Imatinib (STI571) Inhibits DNA Repair in Human Leukemia Oncogenic Tyrosine Kinase-Expressing Cells

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BCR/ABL oncogene, as a result of chromosome aberration t(9;22), is the pathogenic principle of almost 95% of human chronic myeloid leukemia (CML). Imatinib (STI571) is a highly selective inhibitor of BCR/ABL oncogenic tyrosine kinase used in leukemia treatment. It has been suggested that BCR/ABL may contribute to the resistance of leukemic cells to drug and radiation through stimulation of DNA repair in these cells. To evaluate further the influence of STI571 on DNA repair we studied the efficacy of this process in BCR/ABL-positive and -negative cells using single cell electrophoresis (comet assay). In our experiments, K562 human chronic myeloid leukemia cells expressing BCR/ABL and CCRF-CEM human acute lymphoblastic leukemia cells without BCR/ABL expression were employed. The cells were exposed for 1 h at 37 °C to *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) at 5 μM, mitomycin C (MMC) at 50 μM or to γ-radiation at 15 Gy with or without a 24 h preincubation at 1 μM of STI571. The MTT cells survival after 4 days of culture showed that STI571 enhanced the cytotoxity of the examined compounds in the K562 line. Further it was found, that the inhibitor decreased the efficacy of DNA repair challenged by each agent, but only in the K562 expressing BCR/ABL. Due to the variety of DNA damage induced by the employed agents in this study we can speculate, that BCR/ABL may stimulate multiple pathways of DNA repair. These results extend our previous studies performed on BCR/ABL-transformed mouse cells onto human cells. It is shown that BCR/ABL stimulated DNA repair in human leukemia cells. In conclusion we report that STI571 was found to inhibit DNA repair and abrogate BCR/ABL-positive human leukemia cells therapeutic resistance.

Key words: BCR/ABL, Drug Resistance, Comet Assay

Introduction

The constitutively activated BCR/ABL oncogenic kinase as a result of reciprocal chromosomal translocation between chromosomes 9 and 22 [t(9;22)(q34;q11)] plays a critical role in the pathogenesis of chronic myeloid leukemia (CML). BCR/ABL-dependent phosphorylation of multiple downstream target proteins may result in the activation of mitogenic cellular pathways and therapeutic resistance (Daley *et al.*, 1990; Lugo *et al.*, 1990; Sattler and Salgia, 1998). The induction of G2/M cell cycle delays, elevation of the anti-apoptotic Bcl-xL protein and activation of DNA repair are suggested to be involved in the BCR/ABL drug resistance phenomenon (Lugo *et al.*, 1990; Druker *et al.*, 1996; Sattler and Salgia, 1998; Grumbach *et al.*, 2001; Nagar *et al.*, 2002, 2003). The first-line drug in the treatment of BCR/ABL-positive leukemias is STI571, which selectively inhibits oncogenic kinase activity.

Our recent findings indicated that the repair of DNA-induced lesions might be essential for drug resistance of BCR/ABL-positive cells (Slupianek *et al.*, 2001, 2002; Majsterek *et al.*, 2002, 2003, 2004; Hoser *et al.*, 2003). Previously, we reported the facilitation of homologous recombination repair (HRR) in murine pro-B lymphoid BaF3 mouse cells expressing BCR/ABL, but our latest findings indicated that multi-pathway of DNA repair may be involved in this event (Slupianek *et al.*, 2002; Majsterek *et al.*, 2004). To expand our studies, we examined the kinetics of DNA repair in human CML-derived leukemic K562 cells expressing BCR/ABL exposed to various DNA damaging agents: mitomycin C (MMC), *N*-methyl-*N*'-nitro-

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N-nitrosoguanidine (MNNG) and γ-radiation. We also studied the effect of STI571 on DNA repair kinetics in K562 cells. In order to compare the influence of STI571 on leukemia cells, which are not expressing BCR/ABL, we examined the efficiency of DNA repair in human acute lymphoblastic leukemia cells CCRF-CEM exposed to hydrogen peroxide.

The exposure of a cell to γ-irradiation may result in base damage and/or single- (SSBs) and double-strand breaks (DSBs) due to direct interaction or generation of free radicals (Parshad and Sanford, 2001). The majority of DSBs induced by γ-irradiation is repaired by HRR and non-homologous end-joining (NHEJ) mechanisms (Caldecott, 2001; Khanna and Jackson, 2001). Base modifications are repaired by base excision repair (BER) (Caldecott, 2001; Lindahl, 2001). MNNG, a model direct-acting alkylating mutagen, is known to methylate all oxygen and most nitrogen atoms of DNA introducing missparing in a DNA molecule (Bignami *et al.*, 2000). Mismatch repair (MMR) pathway is capable to recover proper base paring to keep a DNA sequence in frame. In addition, MNNG has been demonstrated to generate reactive MNNG radicals and hydroxyl radicals that are capable of causing oxidative damage to DNA and induce single- and double-strand breaks in DNA (Mikuni and Tatsuta, 2002). Mitomycin C is an alkylating agent used in chemotherapy that mainly induces DNA-DNA intrastrand cross-links. Adducts of MMC are repaired by the nucleotide excision repair (NER) pathway (Aboussekhra and Wood, 1995). Therefore multiple pathways of DNA repair may be involved in the processing of DNA lesions induced by MMC, MNNG, and γ-radiation that required subsequent action at least three main pathways, NER, HRR and MMR. We investigated two different cell lines, BCR/ABL-positive K562 cells and BCR/ABL-negative CCRF-CEM cells, and found that STI571 might inhibit the DNA repair pathway selectively in cells that are expressing BCR/ABL.

Materials and Methods

Chemicals

STI571 (2-phenylaminopyrimidine) was kindly provided by Novartis Pharma (Basel, Switzerland); MNNG (*N*-methyl-*N*'-nitro-*N*-nitrosoguanidine), MMC (mitomycin C), Tris [2-amino-2-(hydroxymethyl)-1,3-propanediol], RPMI 1640 medium, agarose, low melting point agarose, phosphate buffered saline (PBS), DAPI (4′,6-diamidino-2-phenylindole) and fetal bovine serum (FBS) were obtained from Sigma Chemicals (St. Louis, MO, USA).

Cell culture and treatment

The K562 human chronic myeloid leukemia cells expressing BCR/ABL were obtained form Dr. Malgorzata Czyz (Medical University of Lodz, Lodz, Poland) and CCRF-CEM human acute lymphoblastic leukemia cells not expressing BCR/ABL were obtained from Dr. Blazej Rychlik (University of Lodz, Lodz, Poland). K562 or CCRF-CEM cells were maintained in RPMI 1640 medium supplemented with 10% FBS and an antibiotic mixture (the growth medium) at a final concentration of 1×10^5 cells/ml. The cells were treated for 1 h at 37 °C with 5 μM MNNG or 50 μM MMC, 10 μM hydrogen peroxide (H_2O_2) at 0 °C for 10 min or irradiated with 15 Gy in the growth medium with or without STI571 pre-incubation. γ-Radiation at a dose rate 0.017 Gy/s was carried out using a ^{60}Co source (Technical University of Lodz, Lodz, Poland). The incubation of cells in culture medium with STI571 at 1 μM was performed at 37 °C in an atmosphere of 5% CO_2 for 24 h. The control cells received only RPMI medium. The cell viability was evaluated by trypan blue exclusion.

Drug resistance

MMC and MNNG at a final concentration from 0.01 μM to 200 μM were added to the cells (1.5×10^6 ml^{-1}) in the growth medium or cells were exposed to γ-radiation from the range 2 Gy to 30 Gy with or without STI571 pre-incubation for 24 h at 1 μM. 4 d later the viability of cells was evaluated by the MTT assay. Briefly, cells were plated onto 96-well plates in 200 μl growth medium and 20 μl of 10 mg/ml MTT reagent were added to each well. After incubation at 37 °C for 4 h, the supernatant was removed and 200 μl of an aliquot solution containing 10% SDS and 0.04 M HCl were added to dissolve the water-insoluble formazan salt. One hour later, the difference ($OD_{650\,nm} - OD_{570\,nm}$) was measured with an ELISA reader (Bio-Rad). Drug and radiation resistance were presented as percentage of viable cells in suspension culture in comparison to the untreated control cells.

DNA repair

To examine DNA repair the cells (1.5×10^6 ml^{-1}) were treated with MNNG, MMC, H_2O_2 or γ-irradiated with or without pre-incubation with STI571, washed and re-suspended in a fresh, drug-free growth medium. Aliquots of cell suspensions were taken immediately (time 0) and 30, 60 and 120 min or 4, 16 and 24 h thereafter and placed on ice to stop the repair reaction. Cells exposed to 10 μM hydrogen peroxide for 10 min at 4 °C served as a positive control and negative control cells were incubated with growth medium only.

Comet assay

The comet assay was performed under alkaline conditions essentially according to the procedure of Singh *et al.* (1988) with some modification (Klaude *et al.*, 1996). A freshly prepared cell suspension in 0.75% low melting point agarose dissolved in PBS was placed onto microscope slides pre-coated with 0.5% normal melting agarose. The cells were then lysed for 1 h at 4 °C in a buffer consisting of 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM Tris, pH 10. After the lysis, the slides were placed in an electrophoresis unit, DNA was allowed to unwind for 40 min in an electrophoretic solution containing 300 mM NaOH, 1 mM EDTA, pH > 13. Electrophoresis was conducted at 4 °C (the temperature of the running buffer not exceeding 12 °C) for 30 min at an electric field strength of 0.73 V/cm (30 mA). The slides were then neutralized with 0.4 M Tris, pH 7.5, stained with 2 μg/ml DAPI and covered with cover slips. To prevent additional DNA damage all steps were conducted under dimmed light or in the dark.

The analysis of repair of cross-links generated by MMC was performed with modification according to McKenna *et al.* (2003). After lysis, the slides were irradiated with 10 Gy radiation at the dose rate 0.017 Gy/s using a ^{60}Co source to induce strand breaks. In each experiment, control slides were prepared with cells, which were not treated with MMC and subsequently received either no radiation (negative control) or 10 Gy radiation only (positive control). After lysis and irradiation, the slides underwent the standard procedure of comet assay described above.

Data analysis

The slides were examined at 200× magnification in an Eclipse fluorescence microscope (Nikon, Tokyo, Japan) attached to a COHU 4910 video camera (Cohu, Inc., San Diego, CA, USA) equipped with a UV filter block containing an excitation filter (359 nm) and barrier filter (461 nm) and connected to a personal computer-based image analysis system, Lucia-Comet v. 4.51 (Laboratory Imaging, Prague, Czech Republic). One hundred images were randomly selected from each sample and the % tail DNA (the % of DNA in comet tail) was measured. Two parallel tests with aliquots of the same sample were performed for a total of 200 cells and the mean % tail DNA was calculated. Each experiment was repeated in triplicate. The % tail DNA is positively correlated with the level of DNA breakage in a cell (Druker, 2003). A mean value of % tail DNA in a particular sample was taken as an indicator of DNA damage in this sample. In the experiment with MMC, tail DNA was expressed as % relative to damage induced by 15 Gy γ-radiation, indicated by the line at 100%.

Results

Fig. 1 (4-set) shows the percentage of the viability of K562 cells after a 4-days treatment with MNNG, MMC or γ-radiation with or without STI571 pre-incubation measured by the MTT assay. In each experiment the viability of cells taken to experiments before treatment was greater than 95% as evaluated by trypan blue exclusion. It was found that pre-incubation with STI571 at 1 μM caused cell killing over the level observed after MNNG, MMC or γ-radiation treatment alone, respectively. STI571 had no effect on the viability of CCRF-CEM cells.

Fig. 2 shows the DNA damage measured as % tail DNA in K562 cells exposed to MNNG (Fig. 2A) or γ-radiation (Fig. 2B), respectively, and analyzed immediately as well as 30, 60 and 120 min thereafter. We observed a decrease of comet tails in time. The K562 cells exposed to MNNG as well as γ-irradiated cells repaired DNA damage within 60 min ($P > 0.05$), while the cells pre-incubated with STI571 did not complete recover even 120 min thereafter ($P > 0.05$). The % tail DNA of the untreated K562 cells was constant, indicating that preparation and subsequent processing of the cells did not introduce significant damage to their DNA.

Table I presents the data of the % tail DNA of K562 cells exposed to MMC and analyzed immedi-

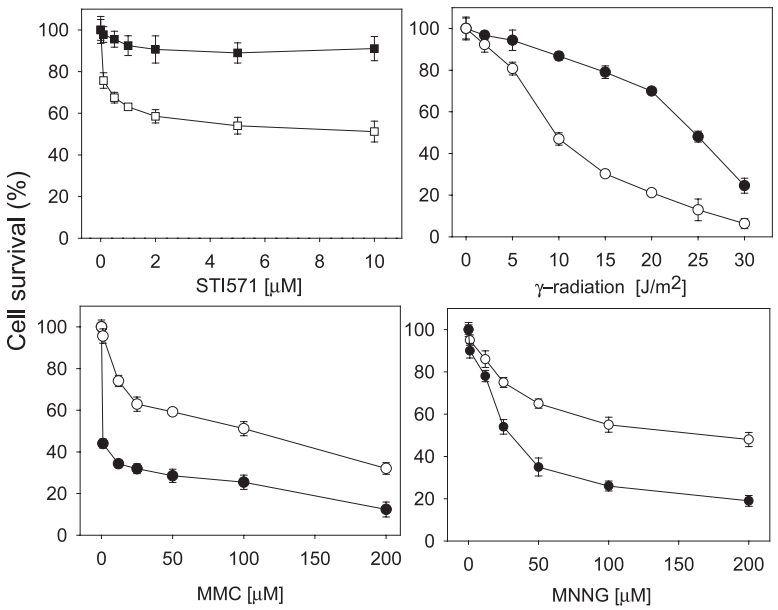

Fig. 1. The MTT percentage of K562 (□) and CCRF-CEM (■) cells survival under an increasing dose of STI571 and the K562 cells after γ-radiation, MMC and MNNG treatment at increasing doses after 4 days of incubation with (●) or without (○) STI571 pre-incubation at 1 μM. Results represent the mean of three independent experiments; error bars denote SEM.

ately as well as 24 h thereafter. After 24 h the MMC-induced DNA cross-links were sufficiently removed for the K562 cells to display comparable levels of damage to that shown by cells, which received post-lysis radiation only. After repair incubation of K562 cells showed growing tails as the repair time increases, however with STI571 pretreated K562 cells inhibited MMC-adducts excision. Values of % tail DNA were significantly higher for K562 cells as compared to their STI671-incubated counterparts (24 h; $P < 0.001$) and did not reach the level of controls received post-lysis radiation only.

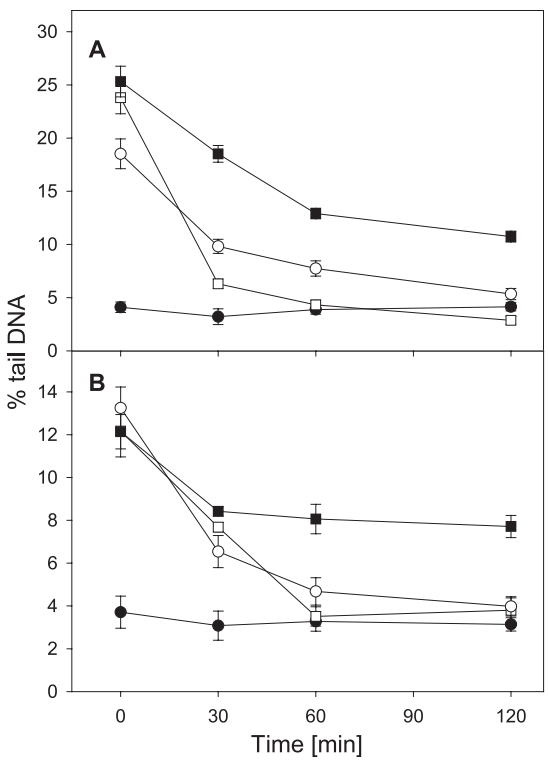

Fig. 2. Time course of DNA damage repair in K562 cells induced by 5 μM MNNG (A) or 15 Gy radiation (B) in the absence (□) or in the presence (■) of STI571 inhibitor at 1 μM compared to untreated controls (●). The positive controls were cells treated with hydrogen peroxide at 10 μM for 10 min on ice (○). DNA damage was evaluated as the percentage of DNA in the tail (% tail DNA) in the alkaline comet assay. The number of cells scored in each treatment was 100. Results represent the mean of three independent experiments; error bars denote SEM.

Fig. 3 shows the % tail DNA of K562 and CCRF-CEM cells exposed for 10 min at 0 °C to 10 μM hydrogen peroxide without or after pre-incubation with STI571 at 1 μM analyzed immediately as well as 30, 60 and 120 min thereafter. After pre-incubation with STI571, the K562 cells repaired DNA damage significantly slower (after 30 and 60 min; $P < 0.001$) (Fig. 3A). However, STI571 had no effect on DNA repair of CCRF-CEM cells exposed to H_2O_2 ($P > 0.05$) (Fig. 3B).

Table I. Data of DNA cross-links repair in K562 cells after MMC treatment at 50 μM as compared to K562 cells non-treated with MMC (positive control) in the absence or in the presence of STI571 inhibitor at 1 μM concentration. The cells were analyzed immediately or after 24 post-incubation repairs. After lysis sample cells and positive control cells were irradiated with 10 Gy to induce strand breaks. In each experiment, negative control was prepared with cells treated neither with MMC nor with γ-radiation. DNA damage was analyzed as the percentage of DNA in the tail (% tail DNA) in the alkaline comet assay. The number of cells scored in each treatment was 100. Results represent the mean of three independent experiments; ± denotes SEM.

	−STI571		+STI571	
Time [h]	0	24	0	24
Negative control (% tail DNA)	1.56 ± 0.25	1.89 ± 0.35	2.64 ± 0.30	2.82 ± 0.61
Positive control (% tail DNA)	39.50 ± 2.98	43.50 ± 3.34	46.17 ± 3.98	48.39 ± 3.34
Sample (% tail DNA)	8.15 ± 1.30	30.17 ± 1.93	9.67 ± 1.34	18.36 ± 1.38

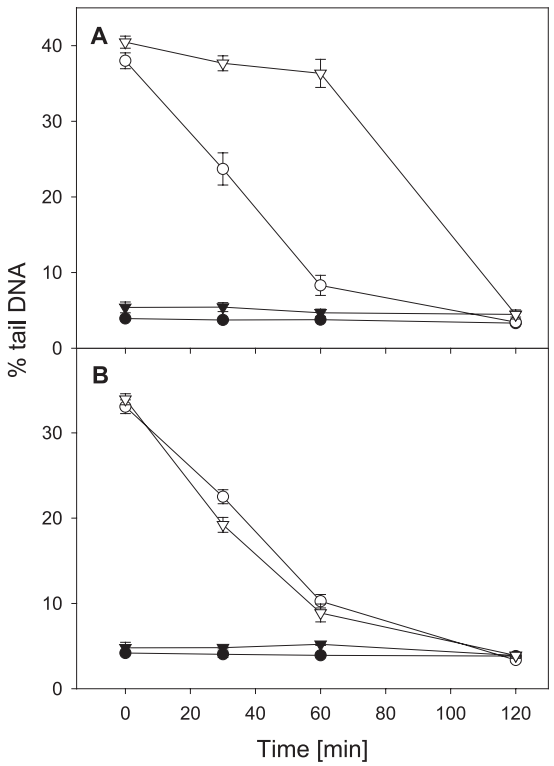

Fig. 3. Time course of DNA damage repair in K562 cells (A) and CCRF-CEM cells (B) induced by 10 μM hydrogen peroxide in the absence (○) or in the presence (▽) of STI571 inhibitor at 1 μM compared to untreated controls (●) and cells incubated with STI571 alone (▼). DNA damage was evaluated as the percentage of DNA in the tail (% tail DNA) in the alkaline comet assay. The number of cells scored in each treatment was 100. Results represent the mean of three independent experiments; error bars denote SEM.

Both K562 and CCRF-CEM cells exposed to H2O2 completely repaired DNA damages within 120 min as compared to untreated controls (no differences; $P > 0.05$).

Discussion

STI571 that we used in experiments with human leukemia cells is a BCR/ABL-selective inhibitor, which exhibits its activity by blocking of the tyrosine kinase ATP binding site (Deininger and Druker, 2003; Knight and McLellan, 2004). Extensive clinical studies have established the high efficacy of this agent in the treatment of CML patients, including those in the chronic phase, as well as patients in the accelerated or blast phases of this malignancy (Mauro *et al.*, 2002; Druker, 2003; O'Brien *et al.*, 2003). Our results indicated that STI571 significantly slowed down the kinetics of DNA repair in both γ-irradiated as well as MNNG-treated K562 human leukemia cells measured by the standard alkaline comet assay. In this study for cross-links repair the K562 cells were treated with MMC followed by post-lysis irradiation and allowed to repair for up to 24 h. The examination of DNA repair kinetics showed that MMC-induced cross-links take at least 24 h to repair them completely in K562 cells but STI571 slowed down MMC adducts excision. In this modified NER-related procedure the comet tails increased as post-treatment incubation time increased indicating repair response to cross-linking agents. The final evidences came from experiments that compared STI571 activity in K562 and in CCRF-CEM leukemia cells. CCRF-CEM cells derived from human acute leukemia are not expressing BCR/ABL and we demonstrated that

STI571 had no effect on DNA repair after hydrogen peroxide treatment. CCRF-CEM cells were able to recover within 60 min of repair incubation independently of STI571 activity, while STI571 elongated the recover of K562 cells expressing BCR/ABL to 120 min. These results confirmed our previous findings which suggested DNA repair inhibition by STI571 in mouse BaF3 BCR/ABL-transformed cells, and finally extended the observation on human leukemia cell lines (Majsterek *et al.*, 2004).

Our previous studies revealed that BCR/ABL might stimulate strand breaks repair by DNA homologous recombination (Slupianek *et al.*, 2001). This phenomenon, which is dependent on BCR/ABL-induced elevation of the level of the RAD51 protein, plays an essential role in homologous recombination. RAD51 in conjunction with Bcl-xL and G2/M arrest has been reported to be responsible for the drug resistance in BCR/ABL-positive cells (Majsterek *et al.*, 2002). Russell *et al.* (2003) showed that STI571-mediated inhibition of RAD51 expression caused the enhancement of tumor cell radiosensitivity (Russell *et al.*, 2003). Further experiments indicated the multi-pathway of repair that may be involved in DNA processing of BCR/ABL-positive mouse hematopetic cells (Canitrot *et al.*, 2003; Majsterek *et al.*, 2004). In the present work we showed that STI571 enhanced the sensitivity to MNNG, γ-radiation or MMC which is correlated with accelerated DNA repair in human leukemia cells derived from CML patients. This suggests that at least three main pathways: MMR, HRR and NER that may correspond to DNA repair are observed, because MNNG introduces missparing, γ-radiation generates strand breaks or reactive oxygen species (ROS) and MMC causes DNA cross-links (Parshad and Sanford, 2001). Also, base excision repair (BER) might have contribution since it is known to be involved in DNA processing of BaF3 BCR/ABL-transformed mouse pro-B lymphoid cells (Majsterek *et al.*, 2004). However, recent studies have shown that in the absence of an efficient BER pathway, the formation of BER intermediates can trigger HRR, a mechanism that depends on BCR/ABL activation (Sobol *et al.*, 2003). It seems unlikely that NHEJ might be activated because BCR/ABL inhibits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) activity by proteasome-dependent degradation (Deutsch *et al.*, 2001). In this aspect regulation of the balance between HRR and NHEJ can be modified by BCR/ABL activity.

Conclusion

The main conclusion of this work is that STI571 might selectively inhibit DNA repair in cells expressing BCR/ABL. After incubation of two different human leukemia cell lines with STI571, we observed inhibition of DNA damage repair induced by hydrogen peroxide only in K562 leukemia cells, but no effect was found in CCRF-CEM human acute leukemia cells. This suggests that BCR/ABL might determinate cellular response to DNA damaging agents that promotes K562 cells survival. We postulate that multi-pathway of DNA repair represents a mechanism dependent on BCR/ABL, which in turn may overlay STI571 activity in human leukemia cells and may contribute to abrogation of drug and radiation resistance during pharmacological treatment.

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