ORIGINAL ARTICLE

Imatinib-induced apoptosis: a possible link to topoisomerase enzyme inhibition

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SUMMARY

What is known and Objective: **Imatinib is a specific BCR/ABL inhibitor, commonly used for the treatment of chronic myeloid leukaemia (CML), a hematological malignancy resulting from a chromosomal translocation that generates the BCR/ABL fusion protein. Recent studies showed that the imatinib has cytotoxic and apoptotic effects on many BCR/ABL-negative cancers. Numerous compounds with cytotoxic potential exert their functions by interfering with the DNA topoisomerase. In this study, we examined the effects of imatinib on tumour cell-killing in relation to DNA topoisomerase enzyme inhibition.**

Methods: **We determined the cytotoxicity by cell proliferation assay (XTT; tetrazolium hydroxide), using the human K562 CML cells, and loss of mitochondrial membrane potential by monitoring the changes in caspase-3 enzyme activity. Type I and II topoisomerase activities were measured by supercoiled plasmid relaxation and minicircle DNA decatenation assays respectively.**

Results and Discussion: **Imatinib-induced apoptosis and inhibited cell proliferation in a dose-dependent manner. We also found that the imatinib was effective in both type I and type II topoisomerase reactions to a varying degree between 94% and 7% for the concentration range of 1 mM–0·02 mM in a dose-dependent manner.**

What is new and Conclusion: **Our results suggest that the inhibition of topoisomerases may be a significant factor in imatinib-induced apoptosis in CML.**

Keywords: apoptosis, BCR/ABL, chronic myeloid leukaemia, imatinib, topoisomerase

WHAT IS KNOWN AND OBJECTIVE

Chronic myeloid leukaemia (CML) is a hematopoietic stem cell disorder, characterized by an increased number of immature white blood cells (1, 2). CML is diagnosed by the presence of BCR/ABL oncogene, resulting from a reciprocal translocation between the long arms of chromosomes 9 and 22, and formation of the diagnostic Philadelphia chromosome (Ph). The *BCR*/ABL hybrid gene encodes a fusion protein with constitutive tyrosine kinase activity that induces leukaemogenesis (3). Imatinib is the first target-specific anticancer agent for the treatment of cancers including Ph-positive CML and gastrointestinal stromal tumours (4). It specifically recognizes and binds to the ATP binding site of BCR/ABL and inhibits its activity (5). Inhibition of BCR/ABL activity modulates the expression of several genes involved in the regulation of cell growth, proliferation, apoptosis, cell cycle, cell adhesion and differentiation (6).

Platelet-derived growth factor receptors (PDGFR), colony stimulating factor 1 receptor (CSF1R) and c-KIT are also the targets of imatinib. These molecules are highly expressed in many BCR/ABL-negative cancer types such as dermatofibrosarcoma protuberans (DFSP), melanoma, chordoma and aggressive fibromatosis, and gastrointestinal stromal tumours (GIST). In GIST, imatinib

blocks the activity of mutant or wild-type c-KIT tyrosine kinase (7, 8). We have previously reported that imatinib induces apoptosis by increasing the intracellular concentrations of apoptotic ceramides via generation and accumulation of ceramides by upregulating ceramide synthase genes and down-regulating the anti-apoptotic sphingosine kinase-1 gene (9). We have evidence indicating that imatinib induces autophagy and programmed cell death-II, by increasing the expression levels of Atg5, Atg6 and LC-3 genes in a dose-dependent manner (unpublished data). However, the mechanisms of imatinib-induced apoptosis in BCR/ABL-negative cancers are yet to be clarified.

Numerous naturally occurring and synthetic compounds with cytotoxic and anticancer properties interfere with the normal DNA topoisomerase functions (10–16) and DNA-protein interactions (17). Topoisomerases are essential enzymes playing important roles in many genetic processes, including the DNA replication, transcription, recombination and transposition (18). There are two types of topoisomerases; Type I topoisomerases (topo I) make a single-stranded break in a DNA duplex, mediate passage of the intact strand through the break, and then reseal it. Type II topoisomerases, on the other hand, create transient breaks in both the strands of a duplex, pass an intact DNA segment through the break and then reseal the cleavage site (19, 20). All known type II topoisomerases require ATP for their catalytic activities in topoisomerization reactions.

In this study, we have determined the cytotoxicity and loss of mitochondrial membrane potential using imatinib. As topoisomerases have been recognized as an effective target for the development of chemotherapeutics, we extended our research to cover the mammalian DNA topoisomerase assays for assessing the biological activities of imatinib.

METHODS

Cell culture

Human K562 CML cells were obtained from German Collection of Microorganisms and Cell Cultures and maintained in RPMI1640 medium containing 10% foetal bovine serum and 1% streptomycin–penicillin at 5% CO_2 incubator at 37 °C (NuAire Laboratory Equipment Supply, MN, USA).

XTT Cell proliferation assay

Cell proliferations were determined by XTT cell proliferation assay (Biological Industries, Kibbutz Beit Haemek, Israel) as described (21). 2×10^4 cells were grown in a 96-well plates containing 200 μL growth medium per well in the absence or presence of increasing concentrations of imatinib and incubated at 37 °C in 5% CO_2 incubator. Following 72 h incubation, 50 μL XTT reagent was added to each well and then incubated for another 4 h. Finally, the absorbance was measured by ELISA reader under 490 nm wavelength of light (Thermo Electron Corporation Multiskan Spectrum, Vantaa, Finland).

Detection of apoptosis by caspase-3 enzyme activity and mitochondrial membrane potential (MMP)

Changes in caspase-3 enzyme activity in response to imatinib were measured by caspase-3 colorimetric assay as described (R&D Systems, Minneapolis, USA) (21). Briefly, the K562 cells were treated with different concentrations of imatinib and incubated for 72 h. The cells were collected by centrifugation at 800 g for 10 min. The pellets were lysed on ice for 10 min, centrifuged at 10 000 g for 1 min and supernatants were transferred into a new tube. To determine caspase-3 enzyme activity, 50 μL of reaction buffer (5X), 5 μL of caspase-3 colorimetric substrate, and 50 μL of the samples were applied to the cell suspensions in 96-well plate and incubated for 2 h at 37 °C in 5% CO_2 incubator. Absorbance of the samples was read under 405 nm wavelength of light via the ELISA (Thermo Electron Corporation Multiskan Spectrum, Vantaa, Finland). Protein concentrations were determined by Bradford assay and caspase-3 enzyme activity levels were normalized to protein concentrations.

Loss of mitochondrial membrane potential (MMP)

The mitochondrial membrane potential was detected by JC-1 MMP kit as described by the manufacturer (Cell Technology, CA, USA). K562 cells were treated with imatinib as described above. The pellets were collected by centrifugation at 800 g for 10 min and then treated with 500 μL of JC-1 dye before their incubation at 37 °C in 5% CO_2

for 15 min. These mixtures were centrifuged at 800 **g** for 5 min, and then mixed with the 2 mL of blank media. After centrifugation at 800 **g** for 5 min, the pellets were dissolved in 500 µL of blank media and 150 µL from each sample was transferred into 96-well plates. The aggregate red form of the dye which is found in inner mitochondria has absorption/emission maxima of 585/590 nm, and the monomeric green form of the dye which was released to the cytoplasm due the loss of the MMP has absorption/emission maxima of 510/527 nm. The plate was read at these wavelengths by fluorescence Elisa reader ELISA (Thermo Electron Corporation Multiskan Spectrum, Vantaa, Finland). Finally, green/red (510/585) values were calculated to determine the changes in MMP.

Topoisomerase I supercoiled plasmid relaxation assay

Supercoiled plasmid relaxation assays were carried out as previously described (14, 22). Briefly, 20 µL of reaction mixture contained 500 ng of superhelical circular (sc) plasmid DNA, one unit of calf thymus topo I (TAKARA, Otsu-Shiga, Japan) in the presence or absence of the Imatinib in the reaction buffer (35 mM Tris–HCl (pH 8·0), 72 mM KCl, 5 mM MgCl$_2$, 5 mM DTT, 5 mM spermidine and 0·1% bovine serum albumin). Relaxation products were separated on 1% agarose gels in TBE buffer (45 mM Tris–Borate and 1 mM EDTA, pH 8·0) in a horizontal electrophoresis apparatus (5 V/cm) (Thermo EC250, Waltham, MA, USA). Electrophoresis was continued until the bromophenol blue dye had migrated 75% down the gel and photographed under UV light after staining in ethidium bromide (Etd-Br) solution (0·5 µg/mL). The relationship between the binding of Etd-Br and the amount of fluorescence given by sc and relaxed DNA (rlx DNA) under UV light was carried out as described (23, 24). DNA bands were quantified from gel photo images using BioRad Multianalyst (ver. 1·1). Average band intensities were calculated from three independent reactions.

Topoisomerase II decatenation assays

Decatenation assays were carried out using 0·2 µg kinetoplast DNA (K-DNA) substrate in a final volume of 20 µL in topo II reaction buffer (50 mM Tris–Cl, pH 8·0, 120 mM KCl, 10 mM MgCl$_2$. 0·5 mM ATP, 0·5 mM dithiothreitol) (25). Reactions were terminated with stop buffer (5% sarkosyl, 0·0025% bromophenol blue, 25% glycerol) and electrophoretic analyses of K-DNA were performed using standard agarose gel containing 0·5 µg Etd-Br/mL. Resolution of decatenated DNA products were monitored as stated above except the gels were destained in water prior to the photography. Etoposide, a well-known topoisomerase II inhibitor was used as a control.

RESULTS AND DISCUSSION

Imatinib inhibits proliferation of K562 cells in a dose-dependent manner

We examined the proliferation rates of K562 cells in response to increasing concentrations of imatinib for 72 h by XTT assay. The results revealed that there were 9%, 14%, 22%, 27%, 35% and 87% decreases in cell proliferation in response to 0·5, 5, 50, 100, 200 and 600 nM imatinib respectively, as compared with untreated controls. We could not detect any significant change in the proliferation of K562 cells in response to 0·1 nM imatinib as compared with the untreated controls whereas 600 nM imatinib showed the highest anti-proliferative effect (Fig. 1). IC50 (drug concentrations that
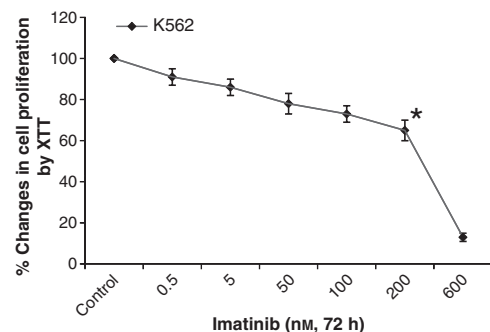

Fig. 1. Effects of imatinib on the growth of K562 cells. The IC50 concentration of imatinib was calculated from cell proliferation plots. The XTT assays were performed using triplicate samples in at least two independent experiments. The error bars represent the SD, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using ANOVA, and (P < 0·05) was considered significant. [Correction made on 12 October 2011, figure 1 has been replaced with the corrected figure.]

inhibits 50% of cell proliferation) value of imatinib, calculated from cell proliferation plots was 310 nм (Fig. 1). [Corrections added on 12 October 2011, after online publication: The sentence 'The results revealed that there were 6%, 12%, 16%, 26%, 80% and 84% decreases in cell proliferation in response to 0·1, 1, 10, 100, 500 and 1000 nм imatinib respectively, as compared with untreated controls (Fig. 1)' was corrected to 'The results revealed that there were 9%, 14%, 22%, 27%, 35% and 87% decreases in cell proliferation in response to 0·5, 5, 50, 100, 200 and 600 nм imatinib respectively, as compared with untreated controls', '500 nм imatinib' was corrected to '600 nм imatinib' and 280 nм was corrected to 310 nм.]

Imatinib induces apoptosis in a dose-dependent manner

To examine the apoptotic effects of imatinib on K562 cells, we incubated the cells with four different concentrations of imatinib and examined the changes in caspase-3 enzyme activity and loss of mitochondrial membrane potential. We choose IC50 and lower concentrations of imatinib (50, 150, 250 and 300 nм) to assess the strength of apoptotic effects of imatinib. The results revealed that incubation of K562 cells in the presence of 50, 150, 250 and 300 nм imatinib resulted in 13%, 37%, 40% and 54% increases in caspase-3 enzyme activity compared with the untreated control group (Fig. 2), To confirm these results, we examined the changes in MMP and observed that the same doses of imatinib caused 14%, 28%, 46% and 51% increases in cytoplasmic/monomeric JC-1 relative to the untreated control cells (Fig. 3). The observed differences seen with both caspase-3 enzyme activity and mitochondrial membrane potential were statistically significant ($P > 0·05$). [Corrections added on 12 October 2011, after online publication: 'incubated the cells with two different concentrations of imatinib' was corrected to 'incubated the cells with four different concentrations of imatinib', the sentence 'We choose IC50 dose, 280 nм and 10 nм to assess the strength of apoptotic effects of imatinib' was corrected to 'We choose IC50 and lower concentrations of imatinib (50, 150, 250 and 300 nм) to assess the strength of apoptotic effects of imatinib', the sentence 'The results revealed that incubation of K562 cells in the presence of 10 and
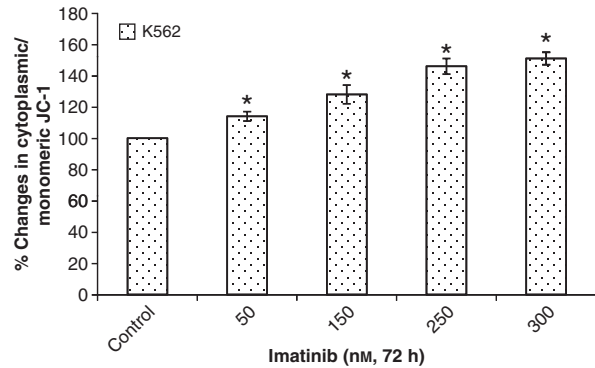

Fig. 2. Percent changes in caspase-3 enzyme activity in imatinib exposed K562 cells. The results are the means of two independent experiments. The error bars represent the SD. Statistical significance was determined using ANOVA, and ($P < 0·05$) was considered significant. [Correction made on 19 January 2011, figure 2 has been replaced with the corrected figure.]

280 nм imatinib resulted in 27% and 58% increases in caspase-3 enzyme activity compared with the untreated control group (Fig. 2) was corrected to 'The results revealed that incubation of K562 cells in the presence of 50, 150, 250 and 300 nм imatinib resulted in 13%, 37%, 40% and 54% increases in caspase-3 enzyme activity compared with the untreated control group (Fig. 2) and '31% and 50% increases' was corrected to '14%, 28%, 46% and 51% increases']
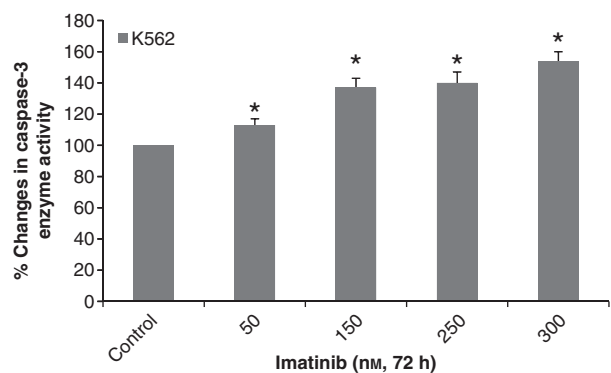

Fig. 3. Percent changes in cytoplasmic/monomeric JC-1 in imatinib treated K562 cells. The results are the means of two independent experiments. The error bars represent the SD. Statistical significance was determined using ANOVA, and ($P < 0·05$) was considered significant. [Correction made on 12 October 2011, figure 3 has been replaced with the corrected figure.]
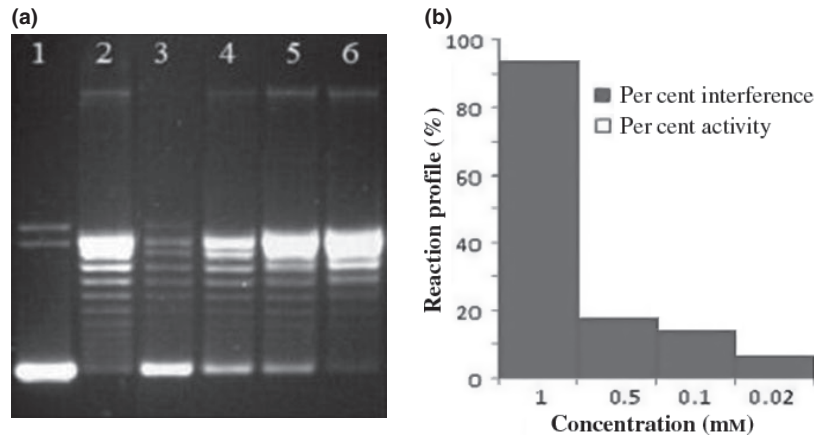
Fig. 4. The effect of imatinib on mammalian DNA topoisomerase I activity. (a) A representative agarose gel photograph of supercoil relaxation in the presence of varying concentrations of imatinib; lane 1, pBR322; lane 2, pBR322 with 1 U of DNA topoisomerase I; lanes 3–6, same as lane 2 plus 1 mM, 0·5 mM, 0·1 mM and 0·02 mM Imatinib respectively. (b) Quantitative assessment of the reaction profile corresponding to samples from lanes 3 to 6 (see 'Materials and methods' for the details).

Imatinib interferes with both type I and type II topoisomerase reactions

We carried out plasmid DNA nicking and DNA decatenation assays to monitor the effect of imatinib on topoisomerase I and topoisomerase II reactions respectively. Topoisomerase I assay employs supercoiled plasmid DNA (sc DNA) and relies on the ability of the enzyme to convert sc DNA to its relaxed form, which can be separated as discrete bands using gel electrophoresis. An inhibition of relaxation activity due to the presence of a particular inhibitor was monitored in the form of an accumulated faster-migrating sc DNA. On the other hand, topoisomerase II assay was based on the decatenation of K-DNA, minicircles from the insect trypanosome *Crithidia fasciculata* (25). As K-DNA is an aggregate of interlocked DNA mini-circles, resulting high molecular weight would prevent the substrate population from entering the gel, unless the K-DNA is decatenated by the enzyme, topoisomerase II. Inclusion of Etd-Br in the gel enabled us to monitor open-circular monomers and covalently closed circular DNA bands.

A representative assay using various concentrations of imatinib is given in Fig. 4. As seen in Fig. 4A, sc plasmid substrate, pBR322 (Fig. 4A, lane 1) is relaxed by topo I (Fig. 4A, lane 2) in the absence of imatinib. However, the reaction was profoundly affected by 1 mM imatinib (Fig. 4A,

lane 3). The interference by imatinib gradually decreased upon the dilution to imatinib to 0·5 mM and 0·1 mM (Fig. 4A, lanes 4 and 5 respectively). This effect completely disappeared when the concentration of imatinib was lowered to 0·02 mM (Fig. 4A, lane 6). At this level, only a very faint sc DNA band with an accumulated lower migrating relaxed DNA (rlx DNA) was seen. Densitometric calculations of relative band intensities showed that the interference was 94% at 1 mM imatinib concentration and this value gradually decreased
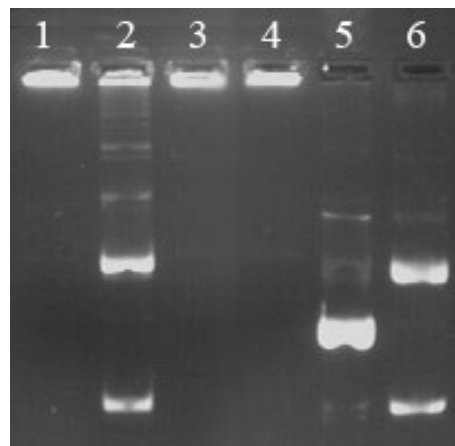

Fig. 5. Decatenation assays by topoisomerase II in the presence or absence of imatinib. Lane 1, K-DNA; lane 2, K-DNA with 1 U of DNA topoisomerase II; lane 3, same as lane 2 plus 0.5 mM etoposide; lane 4, same as lane 2 plus 1 mM Imatinib; lane 5, linear DNA marker; lane 6, decatenated DNA marker.

to 18%, 14% and 7% upon serial dilutions (Fig. 4B). Residual nicked circular DNA present in the plasmid preparation was taken into account in the quantitative evaluations. We next employed the decatenation assays by topoisomerase II in the presence or absence of imatinib (Fig. 5). As seen in Fig. 5, the K-DNA was too large to enter the gel (Fig. 5, lane 1) whereas topoisomerase II yielded monomeric DNA rings of two species, OC-DNA and CCC-DNA (Fig. 5, lane 2). The reaction was completely abolished in the presence of etoposide (Fig. 5, lane 3). Likewise, 1 mM imatinib interfered with the decatenating activity of topoisomerase II (Fig. 5, lane 4) leaving catenated DNA molecules. The last two lanes indicate the linear (Fig. 5, lane 5) and catenated DNA (Fig. 5, lane, 6) markers.

Our results revealed that imatinib-induced apoptosis and inhibited cell proliferation in a dose-dependent manner. Moreover, we also showed that the imatinib inhibited topoisomerase I and II enzymes activities in a dose-dependent manner, *in vitro*. The pharmacological significance of topoisomerases in anti-cancer drug development is well-known (10–16). However, the compounds targeting these enzymes are structurally different, which suggests that there is not a unique mechanism for topoisomerase inhibition. Several studies in mammalian cells, employing cytotoxic agents with topoisomerase-targeting properties, suggest that the cellular responses were related to the formation of a drug/enzyme/DNA cleavable complex. Such a cleavable complex is formed, not through the inhibition of the catalytic activity of topoisomerases but through a mechanism involving the DNA damage (19). All known topoisomerase-targeting drugs, specifically and reversibly block the DNA rejoining, and results in the trapping of a covalent enzyme/DNA intermediate (26, 27). The reversibility of the damage is a main difference between the actions of imatinib on topoisomerase reactions and apoptosis. Although our results showed that the imatinib promotes apoptosis through increasing caspase-3 enzyme activity and loss of mitochondrial membrane potential as well as interfering with the topoisomerase activities as shown in Figs 4 and 5, the concentrations used in two assays were different. As in the former test, an irreversible-damage is assessed, the concentrations of imatinib, expected to inhibit topoisomerase reactions and apoptosis, would not be expected to be the same.

WHAT IS NEW AND CONCLUSION

Unlike type I enzymes, identification of topoisomerase II-targeting chemicals is relatively recent. Our results demonstrate that imatinib blocked the decatenation activity of topo II, thereby suggesting a possible mechanism for imatinib-induced tumour cell-killing.

ACKNOWLEDGEMENTS

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