Inhibition of the Bcl-x\textsubscript{L} Deamidation Pathway in Myeloproliferative Disorders


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**BACKGROUND**

The myeloproliferative disorders are clonal disorders with frequent somatic gain-of-function alterations affecting tyrosine kinases. In these diseases, there is an increase in DNA damage and a risk of progression to acute leukemia. The molecular mechanisms in myeloproliferative disorders that prevent apoptosis induced by damaged DNA are obscure.

**METHODS**

We searched for abnormalities of the proapoptotic Bcl-x\textsubscript{L} deamidation pathway in primary cells from patients with chronic myeloid leukemia (CML) or polycythemia vera, myeloproliferative disorders associated with the BCR-ABL fusion kinase and the Janus tyrosine kinase 2 (JAK2) V617F mutation, respectively.

**RESULTS**

The Bcl-x\textsubscript{L} deamidation pathway was inhibited in myeloid cells, but not T cells, in patients with CML or polycythemia vera. DNA damage did not increase levels of the amiloride-sensitive sodium–hydrogen exchanger isoform 1 (NHE-1), intracellular pH, Bcl-x\textsubscript{L} deamidation, and apoptosis. Inhibition of the pathway was reversed by enforced alkalinization or overexpression of NHE-1, leading to a restoration of apoptosis. In patients with CML, the pathway was blocked in CD34+ progenitor cells and mature myeloid cells. Imatinib or JAK2 inhibitors reversed inhibition of the pathway in cells from patients with CML and polycythemia vera, respectively, but not in cells from a patient with resistance to imatinib because of a mutation in the BCR-ABL kinase domain.

**CONCLUSIONS**

BCR-ABL and mutant JAK2 inhibit the Bcl-x\textsubscript{L} deamidation pathway and the apoptotic response to DNA damage in primary cells from patients with CML or polycythemia vera.
CHRONIC MYELOID LEUKEMIA (CML) AND polycythemia vera are clonal myeloproliferative disorders that are associated with the activation of distinct tyrosine kinases, the BCR-ABL fusion kinase and the Janus tyrosine kinase 2 (JAK2) mutation. In both disorders, patients usually present with chronic disease, which is readily controlled. However, for reasons that are unclear, both diseases carry a risk of progression to a blastic phase resembling acute leukemia that resists further therapy. The cellular prosurvival protein Bcl-xL is up-regulated in patients with CML and polycythemia vera and is thought to inhibit apoptosis. Moreover, BCR-ABL protein expression is associated with a reduced apoptotic response to genotoxic drugs, and quiescent CML stem cells, thought to be responsible for residual disease, are resistant to the apoptosis that tyrosine kinase inhibitors induce.

A pathway regulating the function of Bcl-xL has been described in several studies. In normal mouse thymocytes, DNA damage increases the activity of the amiloride-sensitive sodium–hydrogen exchanger isofrom 1 (NHE-1), thus raising the intracellular pH, which in turn causes nonenzymatic deamidation of Bcl-xL. This pathway to DNA damage was abolished in a mouse model of T-cell lymphoma. Thymocytes that were transformed by an activated Lck tyrosine kinase were unable to respond to DNA damage by increasing NHE-1 levels, Bcl-xL deamidation, or apoptosis. Inhibition of the NHE-1–Bcl-xL pathway does not appear to be a general feature of cancer, since Bcl-xL deamidation that is induced by damaged DNA is intact in cell lines of osteosarcoma and cervical, bladder, and ovarian cancers and in primary chronic lymphocytic leukemia cells. Because the relevance of Bcl-xL deamidation for human cancers associated with activated tyrosine kinases remains unclear, we examined the Bcl-xL deamidation pathway in cells from patients with CML and polycythemia vera.

## METHODS

### REAGENTS AND ANTIBODIES

We obtained etoposide, propidium iodide, and nigericin from Sigma; SNARF-1 from Molecular Probes; imatinib from Novartis; JAK inhibitor 1 from Calbiochem; and JAK2 inhibitors TG101209 and AT9283 from Astex Therapeutics. For Western blot analysis, we used antibodies Bcl-xL, Bcl-2, and AT9283 from Astex Therapeutics. For flow cytometry, we used antibodies CD2-FITC (product code, CD0201), CD3-FITC (product code, MHCD1304), and CD14-APC (product code, MHCD1405) (Caltag).

### PATIENTS

We collected peripheral-blood samples from patients with either CML or polycythemia vera and from healthy control subjects. All subjects provided written informed consent. The study was approved by the Cambridge and Eastern Region ethics committee. Of the 10 CML samples for which data are presented in Figure 1, 6 were from patients with newly diagnosed chronic-phase CML, 1 was from a patient in the accelerated phase, and 3 were from patients in the chronic phase who were receiving therapy. All patients with polycythemia vera whose data are presented in Figure 2 had stable, nontransformed disease and were receiving hydroxycarbamide therapy at the time of blood sampling. The six patients with CML whose data are presented in Figure 3 had newly diagnosed chronic-phase CML, and the patient with the imatinib resistance mutation (E255V) in Figure 4 was in the accelerated phase. All patients with CML had the BCR-ABL rearrangement; patients with polycythemia vera and idiopathic myelofibrosis had received a diagnosis on the basis of criteria that have been reported previously. The quantitative pyrosequencing assay for the JAK2 V617F mutation was performed as described previously.

### CELL PURIFICATION

Peripheral-blood samples from the patients and control subjects were centrifuged through Lymphoprep (Axis-Shield PoC), and the cells in the pellets (granulocytes) or the interphase (peripheral-blood mononuclear cells [PBMCs]) were then harvested for subsequent experiments. The myeloid origin

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of PBMCs and the purity of the granulocyte populations were checked by staining with CD2, CD3, and CD19 monoclonal antibodies, conjugated with fluorescein isothiocyanate, together with CD13-PE and CD14-APC, followed by flow cytometry. Of the PBMCs, 85 to 95% were of myeloid origin, and the granulocytes were more than 90% pure. (Representative flow cytometric data of cell preparations are shown in Fig. 1 in the Supplementary Appendix, available with the full text of this article at www.nejm.org.) Mobilized peripheral-blood samples from control subjects and samples of CML peripheral blood were used to purify CD34+ cells, first using a MACS CD34 MicroBead Kit (Miltenyi Biotec), followed by cell sorting to obtain 95% pure CD34+ cells maintained in RPMI medium with 10% fetal-calf serum.

**Cell Lines**

We used human hematopoietic cancer-cell lines K562, HEL, Daudi, DU528, Jum2, Karpas299, OPM2, and DOHH2 (for details, see Table 1 in the Supplementary Appendix). Cells were cultured in RPMI medium with 10% fetal-calf serum. Murine BaF3 cells expressing the thrombopoietin receptor (BaF3–TpoR) were cultured in RPMI medium with 10% fetal-calf serum containing 1 ng per milliliter of recombinant interleukin-3.
DNA Damage Treatments

Cells were irradiated with 5 Gy with the use of a cesium source or treated with 50 μM of etoposide in dimethyl sulfoxide (DMSO) for the times indicated. Carrier DMSO was added to control cells.

Immunoblotting and Measures of pH and Apoptosis

These analyses were performed as described previously. Intracellular pH was measured with the use of a pH-sensitive dye in conjunction with flow cytometry.

Transfection and Retroviral Transduction

Peripheral-blood mononuclear cells from patients with CML were transfected with plasmid NHE-1–internal ribosome entry site–enhanced green fluorescent protein (pNHE-1–IREs–EGFP) with the use of a Nucleofector kit (K562) (Amaza Biosystems). GFP-positive cells were sorted by flow cytometry with the use of a FACSAria flow cytometer (BD Biosciences). Murine stem-cell virus–IRES–GFP–based retroviral vectors MIG-BCR-ABL, MIG-Jak2V617F, and MIG-NPM-ALK were transfected into the Phoenix cell line with the use of Lipofectamine (Invitrogen), and culture supernatants were harvested 24 hours later. Viral infection of Baf3–TpoR cells was performed by spinoculation (1200 g for 90 minutes at 30°C). GFP-positive cells were cultured in RPMI medium with 10% fetal calf serum and sorted 2 days later by flow cytometry with the use of a FACSAria flow cytometer.

Statistical Analysis

We performed all statistical analyses using Student’s t-test. All P values are two-sided. A P value of less than 0.05 was considered to indicate statistical significance.
reproducible changes in basal NHE-1 levels were apparent (Fig. 1A, and Fig. 2 in the Supplementary Appendix). In marked contrast, we did not see such responses to DNA damage in granulocytes from patients with CML carrying the BCR-ABL tyrosine kinase (Fig. 1A and 1B). Both etoposide and irradiation caused significantly less apoptosis in CML granulocytes than in normal granulocytes (Fig. 1C), which was consistent with the genotoxic resistance for BCR-ABL–positive cells reported previously. In contrast, T lymphocytes from the same patients with CML did not resist alkalinization,  

Figure 2. Inhibition of the NHE-1–Bcl-xL Deamidation Pathway in Polycythemia Vera Cells Induced by DNA Damage. In Panel A, NHE-1 up-regulation induced by DNA damage is inhibited in polycythemia vera (PV) cells. Purified granulocytes (from three control subjects and eight patients with polycythemia vera) that were cultured in RPMI with 10% fetal-calf serum were treated with etoposide (Etop) for 3, 6, or 9 hours, as shown. Cells were lysed and subjected to immunoblotting for NHE-1 or tubulin. A representative blot is shown. NHE-1 relative intensities that were normalized for protein loading are shown below the immunoblots with untreated controls set at a value of 1.0. In Panel B, DNA damage–induced intracellular alkalinization is inhibited in polycythemia vera cells. Purified granulocytes that were cultured in RPMI with 10% fetal-calf serum were either treated with etoposide for 24 hours or exposed to 5 Gy of irradiation (IR) and then cultured for 24 hours. Intracellular pH was measured by flow cytometry. The mean (±SD) values of intracellular pH from three control subjects and eight patients with polycythemia vera are shown. Single asterisks indicate P>0.05, and double asterisks P<0.001. In Panel C, DNA damage–induced Bcl-xL deamidation is inhibited in polycythemia vera cells. Purified granulocytes from control subjects and patients with polycythemia vera were treated and analyzed, as described in Figure 1A. Representative blots from control subjects and patients with polycythemia vera are shown. In Panel D, DNA damage–induced apoptosis is inhibited in polycythemia vera cells. Apoptosis in purified granulocytes from control subjects and patients with polycythemia vera was analyzed, as described in Figure 1C. The single asterisk indicates P>0.05, and double asterisks indicate P<0.001.
Bcl-x<sub>L</sub> deamidation, and apoptosis induced by damaged DNA (Fig. 3 in the Supplementary Appendix).

To investigate the level at which the NHE-1–Bcl-x<sub>L</sub> deamidation pathway was blocked in CML cells, we exposed granulocytes from patients with CML to varying levels of external pH. Enforced alkalinization reversed the inhibition and restored Bcl-x<sub>L</sub> deamidation and apoptosis even in the absence of DNA damage, suggesting that the block was at the level of the NHE-1 antipporter (Fig. 4 in the Supplementary Appendix). Consistent with this interpretation, transfection of NHE-1 complementary DNA (cDNA) into CML cells resulted in an increase in NHE-1 levels by a factor of 2 to 3 and was accompanied by increased intracellular pH, Bcl-x<sub>L</sub> deamidation, and apoptosis (Fig. 1D to 1F).

We next studied patients with polycythemia vera, a disease that is associated with a gain-of-function point mutation in the cytoplasmic tyrosine kinase JAK2. Since peripheral-blood granulocytes and bone marrow progenitors from many patients with polycythemia vera contain a mixture of normal and mutant cells, we selected patients in whom JAK2 pyrosequencing demonstrated that the majority of peripheral-blood granulocytes carried the JAK2 mutation (Fig. 5 in the Supplementary Appendix). In contrast to granulocytes from control subjects, those from all eight patients with polycythemia vera who fulfilled these criteria consistently did not have a response to DNA damage by increasing NHE-1 levels, intracellular pH, or Bcl-x<sub>L</sub> deamidation (Fig. 2A to 2C). In addition, both etoposide and irradiation produced significantly less apoptosis in polycythemia vera granulocytes than in normal granulocytes (Fig. 2D). In contrast to the granulocytes, T cells from the same patients showed no defect in DNA damage–induced Bcl-x<sub>L</sub> deamidation, intracellular alkalinization, or apoptosis (Fig. 3 in the Supplementary Appendix).

As with CML cells, enforced alkalinization of polycythemia vera granulocytes overcame inhibition of the NHE-1–Bcl-x<sub>L</sub> deamidation pathway and was accompanied by increased Bcl-x<sub>L</sub> deamidation and apoptosis (Fig. 6 in the Supplementary Appendix). The Bcl-x<sub>L</sub> deamidation pathway was also inhibited in granulocytes from two patients with idiopathic myelofibrosis with the JAK2 V617F mutation but not in two patients with this disease without the JAK2 mutation (Fig. 7 in the Supplementary Appendix).

To elucidate further the correlation between tyrosine kinase expression and inhibition of the NHE-1–Bcl-x<sub>L</sub> pathway, we investigated eight cell lines representing different hematologic cancers associated with distinct molecular mechanisms (Table 1 and Fig. 8 in the Supplementary Appendix). The NHE-1–Bcl-x<sub>L</sub> pathway was inhibited in K562 cells carrying BCR-ABL and derived from a patient with CML in blast crisis and also in human erythroleukemia (HEL) cells, which carry the JAK2 V617F mutation and were derived from a patient with acute myeloid leukemia. These data suggest that the pathway remains inhibited after leukemic transformation, which was confirmed using blasts from a patient with CML in blast crisis (data not shown). In contrast, the pathway was intact in four other cell lines that are not thought to express oncogenic tyrosine kinases (Table 1 and Fig. 8 in the Supplementary Appendix). The pathway was also intact in a T-lymphoma cell line (Karpas-299), which expresses the NPM-ALK tyrosine kinase fusion protein, and in a myeloma cell line (OPM-2), which overexpresses the fibroblast growth factor receptor 3 (FGFR3) tyrosine kinase. These results suggest that inhibition of the Bcl-x<sub>L</sub> deamidation pathway is not a general feature of hematologic cancers and is mediated by a subgroup of tyrosine kinases or is dependent on a particular cellular context.

To address whether inhibition of the NHE-1–Bcl-x<sub>L</sub> pathway is influenced by kinase strength, we performed a series of experiments using a BaF3–TPoR cell line transduced with BCR-ABL, JAK2 V617F, or NPM-ALK tyrosine kinases, giving rise to several cell populations with varying kinase expression levels (Fig. 9 in the Supplementary Appendix). Levels of BCR-ABL and JAK2 V617F expression correlated well with the degree of inhibition of the Bcl-x<sub>L</sub> deamidation pathway, whereas NPM-ALK caused no inhibition even at its highest expression level.

Both CML and polycythemia vera are thought to arise from a transformed multipotent stem cell. BCR-ABL is expressed at high levels in stem and progenitor cells and induces expansion of the progenitor compartment during chronic-phase CML. We therefore assessed the status of the NHE-1–Bcl-x<sub>L</sub> pathway in normal and CML progenitors that expressed the CD34 stem-cell antigen. In normal CD34+ cells, but not in those derived from patients with CML, etoposide treatment increased NHE-1 levels, Bcl-x<sub>L</sub> deamidation,
intracellular alkalinization, and apoptosis (Fig. 3A to 3C). Furthermore, in normal CD34+ cells, apoptosis that was triggered by DNA damage was significantly inhibited by dimethylamiloride, a selective NHE-1 antiport inhibitor (Fig. 10 in the Supplementary Appendix). These data demonstrate that the Bcl-xL deamidation pathway operates in normal CD34+ cells and is inhibited in CD34+ cells from patients with CML.

We next addressed whether inhibition of the Bcl-xL deamidation pathway was dependent on aberrant kinase activity. Granulocytes from three patients with polycythemia vera were exposed to three different JAK2 inhibitors. All three inhibitors reversed the block of the pathway: Bcl-xL deamidation, intracellular pH, and apoptosis all increased in response to DNA damage (Fig. 3D, and Fig. 11 in the Supplementary Appendix).
Imatinib and the JAK2 inhibitors inhibit the activity of several kinases in addition to BCR-ABL and JAK2, respectively, raising the possibility that inhibition of other kinases may contribute to the observed effects. We therefore studied a patient who had become resistant to imatinib as a consequence of an E255V mutation in the BCR-ABL kinase domain. Imatinib-treated PBMCs from this patient did not have increased NHE-1 levels in response to etoposide or Bcl-xL deamidation in response to either etoposide or irradiation (Fig. 4A and 4B). However, the effects of imatinib resistance could be bypassed by NHE-1 overexpression, which caused increased intracellular pH and apoptosis (Fig. 4C and 4D), or by enforced alkalization, which caused increased Bcl-xL deamidation and apoptosis (Fig. 12 in the Supplementary Appendix). These data demonstrate that BCR-ABL kinase activity is essential for inhibition of the Bcl-xL deamidation pathway in CML cells.

**DISCUSSION**

Deamidation of internal asparagine or glutamine residues can have a profound influence on protein function and has been implicated in a wide range of biologic processes. Rates of asparagine deamidation were initially thought to be fixed and determined solely by the structural context of a given asparagine residue, but an active role in the regulation of biologic processes was suggested by the observations that DNA damage can trigger rapid deamidation of Bcl-xL and that Bcl-xL deamidation plays a central role in the apoptotic response of normal mouse thymocytes to DNA damage.

In our study, we found that the signaling pathway leading from DNA damage to Bcl-xL deamidation and consequent apoptosis is inhibited in two myeloproliferative disorders associated with different tyrosine kinases and activated by distinct mechanisms (Fig. 5). We demonstrated this defect in cells from all 20 patients bearing either BCR-ABL or the JAK2 V617F mutation, whereas no defect was observed in cells expressing other oncogenic tyrosine kinases, such as NPM-ALK. To suppress the apoptotic response to DNA damage, it is insufficient for BCR-ABL or mutant JAK2 merely to up-regulate or maintain Bcl-xL expres-
sion levels. In addition, both oncogenic tyrosine kinases must prevent deamidation of Bcl-xL to preserve its antiapoptotic function. These observations not only shed light on the accumulation of DNA damage that is characteristic of these cancers but also have potential therapeutic relevance.

CML and polycythemia vera are associated with an increased risk of leukemic transformation, which is thought to reflect the accrual of additional genetic lesions. However, it is not clear why stem cells from patients with chronic-phase CML and polycythemia vera are prone to accumulate DNA damage. Normal cells undergo many DNA strand breaks per genome per cell division, and adequate DNA repair mechanisms, combined with the removal of damaged cells by apoptosis, are therefore essential for homeostasis. Inhibition of the Bcl-x deamidation pathway in chronic-phase CML and polycythemia vera...
provides a mechanism for circumventing the apoptotic response and permitting accumulation of DNA damage within the malignant clone.

It has been reported that cells expressing oncogenic tyrosine kinases, including BCR-ABL, are resistant to DNA damage.\(^{32-34}\) Resistance to DNA-damaging agents depends on BCR-ABL catalytic activity.\(^{32}\) Consistent with this finding, the combination of antileukemic chemotherapy with the tyrosine kinase inhibitor imatinib produces increased or synergistic apoptosis.\(^{35-38}\) Our results shed light on the molecular basis for the effectiveness of such combination therapies. Inhibition of BCR-ABL by imatinib is associated with increased levels of proapoptotic BH3-only proteins, such as Bim and Bad.\(^{39-41}\) The activity of these molecules is constrained by their binding to Bcl-x\(_L\). Optimal apoptosis in response to DNA damage requires that imatinib restore the normal Bcl-x\(_L\) deamidation pathway, thus minimizing sequestration by Bcl-x\(_L\) and maximizing apoptosis in response to DNA-damaging agents.

The use of tyrosine kinase inhibitors in CML faces two main challenges. Acquired resistance to imatinib therapy can result in relapse, often as a consequence of kinase-domain mutations in BCR-ABL.\(^{42}\) In addition, treatment of CML with tyrosine kinase inhibitors is usually associated with persistence of residual disease, which is thought to reflect quiescent BCR-ABL–positive stem cells that resist current tyrosine kinase inhibitors.\(^{9-11}\) It is therefore notable that an increase in the expression of NHE-1 by a factor of 2 to 3 was sufficient to increase Bcl-x\(_L\) deamidation and triple the level of apoptosis in imatinib-resistant CML cells (Fig. 4D). Therefore, targeted stimulation of Bcl-x\(_L\) deamidation provides a potential route for circumventing resistance to tyrosine kinase inhibitors and perhaps also for eradicating leukemic stem cells.

The NHE-1 antiport itself represents a potential therapeutic target. Small-molecule inhibitors already exist, although the development of agonists may be more challenging. The NHE-1 anti-
port can also be activated by phosphorylation, which suggests the possibility of other therapeutic approaches. The fact that modulation of the NHE-1–Bcl-xL signaling pathway can bypass resistance to apoptosis in patients with CML and polycythemia vera raises the possibility of new therapeutic approaches that could be of general relevance to any cancer in which Bcl-xL plays an important role in genotoxic resistance. Indeed, Bcl-xL expression in a wide range of cancers has a striking correlation with resistance to genotoxic compounds, which suggests that our findings are likely to have relevance well beyond the myeloproliferative disorders.

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