BCR/ABL kinase induces self-mutagenesis via reactive oxygen species to encode imatinib resistance

Running head: BCR/ABL-induced mutagenesis and imatinib resistance

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Abstract

Mutations in the BCR/ABL kinase domain play a major role in resistance to imatinib mesylate (IM). We report here that BCR/ABL kinase stimulates reactive oxygen species (ROS), which cause oxidative DNA damage resulting in mutations in the kinase domain. The majority of mutations involved A/T→G/C and G/C→A/T transitions, a phenotype detected previously in patients, which encoded clinically relevant amino acid substitutions causing IM resistance. This effect was reduced in cells expressing BCR/ABL[Y177F] mutant, which does not elevate ROS. Inhibition of ROS in leukemia cells by the anti-oxidants pyrrolidine dithiocarbamate (PDTC), N-acetylcysteine (NAC), and vitamin E (VE) decreased the mutagenesis rate and frequency of IM resistance. Simultaneous administration of IM and an anti-oxidant exerted better anti-mutagenic effect than an anti-oxidant alone. Therefore, inhibition of ROS should diminish mutagenesis and enhance the effectiveness of IM.

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Introduction

The BCR/ABL gene is derived from the relocation of a portion of c-ABL gene from chromosome 9 to the portion of BCR gene locus on chromosome 22 [t(9;22), Philadelphia chromosome = Ph], and is present in most chronic myeloid leukemia (CML) and in a cohort of acute lymphocytic leukemia (ALL) patients. BCR/ABL oncogenic tyrosine kinase (a product of BCR/ABL chimeric gene) exhibits two complementary roles in cancer. The first and best-characterized is stimulation of signaling pathways that eventually induce growth factor independence and affect the adhesive and invasive capability of leukemia cells. The second is the modulation of responses to DNA damage, rendering cells resistant to genotoxic therapies, and causing genomic instability. Clinical observations and experimental findings suggest that BCR/ABL-induced genomic instability may lead to mutations and chromosomal translocations frequently observed during the transition from a relatively benign CML chronic phase (CML-CP) to an aggressive blast crisis (CML-BC). In addition, genomic instability is also manifested by numerous mutations detected in the BCR/ABL gene encoding for resistance to imatinib mesylate (IM).

IM, a selective inhibitor of ABL kinase activity, revolutionized the treatment of BCR/ABL-positive leukemias. Unfortunately, clinical and experimental observations reveal that resistance to IM is increasingly problematic. Although the rate of progression of newly diagnosed CML-CP patients on IM is about 4% per year, IM resistance obscures this otherwise very successful oncogene-targeted therapy.

BCR/ABL kinase mutations appear to be the most frequent cause of acquired resistance to IM; resistant cells may also exhibit genomic amplification of non-mutated BCR/ABL, and BCR/ABL independence due to overexpression of LYN kinase. Mutations were also detected
in CML-CP patients before IM treatment, thus arguing for genetic instability early in the disease. Therefore, the BCR/ABL gene appears to be a casualty of genomic instability promoted by its own product – the BCR/ABL kinase.

Mutations usually result from enhanced DNA damage and/or deregulated mechanisms responsible for DNA repair. Much endogenous DNA damage arises from intermediates of oxygen reduction. Oxygen is metabolized inside the cell by a series of one-electron reductions with the generation of reactive and potentially damaging intermediates called reactive oxygen species (ROS), primarily generated by the mitochondrial respiratory chain (MRC). ROS units are usually short-lived and strike only molecules that are close in space and time, such as free nucleotides, which are subsequently incorporated into DNA during replication by unfaithful polymerases. Examples of ROS derivatives include 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxoG), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy), thymidine glycol, and 5-hydroxycytosine.

BCR/ABL-mediated generation of ROS by MRC combined with aberrant regulation of DNA repair pathways may contribute to the mutator phenotype displayed by BCR/ABL cells. Therefore, the probability of accumulating DNA errors appears to be high in BCR/ABL-positive cells, because of enhanced “spontaneous” DNA damage and unfaithful repair mechanisms leading to misrepair. Thus, we investigated whether or not ROS-mediated DNA damage generates mutations in the BCR/ABL gene, leading to IM resistance.
Materials and methods

Cells

32Dcl3 murine growth factor-dependent hematopoietic cell line was transfected with pSRα−neo retroviral construct containing p210BCR/ABL, p190BCR/ABL, p190BCR/ABL(Y177F) mutant cDNA, or empty vector. Clones expressing similar amounts of BCR/ABL were used for the experiments 2 or 10 weeks after transfection. Mononuclear fractions of human normal bone marrow cells (hBMC from 4 donors), 4 patients in CML-CP and 4 patients in myeloid CML-BC were obtained after informed consent and collected after centrifugation on Histopaque-1077 (Sigma, St. Louis, Missouri). Murine cell lines and primary cells were cultured in the presence of IL-3 and SCF + GM-CSF, respectively, in pre-tested minimal concentrations required to maintain the proliferation of normal cells.

ROS assay

Levels of intracellular ROS were analyzed using the redox-sensitive fluorochrome 2’,7’-dichlorofluorescin-diacetate (DCFDA) (Sigma) 14. Briefly, 2x10^5 cells/ml of culture medium were incubated with 5μM DCFDA (5mM stock in DMSO, stored in -20°C) for 15 minutes in 37°C. Next samples were washed, re-suspended in PBS and analyzed using flow cytometer EPICS XL (Beckman-Coulter, Inc., Miami, FL). The oxidized form of DCFDA, carboxy-DCFDA (Molecular Probes, Inc., Eugene, Oregon), was used as a control for uptake, retention, and decay.
In vitro mutagenesis assay

The following compounds were added to tissue culture: 2μM ABL-tyrosine kinase inhibitor imatinib mesylate (IM) (Novartis Pharma AG, Basel, Switzerland), 0.2μM antioxidant pyrrolidine dithiocarbamate (PDTC), 200μM vitamin E (VE), 200μM N-acetylcysteine (NAC), and 100μM oxidant-inducer buthionine sulfoximine (BSO) (all from Sigma). IM or ouabain resistant cells were identified by clonogenic assay in semisolid methylcellulose medium (StemCell Technologies). 10^6 cells were plated in methylcellulose in the absence of growth factor(s) and the presence or absence of 2μM IM or 1μM ouabain (Sigma); colonies were counted after 7 days. Percentage of resistant cells was calculated as follows: number of colonies in the presence of inhibitor x 100 / number of colonies without an inhibitor. IM-resistant single colonies were selected, expanded, and total RNA was isolated and reverse transcribed using random hexamers. To assess the mutagenicity in BCR/ABL-transformed murine cell lines, the primers specific for human c-ABL kinase domain (gi: 28236) (5’ primer: 5’-CGCAACAAAGCCACTGTC-3’, 3’ primer: 5’-TCCACTTCGTCTGAGATCTGGATT -3’) and murine α subunit of Na+/K+ ATPase (gi: 21450276) (1st pair- 5’ primer: 5’-GGTTGGAGACGACAAGATATGAG-3’, 3’ primer: 5’-GCACGTTGGCTACGATGATA-3’; 2nd pair- 5’ primer: 5’-TGATCCTTGAGTACACCTGGC-3’, 3’ primer: 5’-CCCTCCACAATGATGAGACTGTT-3’; 3rd pair- 5’ prime 5’-TACCACACAGAGATCTGGTTTG-3’, 3’ primer: 5’-GCCGCCTGAGTAATGAGCTT-3’) were used for PCR. cDNA from CML patient cells was amplified using 5’ primer in BCR exon 2 (5’-CACAGCATTCCGCTGACCATCA-3’) and 3’ primer in human c-ABL (see above). Then nested PCR was performed using human c-ABL specific 5’ primer (see above) and 3’ primer (5’-AAATGCCCAGACGTCGGGACTTG-3’). Amplified fragments were cloned using TA cloning
kit (Invitrogen, Carlsbad, California) and sequenced. The mutation rate in IM-resistant cells was determined according to the Luria-Delbruck fluctuation test with modifications 18.

**In vivo mutagenesis assay**

Outbred SCID mice (Taconic Farms, Germantown, New York) were fed with control chow or vitamin E-enriched (VE) diet (Bioserv, Frenchtown, New Jersey) 19 starting from one week before and continuously after intravenous injection of $5 \times 10^4$ 32Dcl3-BCR/ABL cells obtained 2 weeks after transfection. Eight weeks after leukemia cell inoculation, splenocytes (SPL) and bone marrow cells (BMC) were harvested and mononuclear cell (MNC) populations were obtained after centrifugation of Lympholyte-M gradient (Cedarlane, Hornby, Ontario, Canada). MNC were used for ROS and mutagenesis assays as described above. The experiments conform to the regulatory standards and were approved by the Institutional Animal Care and Use Committee at Temple University.

**Comet assay**

The comet assay was performed under alkaline conditions as described 14. For enzymatic treatment, cells were drained in agarose and covered with an enzyme buffer (control) or the enzyme (1 μg/ml of Endo III or Fpg) in buffer, and incubated for 30 min at 37°C.

**Immunofluorescence**

Monoclonal anti-8-oxoG (Chemicon International, Temecula, California) followed by the secondary rabbit anti-mouse-Alexa594 (Molecular Probes, Inc., Eugene, Oregon) antibodies
were applied to detect the nuclear localization of the 8-oxoG lesions by immunofluorescence combined with de-convolution technology, as described 17.

**Linker-ligation PCR (LL-PCR) assay**

Genomic DNA was purified by DNeasy Tissue Kit (Qiagen, Inc., Valencia, California). The protocol for LL-PCR to detect broken-ended double-stranded DNA was performed as described with modifications 14. Briefly, two oligomers BW-1 (5’-GCGGTGACCCGGGAGATCTGAATTC-3’) and BW-2 (5’-GAATTCAGATC-3’) were annealed to form a linker, then stored frozen. Purified DNA (2 μg) was incubated with Klenow polymerase and ligated to the linker. Ligated DNA (200 ng) was used in a 50 μl PCR assay containing 3 pmol of the linker reverse primer (BW-1), human c-ABL-specific 5’ primer: 5’-CGCAACAAGCCACTGTC-3’ and 2 units of Taq polymerase. After two rounds of PCR (2 x 20 cycles), the products were detected by Southern analysis using α-dCTP labeled probe specific for the ABL kinase (5’-TGGAAGAAATACACAGCCTGACG-3’).
Results

ROS-mediated oxidative DNA damage generates mutations in the BCR/ABL kinase domain causing IM resistance in murine leukemia cells in vitro

In vitro models were used before to characterize mutations in BCR/ABL emerging after IM treatment in the absence of growth factor(s) \(^{20,21}\). To investigate the biological relevance of the oxidative DNA damage in Ph-positive leukemias, an experimental model was established where the mutagenic parameters were not affected by selective pressure of IM. Therefore, while previously published reports mimic the IM-resistant mutations emerging during IM treatment, our model is focused on mutagenesis in IM-naive leukemia cells. p210BCR/ABL-32Dc13 clones (3 clones per group) and parental counterparts (transfected with empty vector) were cultured for 2 weeks (B/A-early) and 10 weeks (B/A-late) in the presence of pre-tested concentrations of IL-3 required to maintain proliferation of non-transformed cells.

B/A-early cells did not display full transformation phenotype (e.g. growth factor-independence) whereas B/A-late cells were fully growth factor independent (Figure 1A). Cell cycle analysis did not reveal major differences between parental and BCR/ABL-transformed counterparts in the presence of optimal concentrations of IL-3, although fully transformed B/A-late cells displayed a more abundant tyrosine phosphorylation pattern in comparison to B/A-early cells, as compared by Western analysis (Supplementary Figure 1 online). Our model mimics those described before and indicates that leukemogenesis driven by BCR/ABL kinase is a step-wise process reflecting multistage disease progression \(^{22,23}\). In accordance, CML cells display disease stage-specific differences in growth factor-independent survival \(^{24}\).

ROS was not elevated by BCR/ABL at the early (2 weeks) stage of transformation (Figure 1A, compare bar 3 with 1; and 4 with 2), whereas it was induced at the late (10 weeks)
stage (Figure 1A, compare bar 7 with 5 and 8 with 6). BCR/ABL exerted ~3-times stronger stimulatory effect on ROS levels at the late stage in comparison to the early stage (Figure 1A, compare bar 8 with 4). IL-3 and BCR/ABL seemed to display an additive effect on ROS stimulation (Figure 1A, compare bar 7 with 8 and 5). Thus, our experimental model reflects a step-wise transformation including time-dependent growth factor independence accompanied by an increase of ROS levels. IM (48h incubation with 2 μM) downregulated ROS in B/A-late cells in the presence and absence of IL-3 (compare bars 9 with 7, and 10 with 8, respectively) implicating the kinase-dependent phenomenon. Addition of ROS scavenger, PDTC (48h incubation with 0.2 μM) diminished ROS in B/A-late cells in the presence and absence of IL-3 (Figure 1A, compare bars 11 with 7, and 12 with 8, respectively). The differences in ROS levels presented in Figure 1A do not appear to be affected by DCFDA uptake, cellular esterase activity, and efflux because cells in various groups displayed similar fluorescence intensity when carboxy-DCFDA, a constitutively fluorescent form of the dye, was used (data not shown).

Enhanced 8-oxoG staining was detected in B/A-late cells in the presence and absence of IL-3, whereas reduced staining was observed in parental cells, B/A-early cells and in B/A-late cells after inhibition of the kinase with IM and downregulation of ROS by PDTC (Figure 1B). In order to directly detect oxidative damage of DNA we employed two enzymes, endonuclease III (EndoIII) and formamidopyrimidine-DNA glycosylase (Fpg), which convert oxidative lesions into gaps detectable by the alkaline version of the comet assay $^{25,26}$. Addition of both enzymes detected the presence of more oxidative DNA lesions in fully transformed growth factor-independent B/A-late cells, in comparison to parental counterparts and freshly generated B/A-early cells (Figure 1C, compare groups 7 with group 5 and 3, respectively). This effect is dependent upon the BCR/ABL kinase–induced ROS because inhibition of the kinase by IM and
ROS by PDTC reduces the number of oxidative lesions (**Figure 1C**, compare groups 9 and 11 with group 7, respectively). Removal of IL-3 caused modest, statistically non-significant reduction of the oxidative damage in B/A-late cells (**Figure 1C**, compare groups 8 with 7). Results obtained using buffer only were subtracted from these obtained using enzymes (enzyme treatment usually increased the detection of DNA damage by approximately 2-fold).

Elevated oxygen stress is probably a major source of spontaneous DNA double strand breaks (DSBs) 27. Interestingly, LL-PCR followed by Southern analysis revealed “spontaneous” DSB in a G/C-rich stretch in the sequence encoding BCR/ABL kinase domain in B/A-late cells (**Figure 1D**, lanes 7 and 8). Incubation with IM or PDTC prevented this effect (**Figure 1D**, lanes 9 and 11, respectively), again implicating BCR/ABL kinase and ROS. Altogether, it appears that BCR/ABL kinase-dependent enhancement of ROS induced an excess of oxidative DNA damage, which eventually led to a DSB in the BCR/ABL kinase sequence.

32Dcl3 cells transformed with p190BCR/ABL displayed similar properties: elevated levels of ROS, enhanced nuclear staining for 8-oxoG lesions, and a “spontaneous” DSB in the p190BCR/ABL kinase (data not shown).

B/A-early cells neither contained detectable mutations in the sequence encoding for BCR/ABL kinase domain nor displayed IM resistance. However, about 0.001% (1 x 10^{-5}) of B/A-late cells were resistant to 2 µM IM after 8 weeks of continuous culture in the presence of pre-tested optimal concentrations of IL-3 (**Figure 2A**, % of IM-resistant cells, B/A group). IM resistance was associated with mutations in the region encoding for the BCR/ABL kinase domain; ~65% of them involved G/C→A/T transitions (**Figure 2A**, Mutation rate and Mutation phenotype, respectively). Mutations caused amino acid substitutions (**Figure 2A**, Aa substitutions) including those proven to be clinically relevant for resistance to IM 28-30, and those
identified in BCR/ABL mutagenesis screen using DNA repair deficient bacteria\(^{31}\). Mutagenesis in the sequence encoding BCR/ABL kinase domain was dependent upon BCR/ABL kinase activity because inhibition of the kinase by continuous presence of IM (Supplementary Figure 1 online) abrogated the mutagenic effect (Figure 2A, left panel, compare B/A+IM with B/A).

To determine the role of ROS in mutagenesis of BCR/ABL kinase domain leading to IM resistance, B/A-early cells were cultured for 8 weeks in the presence of pre-tested optimal concentrations of IL-3 and ROS scavenger PDTC, or IM + ROS inducer BSO (to inhibit BCR/ABL kinase activity, while keeping high levels of ROS). ROS were monitored periodically during the experiment to ensure the desirable effect of factors modulating ROS levels: IM inhibited the ABL kinase resulting in downmodulation of ROS (Figure 1A, bar 9), PDTC quenched ROS (Figure 1A, bar 11), and BSO (buthionine sulfoximine) elevated ROS after inhibition of BCR/ABL kinase by IM (data not shown). Addition of PDTC to the culture medium reduced IM resistance frequency by ~5-fold (Figure 2A, left panel, compare B/A+PDTC with B/A). These results suggest that ROS plays a crucial role in BCR/ABL kinase self-mutagenesis leading to IM resistance. When BSO was added together with IM, the IM resistance frequency was increased only by ~2-fold (Figure 2A, % of IM-resistant cells, compare B/A+IM+BSO with B/A+IM), implicating that although ROS is essential for IM resistance, another BCR/ABL kinase-induced mechanism may also play a role. In concordance, mutation rate also was reduced greatly by the presence of PDTC, and only modestly increased when BSO was added to tissue culture containing IM (Figure 2A, Mutation rate).

To determine whether or not ROS-induced mutagenesis is restricted to the BCR/ABL gene, the Na+/K+ ATPase mutagenesis model was employed where amino acid substitutions in the \(\alpha_1\) subunit of the enzyme causes resistance to ouabain\(^{32-34}\). Parental and B/A-early cells did
not display detectable resistance to ouabain; however resistant cells accumulated during cell culture in a time-dependent and accelerated fashion in the BCR/ABL-positive population in comparison to parental counterpart (Supplementary Figure 2 online). The percentage of BCR/ABL cells resistant to ouabain was \(~28\)x higher than that of parental cells after 8 weeks of culture in the presence of IL-3 (Figure 2B, % of ouabain-resistant cells). Sequencing of the \(\alpha_1\) subunit detected a mutation rate increased by \(~400\)x in BCR/ABL cells in comparison to parental counterparts (Figure 2B, Mutation rate). Again, mutation phenotype revealed the prevalence of \(\text{G/C} \rightarrow \text{A/T}\) transitions (Figure 2B, Mutation phenotype); mutations produced some of the previously identified amino acid substitutions, such as \(\text{C111A, D128S and D128E}\) (equivalents of sheep \(\text{C104A, D121S, and D121E}\), which induced resistance to ouabain \(^{32-34}\)). \(\text{Na}^+/\text{K}^+\) ATPase mutagenesis in BCR/ABL cells was inhibited by incubation with IM or PDTC. In conclusion, BCR/ABL-induced ROS-dependent mutagenesis appears not to be restricted to BCR/ABL gene.

BCR/ABL kinase mutagenesis and IM resistance was also examined in cells expressing BCR/ABL[\(\text{Y177F}\)] mutant, which does not elevate ROS \(^{35}\). BCR/ABL[\(\text{Y177F}\)]-32Dcl3 cells displayed lower levels of ROS in comparison to BCR/ABL-32Dcl3 cells when cultured in the presence of pre-tested minimal concentrations of IL-3 necessary to support the proliferation of parental cells (Figure 3A). After 8 weeks of continuous culture, BCR/ABL[\(\text{Y177F}\)]-32Dcl3 cells contained \(~3\)-times less clones resistant to IM; in addition, mutation rate was diminished by \(~1.7\)-fold (Figure 3B). These results support the notion that ROS causes mutations in the BCR/ABL kinase domain and IM resistance.

Next, we determined if simultaneous scavenging of ROS by an anti-oxidant and inhibition of BCR/ABL kinase by IM exerts better anti-mutagenic effect than each agent used
individually. For this reason, B/A-early cells were incubated in the presence of IL-3 for 8 weeks with IM and/or various anti-oxidants such as PDTC, NAC, or VE. As expected, each compound inhibited ROS and reduced the number of IM-resistant clones (Figure 4). VE and NAC appear to be more effective in comparison to IM (Figure 4, compare B/A+NAC and B/A+VE with B/A+IM), probably due to the fact that IM inhibits only BCR/ABL-driven generation of ROS, while anti-oxidants scavenge ROS generated by BCR/ABL and IL-3–dependent mechanisms. PDTC did not exert better effect than IM, probably because of dose limitations (higher concentrations of PDTC exerted a detrimental effect on cell proliferation). Addition of IM did not further diminish ROS levels in leukemia cells treated with anti-oxidants, but it significantly reduced the percentage of cells resistant to IM (Figure 4, compare B/A+IM+PDTC/NAC/VE with B/A+IM). Similar mutations to those described in Figure 2A were detected here in IM-resistant colonies (data not shown). These results suggest that although ROS appears to be necessary to trigger mutagenesis, additional BCR/ABL kinase-dependent mechanism(s) may work in concert with ROS to cause mutations in the kinase.

**ROS-mediated oxidative DNA damage generates mutations in the BCR/ABL kinase domain causing IM resistance in CML primary cells in vitro**

A significant increase of ROS was detected in CML-BC cells, whereas CML-CP cells displayed rather modest elevation of ROS in comparison to cells from healthy donors (Figure 5A). However, the latter population sometimes consisted of two subpopulations displaying low and higher levels of ROS (Supplementary Figure 3 online). CML-BC myeloid bone marrow cells contained, on average, 3.8-times more ROS than normal counterparts (Figure 5A), whereas BCR/ABL-transformed 32Dcl3 cell line displayed approximately only 2-fold increase in
comparison to parental cells (Figure 1A). This discrepancy is probably due to the heterogeneity of primary cell populations and/or slower proliferation rate of cells from normal donors in comparison to those from CML-BC patients; 32Dcl3 cells and BCR/ABL-transformed counterparts represent more homogenous populations with similar proliferation rate. Cells obtained from CML-BC patients in lymphoid blast crisis also contained high levels of ROS (2 patients analyzed, data not shown).

8-oxoG, a frequent DNA lesion resulting from ROS, may be detected by immunofluorescence as nuclear diffuse and focal staining; the latter may be associated with extensive oxidative DNA damage. 8-oxoG staining was readily detectable in CML-BC cells displaying high levels of ROS; conversely, reduced staining was observed in normal hematopoietic counterparts displaying low levels of ROS (Figure 5B). Please note that CML-CP cells displaying weak and elevated 8-oxoG staining were detected, perhaps reflecting two sub-populations of CML-CP cells containing low and higher levels of ROS. Thus, enhanced ROS levels in CML cells appear to be associated with elevated oxidative DNA damage.

CML-BC patient cells (3 patients in myeloid blast crisis), previously not treated with IM, were used to test the role of ROS in generating BCR/ABL kinase mutants resistant to IM in primary cells. These cells did not display detectable IM resistance at the beginning of the experiment and were cultured for 6 weeks in the presence of pre-tested optimal concentrations of SCF+GM-CSF with the addition, or not, of IM or PDTC. IM and PDTC decreased ROS levels during the experiment (Figure 6, Relative fluorescence, compare CML+IM with CML and CML+PDTC with CML), resulting in reduction of the frequency of IM resistance by 8-11 -fold (Figure 6, compare % of IM-resistant cells from CML+IM with CML and CML+PDTC with CML). IM resistance was associated with mutations in the BCR/ABL kinase domain often
involving G/C→A/T transitions (30%), A/T→G/C transitions (25%), and A/T→C/G transversions (25%). Mutations caused amino acid substitutions, including Y253H found in patients 28-30, and S265T and I360F identified in bacterial screen 31. In addition, the following amino acid substitutions were found in the previously identified locations: M237V, M244L, M351L, E373A, and L387W. Moreover, additional 24 amino acid substitutions were found in positions not described before. However, the role of these aberrations in IM resistance is unknown, especially because they often were accompanied by previously characterized mutations. A few IM-resistant colonies grew from IM- or PDTC-treated cells, but we did not obtain enough material to examine the cause of resistance.

**An anti-oxidant reduces ROS-dependent mutations in BCR/ABL kinase and IM resistance in vivo**

BCR/ABL leukemia-bearing SCID mice were employed to determine whether or not anti-oxidants could prevent mutagenesis and IM resistance in vivo. Animals were injected with BCR/ABL-early cells and fed with vitamin E-rich (VE) diet as described by Factor et al. 19. VE diet reduced ROS levels in splenocytes (SPL) and bone marrow cells (BMC) in leukemic mice (Supplementary Figure 4 online). The percentage of IM-resistant BCR/ABL leukemia cells was reduced ~4-times in BMC harvested from the mice fed with VE diet than control chow; this effect was associated with ~2-fold reduction in the mutation frequency (Figure 7, % of IM-resistant cells and Mutation frequency, respectively). In spleenocytes, VE diet caused ~3x reduction of the percentage of IM-resistant leukemia cells, but not of mutation frequency. Mutation frequency was calculated by dividing the number of mutations in experimental group by the total number of sequenced bases. The Luria-Delbruck fluctuation test could not be applied...
here because of the unknown number of cell divisions. Therefore, mutation frequency presented here is higher than mutation rate presented in Figure 2A because these two values were calculated using different tests. Mutation phenotype was also analyzed and showed prevalence of G/C → A/T transitions (Figure 7, Mutation phenotype). Similarly to the in vitro experiment, mutations caused amino acid substitutions previously characterized as responsible for IM resistance (Figure 7, Aa substitutions). This shows that VE diet inhibited BCR/ABL-induced ROS-dependent mutagenesis in vivo.
Discussion

Mutations encoding for IM resistance represent a major cause of disease relapse. They likely arise as a result of genomic instability induced by BCR/ABL kinase. Since genomic instability, like other events, may depend on BCR/ABL expression levels, it is likely that mutations causing IM resistance will occur more often in less differentiated leukemia stem cells such as CD34+Lin- population, which express high levels of BCR/ABL. In agreement with this speculation, cell lines expressing BCR/ABL at the levels found in CML-BC were more likely to develop IM-resistant BCR/ABL mutants than those expressing BCR/ABL at the levels detectable in CML-CP. Mutations detected in CD34+ CML cell population are likely to be passed onto successive generations of leukemia cells. IM-resistant mutations were also detected in Ph-positive ALL cells.

Here we report that BCR/ABL oncogenic tyrosine kinases induce self-mutagenesis in murine leukemia cells and CML primary cells over time. This observation is supported by the clinical results showing no mutations in 40 patients in early CML-CP diagnosis (defined as <12 months from diagnosis), conversely 14 mutations were identified in 64 patients in late CML-CP (>12 months from diagnosis) and in 13 patients in accelerated phase. Mutagenesis is not restricted to the kinase sequence because leukemia cells acquire mutations in other genes such as Na+/K+ ATPase (this work), c-kit, hypoxanthine-guanine phosphoribosyl transferase, green fluorescent protein, and LacI. Mutations in Na+/K+ ATPase arose faster in leukemia cells than normal counterparts indicating that mutagenesis in the former cells is an active process. In accordance with previous reports, IM applied in non-selective conditions (in the presence of growth factors) reduced mutagenesis in BCR/ABL-transfected cell line and in CML primary cells, indicating that BCR/ABL kinase activity is essential for acquiring mutations.
Mutagenic force producing IM resistant clones in SCID mice (~2-4.5 x 10^{-5} reported here) is similar to that (~5-12 x 10^{-5}) described in BCR/ABL transgenic mice using Big Blue mutation detection system \(^\text{16}\). Several lines of evidence associated ROS, which appear to play a major role in genomic instability \(^\text{9}\), with BCR/ABL self-mutagenesis. BCR/ABL kinase can induce ROS in cell lines \(^\text{13}\) and CML patient cells as suggested by previous reports \(^\text{46,47}\) and confirmed here. Furthermore, higher levels of ROS in CML-BC cells in comparison to CML-CP cells implicate the role in accelerated mutagenesis and genomic instability leading to malignant progression of the disease \(^\text{48}\).

We found that cells expressing BCR/ABL[Y177F] mutant, which exerts the kinase activity but does not elevate mitochondrial-derived ROS \(^\text{35}\), displayed reduced frequency of IM resistance and mutagenic rate. In addition, anti-oxidants such as PDTC, NAC, and VE caused a reduction of mutagenesis and IM resistance. Moreover, PDTC reduced the frequency of mutations in the gene encoding Na+/K+ ATPase, indicating that ROS may play a more general role in mutagenesis in Ph-positive leukemias. Treatment with the anti-oxidants exerted stronger anti-mutagenic effect than Y177F amino acid substitution in BCR/ABL. This effect may depend on more general anti-ROS effect of the scavengers in comparison to the mutation, which can ablate ROS generated in mitochondria.

Results discussed above suggest that inhibition of ROS may prevent BCR/ABL mutations encoding for IM resistance. This is further supported by the experimental data showing that inhibition of ROS by VE diet in mice harboring BCR/ABL leukemia cells reduced the generation of IM resistant cells in bone marrow and spleen in comparison to mice fed with control diet. Furthermore, VE diet reduced the mutation frequency in the BCR/ABL kinase in IM resistant clones harvested from bone marrow, but not spleen cell populations. Although the
reason for this effect is unknown, it appears rather unlikely that it may result from the capability of cells carrying some IM resistant BCR/ABL mutants to outgrow those containing wild-type BCR/ABL \(^{49}\), because of the detection of a variety of mutants in bone marrow and spleen cell populations. Therefore, it seems that VE diet displayed slightly better anti-mutagenic effect in bone marrow cells than spleen cells, suggesting that localization of leukemia cells may have an impact on the efficiency of anti-oxidative treatment.

It has been shown that CML patients display significant reduction of VE level in serum \(^{50}\), possibly contributing to elevation of ROS in leukemia cells. Therefore, enhanced VE supply may exert anti-mutagenic effect in Ph-positive patients. This hypothesis is supported by others showing that anti-oxidants, including VE, reduce the mutagenic effects of ROS in humans \(^{51}\).

The majority of mutations detected in this work involve A/T→G/C and G/C→A/T transitions, in accordance with the reports from IM-resistant patients \(^{28,29,52}\). Similar transition phenotypes were often detected in the kinase gene family and in p53 in breast cancer \(^{53}\). Such mutator phenotype might emerge from ROS-mediated oxidative DNA damage \(^{9,54}\). ROS-dependent mutagenesis usually results from inefficient and/or unfaithful repair of oxidized bases and DNA double strand breaks (DSBs). The base excision repair (BER) likely produces G/C→A/T transitions \(^{54}\). In addition, DSBs generated by ROS may occur in BCR/ABL-transformed hematopoietic cells in the regions containing G/C stretches \(^{14,27}\). Interestingly, a G/C-rich “hot-spot” generating ROS-induced DSB in the sequence encoding BCR/ABL kinase is identified here, and A/T→G/C transitions are frequently introduced during homologous recombination repair (HRR) in BCR/ABL-positive leukemia cells \(^{55}\). Therefore, it appears that unfaithful repair of oxidative DNA damage by BER and HRR may contribute to the appearance of point mutations in BCR/ABL kinase causing IM resistance. However, quiescent Ph-positive
cells also accumulate mutations. Since ROS-mediated DSBs and HRR depend on DNA replication, unfaithful/inefficient BER may play a primary role in quiescent leukemia cells.

Although ROS appears to play a major role in elevation of oxidative DNA damage and BCR/ABL kinase mutagenesis, other factors may be important, too. This hypothesis is supported by the observation that increased ROS levels are necessary but may not be sufficient to enhance oxidative DNA damage. For example, we showed that anti-oxidant PDTC inhibited ROS and reduced oxidative DNA damage in BCR/ABL-late cells in the presence and absence of IL-3. However, while BCR/ABL-late cells in the absence of IL-3 displayed similar levels of ROS as parental cells and IM-treated BCR/ABL-late cells in the presence of IL-3, accumulation of oxidized bases was detected only in the former cells. Thus, we speculate that not only BCR/ABL kinase-induced elevation of ROS, but also stimulation of incorporation of oxidized nucleotides into DNA, and/or inhibition of their excision may be responsible for accumulation of oxidative DNA damage in BCR/ABL-late cells.

In addition, mutagenesis in BCR/ABL gene was only modestly induced if ROS was elevated by BSO in the absence of BCR/ABL kinase activity. Moreover, although inhibition of BCR/ABL kinase by IM did not cause significantly stronger inhibition of ROS when used with the anti-oxidants in concentrations applied here, it further reduced the frequency of IM-resistant clones. These observations implicate additional BCR/ABL kinase-dependent mechanisms in mutagenesis. For example, DNA polymerase β is overexpressed in BCR/ABL cells, which may diminish the fidelity of BER and HRR. Moreover, we showed that point mutations might be introduced during HRR in BCR/ABL-positive cells. Therefore, we hypothesize that although ROS may be essential to initiate mutagenesis, inefficient and/or unfaithful DNA repair
mechanisms introduce mutations into the sequence encoding BCR/ABL kinase during attempts of repair of numerous oxidative DNA lesions in leukemia cells.

IM resistance associated with BCR/ABL mutations plague an otherwise very effective anti-leukemia therapy \(^4,5,7\). In addition, resistance to FLT3 and epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors was associated with mutations in the kinase domains \(^57,58\). Novel inhibitors are being generated to overcome IM resistance \(^59-61\), but mutations causing resistance against new drugs are likely to emerge \(^62,63\). Therefore, a rational strategy should also target the mechanisms leading to mutagenesis in order to prevent generation of new mutations during inhibitor(s) therapy. Here we demonstrated that anti-oxidant treatment may reduce/eliminate IM-resistance driven by mutations in the BCR/ABL kinase. Interestingly, cells transformed by BCR/ABL-related fusion tyrosine kinases (FTKs), such as TEL/ABL and TEL/PDGFβR, and those expressing EGFR, HER1-2 kinase, also display elevated levels of ROS \(^64\) (data not shown). Thus, it is conceivable that tumor cells harboring oncogenic tyrosine kinases will be prone to develop resistance to kinase inhibitors. Although inhibition of an oncogenic kinase reduces the frequency of mutations in vitro, anti-mutagenic effect of the inhibitors may be limited in vivo. For example IM does not penetrate to the central nervous system, and also may not achieve effective concentrations in portions of other tissues/organs \(^65\). In addition, non-proliferating CML CD34+ progenitors probably containing leukemia stem cells are resistant to IM \(^66\). Thus, identifying approaches that reduce mutations in the oncogenic kinases may have a great impact on the therapeutic efficiency of IM and other small molecule inhibitors.
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FIGURE LEGENDS

Figure 1. **BCR/ABL induces ROS-mediated DNA oxidative damage in hematopoietic cell lines.**

(A) Parental 32Dcl3 cells freshly transfected with empty plasmid (P) or p210BCR/ABL retroviral construct (B/A) were cultured continuously for 2 weeks (P-early [groups 1,2] and B/A-early [groups 3,4], respectively) or 10 weeks (P-late [groups 5,6] and B/A-late [groups 7,8], respectively) in the presence of IL-3. B/A-late cells were eventually pre-incubated for 48h with IM or PDTC [groups 9 and 10, or 11 and 12, respectively]. ROS was measured by fluorescence in cells starved (-) or not (+) from IL-3 for 12h. Survival of cells was examined after 24h of incubation in the absence (starvation) or presence of IL-3 and represents the percentage of cells excluding Trypan blue. *p<0.05* in comparison to other groups (*), corresponding group incubated without IL-3 (**), and groups 10 and 12 (***)

(B) 8-oxoG staining is detected by immunofluorescence in the nuclei, which borders are marked in blue.

(C) Oxidative DNA lesions were detected after addition of either EndoIII or Fpg to the comet reaction. Bars represent the enzyme-dependent increase of DNA damage over that detected in the undigested samples. *p<0.05* in comparison to groups 1, 3, 4, 5, 9, and 11.

(D) A DSB (depicted by arrow) detected in the BCR/ABL kinase sequence by LL-PCR followed by Southern analysis; parental 32Dcl3-late cells served as negative control. G/C-rich stretch at the predicted DSB site is listed. Numbering is consistent in the panels.
Figure 2. Anti-oxidants prevent BCR/ABL-induced ROS-mediated mutations in BCR/ABL kinase domain and mouse α1 subunit of Na⁺/K⁺ ATPase in vitro.

32Dcl3 parental (P-early) and 2 week-old 32Dcl3-p210BCR/ABL cells (B/A-early, no mutations detected in BCR/ABL kinase and mouse α1 Na⁺/K⁺ ATPase) were cultured for 8 weeks in medium supplemented with IL-3 (B/A) and IM (B/A+IM), PDTC (B/A+PDTC) or IM and BSO (B/A+IM+BSO). The percentage of IM-resistant cells (A, % of IM-resistant cells) and ouabain-resistant cells (B, % of ouabain-resistant cells) was determined by clonogenic assay performed in the absence of IL-3, and the presence or absence of IM or ouabain, respectively. Mutation rate and mutation phenotype in BCR/ABL kinase and mouse α1 subunit of Na⁺/K⁺ ATPase are shown in A and B, respectively. Mutations leading to previously described amino acid substitutions are listed (Aa substitutions in A and B, respectively). *p<0.05 in comparison to B/A group; **p<0.05 in comparison to other groups.

Figure 3. Cells expressing BCR/ABL[Y177F] mutant displayed reduced ROS and mutagenic activity.

Freshly transformed p190BCR/ABL wild-type (wt) and p190BCR/ABL(Y177F) cells were cultured continuously for 8 weeks in the presence of IL-3. (A) ROS was measured by fluorescence. (B) The percentage of IM-resistant cells (% of IM-resistant cells) was determined by clonogenic assay performed in the absence of IL-3, and the presence or absence of IM. Mutation rate in BCR/ABL kinase is shown. *p=0.002 in comparison to wt group.
Figure 4. **IM enhanced the effect of anti-oxidants in prevention of IM resistance.**

B/A-early cells were cultured for 8 weeks in medium supplemented with IL-3 (B/A) and PDTC, NAC, VE, and IM administered individually or in combinations of an anti-oxidant with IM. ROS levels were examined by fluorescence (Relative fluorescence). Cells were plated in methylcellulose in the presence or absence of 2μM IM and absence of growth factors and colonies were counted after 7 days. Results represent % of IM-resistant cells (mean ± SD); *p<0.05* in comparison to other groups (*), B/A+IM group (**), and corresponding group treated with anti-oxidant only (***)

Figure 5. **Primary CML cells contain elevated levels of ROS and oxidative DNA lesions.** (A) ROS levels in mononuclear bone marrow cells from healthy volunteers (N), CML-CP, and CML-BC patients were analyzed by fluorescence; bars show mean ± SD; *p=0.02, **p=0.001* in comparison to N group. (B) 8-oxoG nuclear staining detected by immunofluorescence. The nuclei borders are marked in blue.

Figure 6. **An anti-oxidant prevents mutations in BCR/ABL kinase domain and IM resistance in CML cells.**

Cells from CML-BC patients previously untreated with IM (no resistance detected prior the experiment) were cultured for 6 weeks in medium supplemented with SCF+GM-CSF in the absence (CML) or presence of IM (CML+IM) or PDTC (CML+PDTC) (similarly to that described in **Figure 2**). ROS levels were examined by fluorescence (Relative fluorescence). Cells were plated in methylcellulose without growth factors in the presence or absence of 2μM IM and colonies were counted after 7 days. Results represent
% of IM-resistant cells (mean ± SD). Mutation rate and mutation phenotype in BCR/ABL kinase is shown. Mutations leading to previously described amino acid substitutions are listed (Aa substitutions). *p<0.05 and **p<0.001 in comparison to CML group.

Figure 7. An anti-oxidant inhibits ROS-induced mutations in BCR/ABL kinase domain in vivo.

Leukemia-bearing SCID mice were fed with control chow (transparent bars) or vitamin E enriched diet (VE diet, gray bars). Then, mononuclear cells of SPL and BMC were plated in methylcellulose in the absence of IL-3 (only leukemia cells grow) and the presence or absence of IM, and colonies were counted after 7 days. BCR/ABL kinase domain in IM-resistant cells was amplified by RT-PCR and sequenced. Mutation frequency and the percentages of base substitutions (Mutation phenotype) in the sequence encoding BCR/ABL kinase are shown. Mutations leading to previously described amino acid substitutions in BCR/ABL kinase are listed (Aa substitutions). *p<0.01 in comparison to control chow group.
Figure 1.
Figure 2.
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Figure 7.
BCR/ABL kinase induces self-mutagenesis via reactive oxygen species to encode imatinib resistance

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