Non-homologous DNA End Joining Repair in Normal and Leukemic Cells Depends on the Substrate Ends

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Double-strand breaks (DSBs) are the most serious DNA damage which, if unrepaired or misrepaired, may lead to cell death, genomic instability or cancer transformation. In human cells they can be repaired mainly by non-homologous DNA end joining (NHEJ). The efficacy of NHEJ pathway was examined in normal human lymphocytes and K562 myeloid leukemic cells expressing the BCR/ABL oncogenic tyrosine kinase activity and lacking p53 tumor suppressor protein. In our studies we employed a simple and rapid *in vitro* DSB end joining assay based on fluorescent detection of repair products. Normal and cancer cells were able to repair DNA damage caused by restriction endonucleases, but the efficiency of the end joining was dependent on the type of cells and the structure of DNA ends. K562 cells displayed decreased NHEJ activity in comparison to normal cells for 5′ complementary DNA overhang. For blunt-ended DNA there was no significant difference in end joining activity. Both kinds of cells were found about 10-fold more efficient for joining DNA substrates with compatible 5′ overhangs than those with blunt ends. Our recent findings have shown that stimulation of DNA repair could be involved in the drug resistance of BCR/ABL-positive cells in anticancer therapy. For the first time the role of STI571 was investigated, a specific inhibitor of BCR/ABL oncogenic protein approved for leukemia treatment in the NHEJ pathway. Surprisingly, STI571 did not change the response of BCR/ABL-positive K562 cells in terms of NHEJ for both complementary and blunt ends. Our results suggest that the various responses of the cells to DNA damage *via* NHEJ can be correlated with the differences in the genetic constitution of human normal and cancer cells. However, the role of NHEJ in anticancer drug resistance in BCR/ABL-positive cells is questionable.

Key words: Non-homologous DNA End Joining (NHEJ), BCR/ABL Oncogenic Tyrosine Kinase, Imatinib (STI571)

Introduction

DNA double-strand breaks (DSBs) are the most pronounced DNA damage induced by a variety of different mechanisms including exposure to ionizing radiation and a number of chemicals. Physiological forms of DSBs occur in V(D)J [variable (diversity) joining] recombination playing a pivotal role in immunoglobulin diversification in human lymphocytes and in class switch recombination in lymphocytes as well as in the generation of haploid germ cells. Abnormal forms of some other physiological processes like replication on a DNA template containing single strand break or stabilization of the complex of DNA with DNA topoisomerase II may also lead to the generation of DSBs (see West *et al.*, 2000 for review). DSBs, if not repaired or misrepaired, may lead to mutations and cell death. Therefore the presence of an efficient repair system dealing with such damage is of great importance for all cells carrying genetic information. In principle we can consider two pathways of DSBs repair operating both in pro- and eukaryotic cells: homologous recombination (HR) repair and non-homologous DNA end joining (NHEJ) (see Valerie and Povirk, 2003 for review). Additionally, a shared pathway between HR and NHEJ-single

Abbreviations: BCR, breakpoint cluster region; DNA-PK_{cs}, DNA protein kinase catalytic subunit; DSBs, DNA double-strand breaks; HR, homologous recombination; NHEJ, non-homologous DNA end joining; SCID, severe combined immunodeficiency; SSA, single strand annealing; V(D)J, variable (diversity) joining; WRN, Werner syndrome helicase; XRCC4, X-ray cross complementation 4.

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strand annealing (SSA) or strand exposure and repair, can be considered. The relative contribution of each type of repair in DSB repair in mammals is controversial and it depends on an organism and the cell cycle (Haber, 2000; Johnson and Jasin, 2001). It seems that NHEJ may dominate, at least in some cell cycle phases, in higher eukaryotes (Less-Miller and Meek, 2003; Lieber *et al.*, 2003; Pastwa and Blasiak, 2003).

NHEJ in human involves recognition and processing of the termini of damaged DNA performed by the protein complex containing the multiple Ku heterodimers consisting of Ku70 and Ku86 forming a close-fitting asymmetrical ring that threads onto free termini of the damaged DNA (Walker *et al.*, 2001). The end-processing action of Ku is assisted by the catalytic subunit of DNA protein kinase (DNA-PK_{cs}) and the Artemis nuclease (Leuther *et al.*, 1999; Ma *et al.*, 2002). Other proteins, like XRCC4 (X-ray cross complementation 4) and WRN (Werner syndrome helicase), may be also involved at this stage (Leber *et al.*, 1998; Yannone *et al.*, 2001). The end-processing process depends strongly on the structure of the termini. After this step two fragments of damaged DNA are ligated by DNA ligase IV assisted by the XRCC4 protein (Chen *et al.*, 2000).

Cells deficient in DSBs repair may contribute to many serious human syndromes displaying cancer proneness (De la Torre *et al.*, 2003). Defects in NHEJ may underly human severe combined immunodeficiency (SCID) and hypersensitivity to ionizing radiation as well as lymphomas, solid tumors and degradation of enteric neurons (Less-Miller and Meek, 2003). Moreover, many anticancer drugs and ionizing radiation used for anticancer therapy target DNA and cause DSBs, so their repair should be taken into account in planning chemotherapy, especially in the context of its two main obstacles: adverse side effects in normal cells and resistance of cancer cells. If normal cells are not able to remove DSBs resulting from therapeutic treatment effectively, they may suffer from its consequence including induction of secondary malignances. On the other hand, if target cancer cells can efficiently repair DSBs, they can develop resistance to the drug or/and radiation employed. It is therefore important to evaluate the function of the DSB repair system not only in normal cells, but also in their pathological counterparts. To address this problem in the present study we compared the efficacy of NHEJ in normal human lymphocytes and K562 leukemic cells. The latter are human myeloid leukemia cells expressing the Philadelphia chromosome resulting in the synthesis of fusion oncogenic protein BCR/ABL displaying tyrosine kinase activity (see Mauro and Druker, 2001 for review). These cells have a different genetic constitution than normal human lymphocytes due to the presence of the BCR/ABL fusion gene, which we have shown to be involved in the response of the cell to the DNA-damaging agents (Blasiak *et al.*, 2002a, b; Slupianek *et al.*, 2002). To assess the role of BCR/ABL of K562 cells in NHEJ we employed STI571 (Imatinib), which is a specific ABL family tyrosine kinases inhibitor approved for treatment of leukemias (Druker *et al.*, 1996).

Materials and Methods

Chemicals

RPMI 1640 medium without L-glutamine, phosphate buffered saline (PBS), fetal bovine serum (FBS), penicillin/streptomycin (10,000 U/ml), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). 2-Phenylaminopyrimidine (STI571, Imatinib) was kindly provided by Novartis Pharma (Basel, Switzerland). T4 DNA ligase (1 U/μl) was purchased from Invitrogen (Carlsbad, CA, USA). *Hin*dIII (20 U/μl) and *Hin*cII (10 U/μl) were obtained from Fermentas (St. Leon-Rot, Germany). Vistra Green was obtained from Amersham Biosciences (Little Chalfont Buckinghamshire, UK). Protease inhibitors cocktail was purchased from Roche Molecular Biochemicals (Mannheim, Germany).

Cells

Lymphocytes were isolated from peripheral blood of young, healthy, non-smoking donors. Peripheral blood lymphocytes were isolated by centrifugation in a density gradient of Histopaque (15 min, 230 $\times$ *g*). The final concentration of the lymphocytes was adjusted to $1-3 \times 10^5$ cells/ml by adding the growth medium to the single cell suspension. The human myeloid leukemia cells K562 were maintained in RPMI 1640 medium supplemented with 10% FBS, and 1% streptomycin/penicillin. The viability of the cells was measured by the trypan blue exlusion and was found to be about 99%. In order to obtain whole cell extract the cells were pelleted twice in ice-cold PBS (900 $\times$ *g*) and resuspended in the hypotonic lysis buffer

[10 mM Hepes (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), pH 7.9, 60 mM KCl, 1 mM EDTA, pH 8.0, 1 mM DTT and protease inhibitors cocktail according to the manufacturer's instructions] (min. $4-6\times10^7$ cells/0.5 ml extraction buffer). Then the cells were lysed by three cycles of freeze-thawing in a bath of dry ice and ethanol and in a 37 °C water bath. After the final thawing the extract was clarified by centrifugation at $15{,}000\times g$ for 30 min, removed as supernatant and stored at – 70 °C until needed. Protein determinations were made according to the method of Bradford (1976), using bovine serum albumin as the standard.

STI571 treatment

K562 cells and lymphocytes (10^6/ml) were pre-incubated with ABL kinase inhibitor STI571 at a final concentration of 1 μM or 4 μM for 24 h at 37 °C. After 12 h the drug at the same concentration was added again. The control cells received only RPMI 1640 medium.

DNA preparation

DNA substrate with either 5′ complementary or blunt ends was produced by complete digestion of the pUC19 plasmid with *Hin*dIII or *Hin*cII restriction endonucleases, respectively. Protein was removed by phenol/chloroform extraction and the plasmid DNA was recovered in TE buffer, pH 8.0.

End joining assay

The end joining assay was performed as described previously by Pastwa *et al.* (2001). The repair reactions were conducted in a total volume of 50 μl. The reaction medium contained 50 mM Tris [tris(hydroxymethyl)aminomethane]-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% polyethyleneglycol (PEG) 8000, protease inhibitors cocktail according to the manufacturer's instructions, 100 ng substrate DNA and K562 cells or lymphocytes whole cell extract. The repair was stopped by adding of 0.4% SDS and incubation at 65 °C for 15 min. DNA was recovered by extraction with phenol/chloroform (1:1 v/v) and ethanol precipitation using 0.5 μg tRNA as a carrier. The repair products were identified by gel shift following 1% agarose electrophoresis and staining for 1 h with Vistra Green according to the manufacturer's instructions. The images were digitized with a Gel Doc 2000 system and quantified densitometrically using Quantity One 1-D analysis software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All the values in this study represent the means ± SD for three separate experiments performed in triplicate each. The significance of differences between experimental variables was determined using the Student's *t*-test. If no significant differences between variations were found, the differences between means were evaluated by applying the Anova test.

The data were analyzed using the Statgraphics Plus v. 5.1. software (Statistical Graphics Corporation, Englewood Cliffs, NJ, USA).

Results

NHEJ in normal and cancer cells

We compared the ligation efficiency in whole cell extracts prepared from normal and cancer cells. Fig. 1 shows the ability to join compatible 5′ overhang ends (Fig. 1A) and blunt ends (Fig. 1B) by extract of human lymphocytes and K562 myeloid leukemia cells. Both kinds of cells were able to repair DNA damage caused by *Hin*dIII and *Hin*cII restriction endonucleases, but the efficiency of end joining was different and was dependent on the type of cells used for extraction and the type of DSBs ends generated by restriction enzymes. Significant differences were observed between the ability of extracts from lymphocytes and K562 cells to join DNA ends generated by *Hin*dIII ($p < 0.001$) (Fig. 1C). Extracts from human lymphocytes converted nearly 50% of linear DNA substrate to repair products (dimers, trimers and high molecular weight products), whereas enzymes from K562 cells produced 39% end joined products. There were no significant differences between the efficiency of DNA end joining for these extracts with DNA blunt ends generated by *Hin*cII ($p > 0.05$) (about 5% substrate conversion for both extracts) (Fig. 1D). Our results demonstrate that extracts from lymphocytes and leukemic cells were nearly 10-fold more efficient in NHEJ with compatible 5′ overhang DNA substrates (*Hin*dIII) than those with blunt ends (*Hin*cII) ($p < 0.001$) (Fig. 1E).

The influence of STI571

To determine the role of the BCR/ABL oncogenic tyrosine kinase protein of K562 cells in

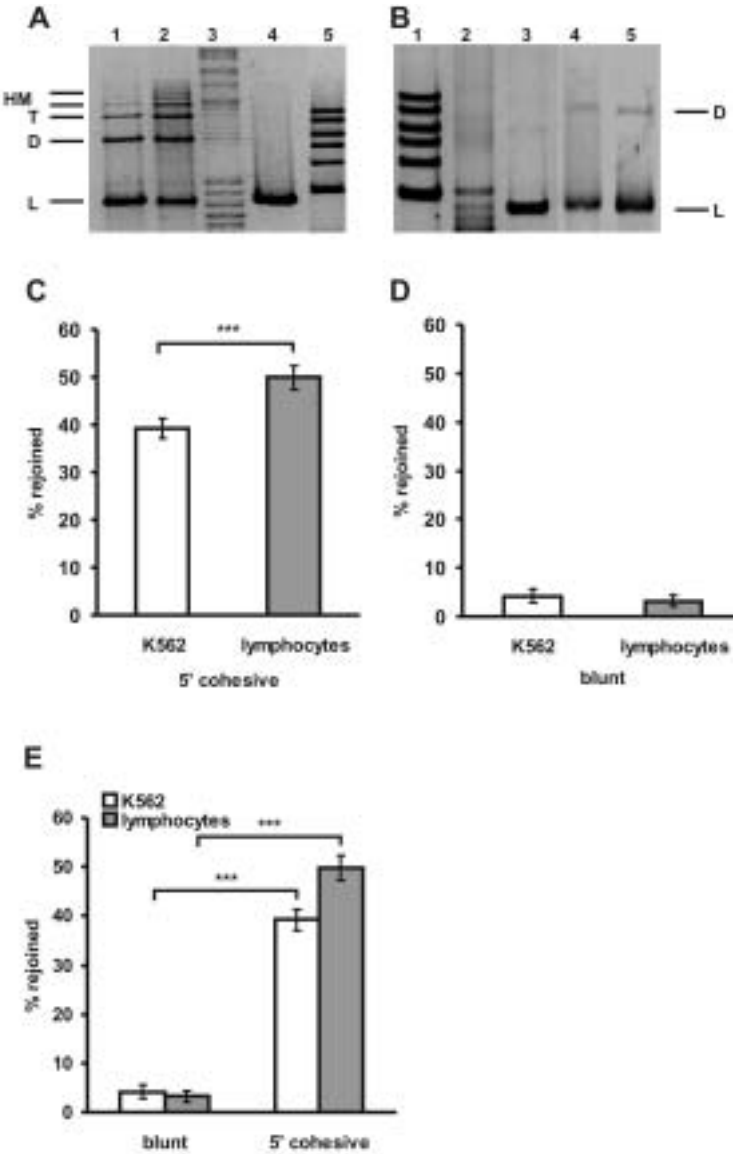

Fig. 1. DNA end joining in human lymphocytes and K562 cells. Standard repair reactions were performed with 100 ng substrate DNA linearized by *Hin*dIII or *Hin*cII digestion and 15 μg proteins, then incubated at 17 °C for 18 h. (A) Agarose gel of ligated pUC19 linearized with *Hin*dIII (5′ overhang ends) in human lymphocytes and K562 cell extracts. The reactions were as follows: lane 1, *Hin*dIII cut DNA + K562 cell extract; lane 2, *Hin*dIII cut DNA + human lymphocyte extract; lane 3, *Hin*dIII cut DNA + T4 ligase positive control; lane 4, *Hin*dIII cut DNA negative control; lane 5, 0.5 μg 1 kb DNA ladder. DNA substrate and product bands are indicated as follows: L, linear DNA; D, dimer; T, trimer; HM, quatramer and larger high molecular weight products. (B) As in (A), but with pUC19 linearized with *Hin*cII (blunt ends). The reactions were as follows: lane 1, 0.5 μg 1 kb DNA ladder; lane 2, *Hin*cII cut DNA + T4 ligase positive control; lane 3, *Hin*cII cut DNA negative control; lane 4, *Hin*cII cut DNA + K562 cell extract; lane 5, *Hin*cII cut DNA + human lymphocyte extract. (C) A comparison of NHEJ efficiency of 5′ overhang ended DNA substrates for both normal and leukemic cells. The data in the gel (A) were plotted as a percentage of linear substrate DNA converted to rejoined products. Results are the mean of three independent experiments; error bars represent ± SD; *** $p < 0.001$. (D) As in (C), but with data from the gel (B) and with blunt ended DNA. (E) As in (C), but with data from the gel (A) and (B) and with both kinds of restriction endonucleases.

NHEJ, its specific inhibitor STI571 was used in our study. Fig. 2 shows the ability to join compatible 5′ overhang ends (Fig. 2A) and blunt ends (Fig. 2B) by human lymphocytes and K562 cells with and without 24 h pre-incubation with STI571 inhibitor. Both kinds of cells were able to repair DNA damage caused by *Hin*dIII and *Hin*cII restriction enzymes in the presence and absence of STI571 with different efficiency depending on restriction enzyme used. However, the treatment of cells with STI571 at different concentrations (1 μM and 4 μM) had no effect on non-homologous DNA end joining efficiency ($p > 0.05$) in BCR/ABL expressing K562 cell lines and human lymphocytes for both complementary 5′ overhang and blunt ends (Figs. 2C and 2D).

Discussion

The efficacy of DSBs repair should be taken into account in planning of anticancer strategy, since most anticancer drugs target DNA. In hu-

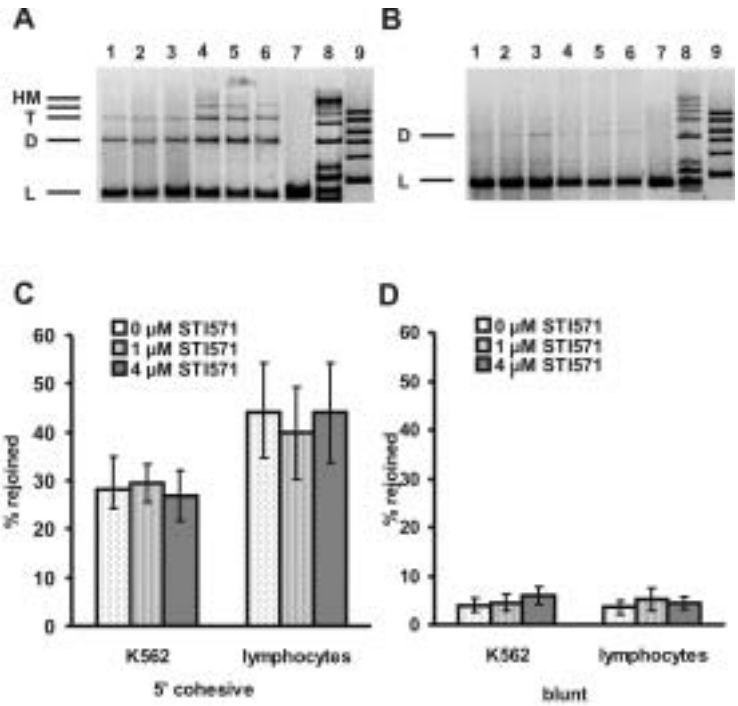

Fig. 2. DNA end joining in human lymphocytes and K562 cells with and without 24 h pre-incubation with STI571 inhibitor. Repair reactions were performed under standard conditions. Before the extraction procedure the cells were pre-incubated with ABL kinase inhibitor STI571 at concentrations of 1 μM or 4 μM for 24 h. (A) Agarose gel of ligated pUC19 linearized with *Hin*dIII (5′ overhang ends) in human lymphocytes and K562 cell extracts with and without 24 h pre-incubation with different concentrations of STI571 inhibitor. The reactions were as follows: lane 1, *Hin*dIII cut DNA + K562 cell extract; lane 2, *Hin*dIII cut DNA + K562 cell extract + 1 μM of STI571; lane 3, *Hin*dIII cut DNA + K562 cell extract + 4 μM of STI571; lane 4, *Hin*dIII cut DNA + human lymphocyte extract; lane 5, *Hin*dIII cut DNA + human lymphocyte extract + 1 μM of STI571; lane 6, *Hin*dIII cut DNA + human lymphocytes extract + 4 μM of STI571; lane 7, *Hin*dIII cut DNA negative control; lane 8, *Hin*dIII cut DNA + T4 ligase positive control; lane 9, 0.5 μg 1 kb DNA ladder. DNA substrate and product bands are indicated as follows: L, linear DNA; D, dimer; T, trimer; HM, quatramer and larger high molecular weight products. (B) As in (A), but with pUC19 linearized with *Hin*cII (blunt ends). The reactions were as follows: lane 1, *Hin*cII cut DNA + K562 cell extract; lane 2, *Hin*cII cut DNA + K562 cell extract + 1 μM of STI571; lane 3, *Hin*cII cut DNA + K562 cell extract + 4 μM of STI571; lane 4, *Hin*cII cut DNA + human lymphocyte extract; lane 5, *Hin*cII cut DNA + human lymphocyte extract + 1 μM of STI571; lane 6, *Hin*cII cut DNA + human lymphocyte extract + 4 μM of STI571; lane 7, *Hin*cII cut DNA negative control; lane 8, *Hin*cII cut DNA + T4 ligase positive control; lane 9, 0.5 μg 1 kb DNA ladder. (C) A comparison of NHEJ efficiency of 5′ overhang ended DNA substrates for both normal and leukemic cells with and without 24 h pre-incubation with different concentrations of STI571 inhibitor. The data in the gel (A) were plotted as a percentage of linear substrate DNA converted to rejoined products. Results are the mean of three independent experiments; error bars represent ± SD. (D) As in (C), but with data from the gel (B) and with blunt ended DNA.

man cells DSBs can be mainly repaired *via* the NHEJ pathway, depended on Ku, DNA-PK$_{cs}$, XRCC4 and ligase IV proteins. To understand the mechanism of DNA metabolism, we compared the efficiency of NHEJ in normal human lymphocytes and K562 leukemic cells. Both kinds of cells have a different genetic constitution. Human myeloid leukemia cells express BCR/ABL fusion oncogenic tyrosine kinase activity, whereas peripheral blood lymphocytyes do not display this activity and were employed as a control. Additionaly, contrary to lymphocytes, K562 cells do not express wild-type p53 tumor suppressor protein, which is involved in DSBs repair. In our study we employed an *in vitro* NHEJ assay, in which fluorescent dye has been used for rapid and direct visualization of rejoining products (dimers, trimers and high molecular weight multimers) in agarose gel (Pastwa *et al.*, 2001). This detection method was at least 50-fold more sensitive than ethidium bro-

mide and required small quantities ($\geq$ 100 ng) of substrate DNA in order to achieve conversion of substrate to end joined products. Moreover, the procedure of whole cell extract preparation was simple and allowed us to recover nuclear and cytoplasmic proteins involved in repair pathways without additional subcellular fractionations steps.

In the first part of our study we showed that myeloid leukemia cells K562 had decreased NHEJ activity as compared to peripheral blood lymphocytes for 5′ complementary overhang ended DNA (Figs. 1A and 1C). For blunt ended DNA we did not observe any significant differences between these cells in end joining efficiency, maybe due to a very low level of NHEJ (only 5%) (Figs. 1B and 1D). Our data, that NHEJ seems to be inhibited by BCR/ABL from K562 cells as compared to the normal cells, suggest a correlation between BCR/ABL oncogenic protein and repair proteins involved in end joining pathway. In fact, the study of Deutsch *et al.* (2001) demonstrates a down-regulation of the major mammalian DNA repair protein DNA-PK_{cs} by BCR/ABL in both BCR/ABL-positive murine and human hematopoietic cells. Gaymes *et al.* (2002) have also studied NHEJ activity in K562 cells and human lymphocytes with DNA substrate linearized by *Eco*RI (5′ complementary overhang ends). Contrary to our results they showed that ligation efficiency was increased 2- to 7-fold in myeloid leukemia cells in comparison to normal peripheral blood lymphocytes. The authors hyphothesise that the DNA-PK_{cs}-independent, error-prone, pathway responds to DSBs in leukemic cells since the DNA-PK_{cs}-dependent, error-free, pathway can be inactivated in these cells according to the important findings of Deutsch *et al.* (2001). Also the recent studies using different NHEJ assays with different cells and different DNA substrates indicated 5-fold increase of NHEJ activity in BCR/ABL wilde-type mouse myeloid cells with respect to parental cells when non-complementary 5′ overhangs as DNA substrate have been used (Nowicki *et al.*, 2004). Taken together, the end joining activities of BCR/ABL positive cells *vs.* normal cells and their relationship to DNA-PK-dependent and independent reactions require further research.

Our observation that both extracts were 10-fold more efficient at joining DNA substrates with compatible 5′ overhangs than those with blunt ends is in agreement with other reports (Baumann and West, 1998; Diggle *et al.*, 2003; Wang *et al.*, 2003).

Another explanation of our results is the possible role of p53 in DSB repair and NHEJ. p53 is a tumor suppressor protein, which is involved in many pathways of signal transduction in stress responses that affect cell cycle regulation, apoptosis and DSB repair (Gebow *et al.*, 2000). The human leukemia K562 cell line does not express wild-type p53 protein, but only truncated p53 protein of 148 amino acids (Usuda *et al.*, 2003). As we showed in this study, normal human lymphocytes possessing p53 protein were able to rejoin 5′ compatible overhang ended DNA *via* NHEJ more efficient than p53-negative cell line K562. Our observations are consistent with several studies showing that p53 directly enhances rejoining of DSB with cohesive ends *via* short homologies in mouse fibroblasts and thyroid cells (Yang *et al.*, 1997; Tang *et al.*, 1999). On the other hand, a recent report using non-homologous overhangs as DNA substrate in human leukemic K562 and lymphoblastoid cells demonstrated inhibition of microhomology-directed NHEJ by p53 (Akyuz *et al.*, 2002). These differences could come from the fact, that for non-homologous DNA overhangs p53 might play a fidelity control function like in HR, either by recognition of heterologies and inhibition of NHEJ or by exonucleolytic proofreading. That is why error-prone NHEJ process could be downregulated by p53 for non-cohesive ends and error-free NHEJ can be stimulated for cohesive ends. Interestingly, physical interactions of p53 with polymerase β, the enzyme participated in gap filling during NHEJ, have been reported, making p53 a candidate to provide a proofreading activity for polymerase β during NHEJ (Zhou *et al.*, 2001).

In the second part of our study we investigated NHEJ in BCR/ABL-positive cells in the presence of its STI571 inhibitor. The signal transduction inhibitor STI571 is a therapeutic drug used in treatment against leukemia (Druker, 2002). It can specifically inhibit the activity of oncogenic tyrosine kinase BCR/ABL, a hallmark of chronic myelonic leukemia, at a concentration of 1 μM (Slupianek *et al.*, 2002). Our recent studies indicate that the drug resistance in BCR/ABL-positive cells in chemotherapy can be caused by stimulation of DNA double-strand break repair by homologous recombination (Slupianek *et al.*, 2002; Blasiak *et al.*, 2002a, b). Elevated levels of RAD51 (essential protein in HR), Bcl-xL (antiapoptotic protein

from fusion tyrosine kinase family) or G_2/M cell cycle arrest could be responsible for this resistance. Moreover, we showed for the first time that an increase in the efficiency of repair could be involved in drug resistance in cells expressing BCR/ABL oncogenic protein (Majsterek *et al.*, 2002). So far, there is no report of direct correlation between STI571 treatment and NHEJ activity in human leukemic cells. That is why it is of interest to study the possible mechanism of inhibition of BCR/ABL *via* STI571 in terms of NHEJ pathway. Our present results demonstrate that STI571 (imatinib), a BCR/ABL tyrosine kinase inhibitor, did not change the response of BCR/ABL-positive K562 cells in terms of non-homologous DNA end joining for both complementary and blunt ends (Fig. 2). The same results were obtained with BCR/ABL-negative normal human lymphocytes treated as control. However, the recent studies indicated that inactivation of the BCR/ABL kinase activity by point-mutation in mouse myeloid cells reduced NHEJ by 2-fold, when non-complementary 5′ overhangs were used (Nowicki *et al.*, 2004). We suggest that the lack of expected NHEJ inhibition in K562 cells in our study can be caused by resistance of these cells to STI571 in lymphoblastic crisis. In conclusion, the present study showed that a different genetic constitution of cancer cells in comparison to normal human lymphocytes could be responsible for the various responses of the cells to DNA damage *via* non-homologous end joining. However, these findings need further investigations. The question whether NHEJ is responsible for anticancer drug resistance in leukemic cells remains still unanswered.

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