and normalized by GAPDH expression. P-value of <0.05 versus control group (one-way ANOVA analysis followed by the Student’s t-test)

Received: October 19, 2014; Published: December 2, 2014