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SHORT COMMUNICATION

Pharmacological inhibition of c-Abl compromises genetic stability and DNA repair in Bcr-Abl-negative cells

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Imatinib inhibits the kinase activity of Bcr-Abl and is currently the most effective drug for treatment of chronic myeloid leukemia (CML). Imatinib also blocks c-Abl, a physiological tyrosine kinase activated by a variety of stress signals including damaged DNA. We investigated the effect of pharmacological inhibition of c-Abl on the processing of irradiation-induced DNA damage in Bcr-Abl-negative cells. Cell lines and peripheral blood mononuclear cells (PBMCs) from healthy volunteers were treated with imatinib or dasatinib before γ-irradiation. Inhibition of c-Abl caused an enhanced irradiation-induced mutation frequency and slowdown of DNA repair, whereas imatinib was ineffective in cells expressing a T315I variant of c-Abl. Mutation frequency and repair kinetics were also studied in c-Abl−/− murine embryonic fibroblasts (MEFs) retransfected with wild-type c-Abl (wt-Abl) or a kinase-defect variant of Abl (KD-Abl). Enhanced mutation frequency as well as delayed DNA repair was observed in cells expressing KD-Abl. These data indicate that pharmacological inhibition of c-Abl compromises DNA-damage response.
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Introduction

The development of imatinib mesylate (STI571, Gleevec) for the treatment of chronic myelogenous leukemia (CML) is one of the most important advances in malignant disease management during recent years (Buchdunger *et al*., 1996; Druker *et al*., 1996). Imatinib directly binds to the kinase domain of Bcr-Abl blocking its activity. In addition, it targets a limited number of other tyrosine kinases such as c-Abl, c-KIT and platelet-derived growth factor receptor (Carroll *et al*., 1997; Heinrich *et al*., 2000). Most patients with newly diagnosed CML achieve durable responses upon imatinib therapy (Druker *et al*., 2006). However, even in patients with complete molecular remission, the Bcr-Abl-positive clone quickly reappears after discontinuation of imatinib (Michor *et al*., 2005). Therefore, prolonged, maybe lifelong therapy is required to control CML with imatinib. As a consequence, it is of great importance to understand the biological effects of imatinib on normal cells. The observation of an unexpected cardiac toxicity related to c-Abl inhibition (Kerkelä *et al*., 2006) demonstrates that pharmacological targeting of physiological c-Abl is of clinical relevance.

c-Abl is a tightly regulated cellular tyrosine kinase involved in several stress signaling pathways. It interacts with many proteins involved in processing and repair of DNA damage such as p53 (Wei *et al*., 2005), p73 (Yuan *et al*., 1999), Mdm2 (Goldberg *et al*., 2002), Rad51 (Yuan *et al*., 1998), DNA-PK (Kharbanda *et al*., 1997), WRN (Cheng *et al*., 2003), CSB (Imam *et al*., 2007) and BRCA1 (Foray *et al*., 2002). In addition, several studies have shown that the activity of c-Abl is upregulated following genotoxic agents or γ-irradiation (Shaul and Ben-Yehoyada, 2005). Therefore, it seems likely that c-Abl plays an important role for the maintenance of genetic stability. This prompted us to investigate whether long-term inhibition of the c-Abl activity affects genetic stability in Bcr-Abl-negative cells. We analyzed the frequency of hypoxanthine-guanine phosphoribosyltransferase (HPRT) loss-of-function mutations in cell lines exposed to imatinib and in murine embryonic fibroblasts (MEFs) expressing either a kinase defect variant of c-Abl (KD-Abl) or wild type c-Abl (wt-Abl). As shown in Figure 1a, significantly more HPRT mutant colonies were observed in a γ-irradiated Bcr-Abl-negative cell line in the presence of imatinib. This contrasts the effect of imatinib observed in Bcr-Abl-positive cells. We and others found that expression of the active Bcr-Abl kinase in CML cells results in genetic instability (Canitrot *et al*., 1999; Salloukh and Laneuville, 2000; van der Kuip *et al*., 2004). This can be partially reversed by imatinib in Bcr-Abl-positive cells

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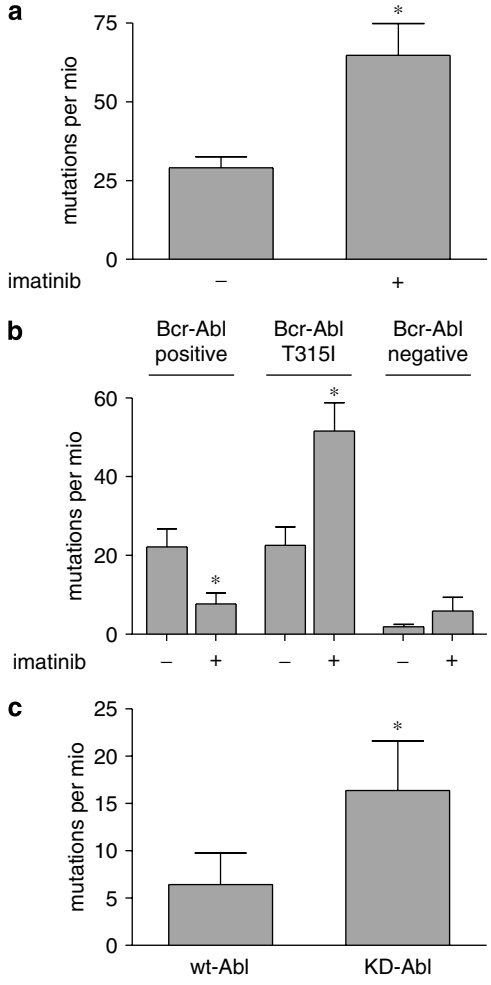

Figure 1 Influence of imatinib on mutation frequencies in Bcr-Abl-positive and Bcr-Abl-negative cells. 32D and BaF3 cell lines are described elsewhere (Moehring *et al*., 2005). The immortalized Abl−/− MEFs were a kind gift from Dr Wang (UCSD, San Diego, CA, USA). The cells were reconstituted with KD-Abl, or wt-Abl by retroviral infection. Cells were cultivated in RPMI1640/10% FCS complemented with 1 ng ml^{-1} mIL-3 for BaF3 and 32D cells. Cells were treated with or without imatinib (Toronto Research Chemicals Inc., North York, Ontario, Canada) and irradiated three times (2 Gy) over a time period of 10 days before 6-TG selection. Imatinib was used at concentrations of 1 or 3 μM. Preliminary experiments showed that these concentrations are sufficient to completely inhibit irradiation-induced c-Abl activity in all cell lines used (not shown). The concentrations are in the range of those found in plasma from patients treated with 400 or 600 mg imatinib (Picard *et al*., 2007). The HPRT mutation frequency was evaluated as described (van der Kuip *et al*., 2004). For each single experiment, the mean value of six-well plates for CE and 12-well plates for selection of 6-TG resistant colonies were used for calculation. (**a**) γ-irradiation induced mutation frequency in BaF3 cells treated with or without 1 μM imatinib. Values reflect the mean ± s.e.m. of six independent examinations. (**b**) HPRT mutation frequency in Bcr-Abl-positive 32D cells (BA+), 32D cells expressing the imatinib-resistant *bcr-abl* mutant T315I (Bcr-AblT315I), and Bcr-Abl-negative 32D cells (BA−) in the presence or absence of 3 μM imatinib. Values reflect the mean ± s.e.m. of six independent examinations. (**c**) Frequency of 6-TG resistant clones in MEFs expressing a KD- or wt-Abl. Values reflect mean ± s.e.m. of six independent examinations. (*$P<0.05$, two-sided paired Student's *t*-test). CE, cloning efficiency; FCS, fetal calf serum; HPRT, hypoxanthine-guanine phosphoribosyltransferase; KD-Abl, kinase defect variant of c-Abl; MEFs, murine embryonic fibroblasts; RPMI, Roswell Park Memorial Institute medium; TG, thioguanine; wt-Abl, wild-type c-Abl.

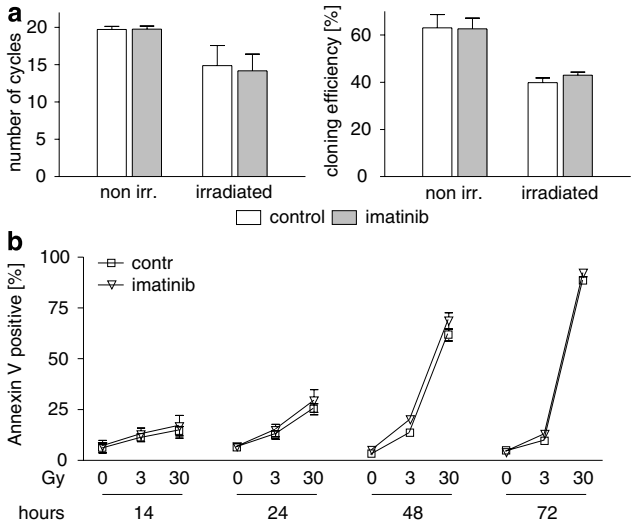

Figure 2 Imatinib treatment of Bcr-Abl-negative cells has no effect on proliferation or induction of apoptosis. (**a**) 32D cells were treated with or without 3 μM imatinib and irradiated three times (2 Gy) over a time period of 10 days. Medium and/or imatinib were changed every other day. Cells were counted every 48 h and the number of cell divisions was calculated (left panel). Cells were resuspended in semisolid RPMI containing 0.3% agar and plated at a density of 200 cells/well (six-well plates). Cells were then incubated for 7 days. Colonies larger than 50 cells were counted to determine CE (right panel). Values reflect mean ± s.e.m. of five independent examinations. (**b**) 32D cells were pretreated with or without 3 μM imatinib for 48 h before irradiation with indicated dosages. Cell death was assessed by FITC-conjugated Annexin V staining (Pharmingen, San Diego, CA, USA) at indicated time points after irradiation. The staining was performed according to manufacturers' instructions and analyzed by flow cytometry. Values reflect mean ± s.e.m. of three independent examinations. CE, cloning efficiency; FITC, fluorescein isothiocyanate; RPMI, Roswell Park Memorial Institute medium.

resulting in mutation frequencies comparable to those seen in imatinib-treated Bcr-Abl-negative cells (Figure 1b). Thus, inhibition of c-Abl causes an increase of the mutation frequency, whereas blocking of Bcr-Abl activity reduces this frequency. We then asked whether c-Abl inhibition might contribute to genetic instability also in Bcr-Abl-positive cells. Therefore, we analyzed mutation frequency in cells transfected with an imatinib-resistant *bcr-abl* mutant (Bcr-AblT315I). As expected, in cells expressing imatinib-resistant Bcr-Abl and imatinib-sensitive c-Abl, the HPRT mutation frequency is significantly enhanced in the presence of imatinib (Figure 1b). To verify that inhibition of c-Abl is the responsible mechanism for the enhanced mutation frequency, we examined MEFs derived from c-Abl−/− mice retransfected with either KD-Abl or wt-Abl. As shown in Figure 1c, HPRT mutation frequency was consistently elevated in cells lacking c-Abl kinase activity. We conclude that inhibition of c-Abl compromises mechanisms responsible for the maintenance of genomic integrity.

It has been shown that activation of c-Abl by many sources of DNA damage is associated with inhibition of cell growth and induction of apoptosis (Kharbanda *et al*., 1998; Wang, 2000). Therefore, we examined

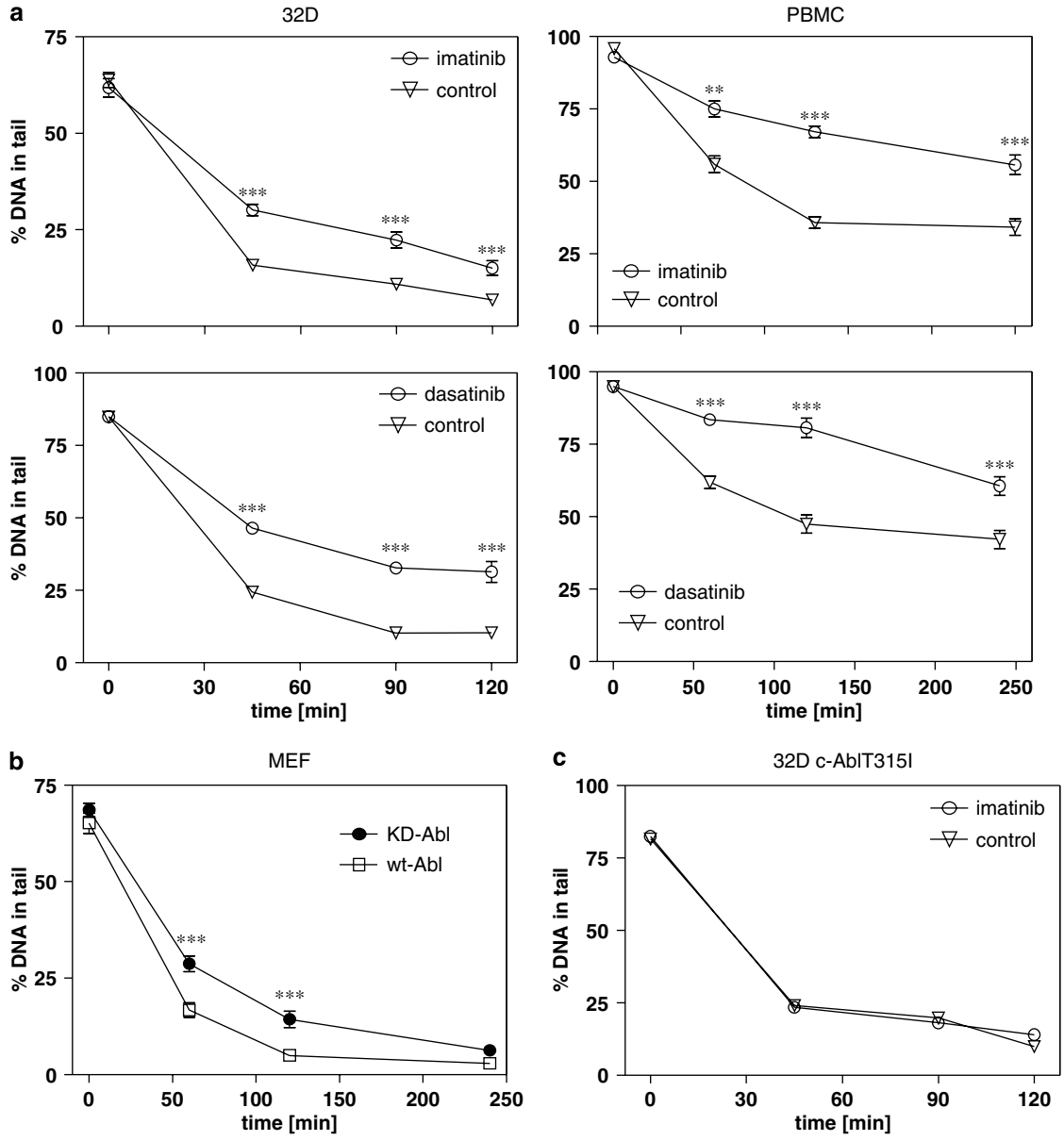

Figure 3 c-Abl activity influences DNA strand break repair kinetics. The time course of repair kinetics was evaluated by comet assay. Following preincubation with/without imatinib or dasatinib (Toronto Research Chemicals Inc., Ontario, Canada) for 48 h, cells were irradiated with 30 Gy. Cells were harvested at indicated time points. The comet assay was performed according to Singh *et al*. (1988). In brief, a suspension of 160 000 cells in 0.5% low melting point agarose was placed onto precoated slides. Lysis was performed for 10 min in 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10. The slides were transferred into electrophoresis buffer (300 mM NaCl, 1 mM EDTA). Electrophoresis was conducted at 4 °C (15 min, 25 V, 300 mA). The slides were neutralized with 0.4 M Tris, pH 7.5 and stained with DAPI-Vectashield (Vector, UK). At least 50 cells of each slide were randomly selected and evaluated using a DMRM microscope (Leica, Germany) and a computer-based image analysis system (TriTec Corp., Virginia, USA). Each experiment was conducted in independent triplicates. The mean value of the percentage of DNA in tail was taken as an indicator of DNA strand breaks. (**a**) 32D cells (left panels) and PBMCs from healthy volunteers (right panels) were treated with or without 3 μM imatinib (upper panels) or 10 nM dasatinib (lower panels) for 48 h before γ-irradiation (30 Gy). At indicated time points after irradiation, cells were harvested and the percentage of DNA in tail was evaluated as equivalent for DNA strand breaks. PBMCs were isolated by Ficoll–Hypaque gradient centrifugation (Seromed, Germany) and grown in RPMI1640/10% FCS supplemented with 2 μg ml^{-1} PHA (Sigma, Germany). (**b**) Immortalized MEFs derived from c-Abl-null mice retransfected with either a kinase-defect variant of c-Abl or a wild type c-Abl were harvested after indicated time points after γ-irradiation (30 Gy) and analyzed using comet assay. (**c**) Imatinib-insensitive 32D c-AblT315I cells were treated with or without 3 μM imatinib for 48 h and harvested at indicated time points after γ-irradiation (30 Gy) for quantification of DNA damage by comet assay. 32D cells were stably transfected with an HA-tagged type IV c-ablT315I cDNA cloned into pcDNA3.1zeo (−) (Invitrogen, Karlsruhe, Germany). The T315I mutation was introduced by site-directed mutagenesis. (**$P<0.001$, ***$P<0.0001$, unpaired Student's *t*-test). EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; HA, hemagglutinin; MEFS, murine embryonic fibroblasts; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin.

whether the effect of c-Abl inhibition on genetic stability is due to differences in cell proliferation and induction of apoptosis. Treatment with imatinib neither affected the total number of cell doublings over a 10-day cultivation period nor long-term clonogenic survival in 32D and BaF3 cells (Figure 2a). In addition, induction of cell death following irradiation was not significantly altered by imatinib in 32D or BaF3 cells (Figure 2b).

Proteins involved in DNA repair are among the well-characterized targets of the c-Abl kinase. To address the question whether imatinib may interfere with the kinetics of γ-irradiation-induced DNA strand break repair, we performed comet assays in murine cell lines and primary human peripheral blood mononuclear cells (PBMCs) from healthy volunteers. The upper panel of Figure 3a shows the kinetics of DNA strand break repair in Bcr-Abl-negative cell lines (left panel) and in PBMCs (right panel) in the presence or absence of imatinib. Imatinib had no effect on the peak amount of irradiation-induced DNA strand breaks (Figure 3a, time 0) or on constitutive breaks in non-irradiated controls (not shown). However, pretreatment with imatinib significantly impaired the efficiency of DNA repair (Figure 3a, upper panel). This phenomenon was also observed in BaF3 cells and with different doses of irradiation (3 and 5 Gy; not shown). We next investigated if other c-Abl inhibitors such as dasatinib also affect DNA repair. As shown in Figure 3a (lower panel), a comparable delay of DNA repair was observed with dasatinib in all cells tested including PBMCs from healthy volunteers (not shown for BaF3 cells). To further verify that this phenomenon is caused by inhibition of c-Abl, we examined the repair kinetics in MEFs expressing either KD-Abl or wt-Abl. Cells expressing the kinase-defect form again showed a significant delay in DNA repair compared to cells transfected with wt-Abl (Figure 3b). To exclude imatinib effects unrelated to c-Abl inhibition, we generated a cell line transfected with an imatinib-resistant c-Abl construct (c-AblT315I). Expression of this c-Abl variant was sufficient to completely abolish the effect of imatinib on the kinetics of DNA repair (Figure 3c).

In conclusion, our experiments demonstrate that inhibition of c-Abl both affects the velocity of DNA repair and the maintenance of genetic stability. These phenomenons are caused by deficient c-Abl kinase activity irrespective whether genetic or pharmacological methods were applied. c-Abl activity plays a role in DNA repair processes, proliferation and induction of apoptosis (Kharbanda *et al*., 1998). The ultimate Abl-dependent response to DNA damage may depend on the cell type as well as on the type of DNA damage induced. Our results suggest that in hematopoietic cells, c-Abl inhibition alters DNA repair processes rather than survival after irradiation. In accordance with this, Uemura and Griffin (2000) found no effects of imatinib on survival of Bcr-Abl-negative hematopoietic cells after ionizing radiation.

The question remains whether our findings are of importance for the further development of c-Abl kinase inhibitors. So far, clinical observations do not support a direct mutagenic effect of imatinib. The increase of chromosomal aberrations in Philadelphia-negative cells reported during early clinical studies of imatinib (Andersen *et al*., 2002; Terre *et al*., 2004) is not observed in patients treated upfront with imatinib. However, agents with mutagenic capacity such as cigarette smoking cause malignant disease not before decades of exposure. Therefore, a carcinogenic risk of imatinib may become evident only after long-term treatment. Consequently, patients treated for many years with imatinib should be closely monitored to timely identify unwanted adverse events.

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