Published in final edited form as:

Oncogene. 2013 April 4; 32(14): 1784–1793. doi:10.1038/onc.2012.203.

Targeting abnormal DNA double strand break repair in tyrosine kinase inhibitor-resistant chronic myeloid leukemias

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Abstract

Resistance to imatinib (IM) and other BCR-ABL1 tyrosine kinase inhibitors (TKI)s is an increasing problem in leukemias caused by expression of BCR-ABL1. Since chronic myeloid leukemia (CML) cell lines expressing BCR-ABL1 utilize an alternative non-homologous end-joining pathway (ALT NHEJ) to repair DNA double strand breaks (DSB)s, we asked whether this repair pathway is a novel therapeutic target in TKI-resistant disease. Notably, the steady state levels of two ALT NHEJ proteins, poly-(ADP-ribose) polymerase 1 (PARP1) and DNA ligase III α were increased in the BCR-ABL1-positive CML cell line K562 and, to a greater extent, in its imatinib resistant (IMR) derivative. Incubation of these cell lines with a combination of DNA ligase and PARP inhibitors inhibited ALT NHEJ and selectively decreased survival with the effect being greater in the IMR derivative. Similar results were obtained with TKI-resistant derivatives of two hematopoietic cell lines that had been engineered to stably express BCR-ABL1. Together our results show that the sensitivity of cell lines expressing BCR-ABL1 to the combination of DNA ligase and PARP inhibitors correlates with the steady state levels of PARP1 and DNA ligase III α , and ALT NHEJ activity. Importantly, analysis of clinical samples from CML patients confirmed that the expression levels of PARP1 and DNA ligase III α correlated with sensitivity to the DNA repair inhibitor combination. Thus, the expression levels of PARP1 and DNA ligase III α serve as biomarkers to identify a subgroup of CML patients who may be candidates for therapies that target the ALT NHEJ pathway when treatment with TKIs has failed.

Keywords

Chronic myeloid leukemia; DNA ligase III α ; PARP1; NHEJ; PARP inhibitors; DNA ligase I and III inhibitors

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Conflict of interest

AET is a co-inventor on a patent application that covers the use of DNA ligase inhibitors as anti-cancer agents.

Introduction

Chronic myelogenous leukemia (CML) is a hematological malignancy characterized by increased and unregulated growth of myeloid cells in the bone marrow (BM), and accumulation of excessive white blood cells(1, 2). In most cases, this is caused by the expression of the BCR-ABL1 fusion protein, a constitutively active tyrosine kinase (TK)(3, 4). The ABL-specific inhibitor, imatinib mesylate (IM), is currently used as first line therapy for CML. Although responses in chronic phase CML tend to be durable, relapse after an initial response is common in patients with more advanced disease (5–11). Approximately 50% of imatinib resistant (IMR) patients have acquired mutations in BCR-ABL1 (12), particularly within and around the ATP-binding pocket of the ABL kinase domain. Although second generation TK inhibitors (TKI)s inhibit all of the BCR-ABL1 mutants except T315I, resistance to these inhibitors is also being reported (13, 14). Thus, the development of novel therapies is critically important for patients with acquired resistance to BCR-ABL1-directed TKIs.

Expression of the BCR-ABL1 kinase induces production of reactive oxygen species (ROS) that, in turn, cause DNA damage including double strand breaks (DSB)s (15–20).

Previously, we have shown that CML cells respond to increasing DNA damage with enhanced DNA repair processes (15, 21). DNA-dependent protein kinase (DNA PK)-dependent nonhomologous end joining (NHEJ) is one of the main pathways for repairing DSBs in mammalian cells. It is initiated by binding of the Ku70/86 heterodimer to DSBs, followed by the recruitment of the DNA PK catalytic subunit to form active DNA PK (22–24). After protein-mediated end-bridging, the DNA ends are processed by a combination of nucleases and polymerases, and then joined by DNA ligase IV in conjunction with XRCC4 and XLF (25–27). Repair of DSBs by this pathway usually results in the addition or loss of few nucleotides at the break site but rarely involves the joining of previously unlinked DNA molecules. In addition to DNAPK-dependent NHEJ, there is a highly error-prone version of NHEJ, alternative (ALT) NHEJ, that is characterized by a high frequency of large deletions, chromosomal translocations, and short tracts of microhomologies at the repaired site (28). We showed recently that the abnormal DSB repair in BCR-ABL1-positive CML was due to reduced activity of DNA PK-dependent NHEJ and increased activity of ALT NHEJ (29). Furthermore, “knockdown” of DNA ligase III α , a participant in ALT NHEJ, resulted in increased accumulation of unrepaired DSBs and reduced survival, suggesting that ALT NHEJ pathway components, such as PARP1 and DNA ligase III α (29–35) may be novel therapeutic targets in cancer cells that are more dependent on ALT NHEJ for DSB repair.

The recent development of PARP inhibitors, which selectively target the DSB repair defect in hereditary breast cancers (36, 37), has stimulated interest in the use of DNA repair inhibitors as cancer therapeutics. Since DNA ligation is the final step of almost all DNA repair pathways, we used a structure-based drug design approach to identify small molecule inhibitors with different specificities for the three human DNA ligases (38, 39). As expected, a subset of these inhibitors potentiated the cytotoxicity of DNA-damaging agents, but, interestingly, this effect was more pronounced in cancer cells (38, 39). Since BCR-ABL1-positive CML cells have abnormal DSB repair (29), we have examined the effect of PARP1 inhibitors on TKI-sensitive and -resistant CML cells in the presence or absence of a DNA ligase inhibitor. Our results provide evidence that targeting ALT NHEJ with a combination of DNA ligase and PARP inhibitors is a potentially novel therapeutic strategy for CML patients who fail TKI therapy.

Results

Generation and characterization of IMR BCR-ABL1-positive cell lines

IMR derivatives of the CML IM sensitive (IMS) cell line K562, and the hematopoietic cell lines, Mo7e-P210 and Baf3-P210 that had been engineered to stably express BCR-ABL1 (Figure S1A–C and Table S1), were selected by growth in IM-containing media. The IMR cell lines, Mo7e-P210 IMR2 and Baf3-P210 IMR, had acquired mutations within BCR-ABL1 resulting in D276G and T315I amino acid changes, respectively. Notably, these amino acid changes have been observed in IMR CML patients (Table S1, 6, 9). While BCR-ABL1 was neither overexpressed nor mutated in the K562 IMR and Mo7e-P210 IMR1 cell lines, the Mo7e-P210 IMR1 cells had increased RAS activation and phosphorylation of AKT compared to Mo7e-P210 (Figure S1D–E), suggesting that activation of parallel signaling pathways may contribute to the IMR of these cells(40). Importantly, our IMR cell lines recapitulate different mechanisms of resistance to TKIs that have been described in IMR CML patients (6, 7, 9).

Altered expression of DNA repair proteins in IMS and IMR BCR-ABL1-positive cell lines

Since we had shown previously that the steady-state levels of the ALT NHEJ protein, DNA ligase III α were higher in K562 leukemia cells compared with B cell lines established from normal individuals (29), we examined the steady state protein levels of key DNAPK-dependent and ALT NHEJ proteins in other cell lines expressing BCR-ABL1. In addition to DNA ligase III α , the steady-state levels of another ALT NHEJ protein, PARP1 (29–35), was also elevated in K562 compared to NC10 cells ($p < 0.05$, Figure 1A–B). The NC10 cells are not genetically related to K562 cells so the alterations in the steady state levels of DNA ligase III α and PARP1 could be due to intrinsic differences between the cell lines rather than BCR-ABL1 expression. However, the steady state levels of DNA ligase III α and PARP1 were also increased in the derivatives of the hematopoietic cell lines, Mo7e and Baf3, that express BCR-ABL1 ($p < 0.05$, Figure 1C) albeit to a lesser extent than in the K562 cells. Thus, we conclude that BCR-ABL1 expression does result in increased steady state levels of DNA ligase III α and PARP1. While the IMR derivative of K562 expressed similar levels of DNA ligase III α and PARP1 compared to parental K562 cells (Figure 1B), the steady-state levels of these proteins were significantly increased in the IMR derivatives of Mo7e-P210 and Baf3-P210 compared with their IMS counterparts ($p < 0.05$, Figure 1C). As we reported previously (29), there was a small reduction in the steady state levels of the DNAPK-dependent NHEJ factors, DNA ligase IV and Ku70, in K562 cells compared to NC10 cells (Figure 1A–B). There was, however, a significant decrease in Ku70 levels in the K562 IMR cells ($p < 0.05$; Figure 1A–B). No significant changes in the levels of DNA ligase IV and Ku70 were observed in the IMS and IMR derivatives of Mo7e and Baf3 expressing BCR-ABL1 compared with their parental cells (Figure 1C).

BCR-ABL1-positive cell lines are hypersensitive to the combination of DNA ligase and PARP inhibitors

Since all the BCR-ABL1-positive cell lines have increased steady state levels of DNA ligase III α and PARP1, we asked whether they exhibited increased sensitivity to inhibitors of these proteins. In the absence of a DNA ligase III α -specific inhibitor, we examined the effects of L67, which inhibits DNA ligases I and III α but not DNA ligase IV (38) and the PARP inhibitor NU1025 (41). None of the hematopoietic cell lines, Mo7e, Mo7e-P210 (Figure S2A–B), Baf3 or Baf3-P210 (data not shown) exhibited significantly increased sensitivity to either L67 or NU1025 alone, with the agents reducing growth and viability of all cell lines with IC50s of about 3.5 μ M and 500 μ M, respectively. This prompted us to examine the effect of lower concentrations of L67 (0.3 μ M) and NU1025 (50 μ M), alone and in combination, in colony survival assays. Under these conditions, NC10, K562 and K562 IMR

were relatively insensitive to L67 alone (Figure 2A) whereas only K562 IMR cells exhibited significantly increased sensitivity to NU1025 ($p < 0.05$; Figure 2A). Notably, both K562 and, to an even greater extent, K562 IMR cells, exhibited significantly increased hypersensitivity to the combination of DNA repair inhibitors compared with either agent alone and NC10 cells ($p < 0.05$; Figure 2A). In similar experiments with the BCR-ABL1-transfected cell lines, both IMR derivatives of Mo7e-P210 and the IMR derivative of Baf3-P210 also exhibited significantly reduced colony survival with the repair inhibitor combination ($p < 0.05$; Figure 2B). To determine whether the activity of L67 can be specifically attributed to its effects on DNA ligase III α , colony survival assays were performed following siRNA knockdown of DNA ligase III α . Reducing the steady state levels of DNA ligase III α by about 50% in combination with PARP inhibitor (Figure 2C) resulted in a similar reduction in colony survival as the treatment with L67 and PARP inhibitor (Figure 2D), demonstrating that the activity of L67 is due to its inhibition of DNA ligase III α .

Combination of DNA repair inhibitors increase DSBs and inhibits ALT NHEJ in BCR-ABL1-positive cell lines

To determine whether the altered levels of NHEJ proteins in cells that express BCR-ABL1 result in abnormal repair of DSBs, we first measured the percentage of cells with more than 3 γ H2AX foci/cell, as an indicator of unrepaired spontaneous DSBs (42). As expected, the cell lines expressing BCR-ABL1 had more spontaneous DSBs than control cell lines (Figure 3A–C,29). Notably, all of the IMR derivatives had significantly higher levels of spontaneous DSBs compared with IMS cell lines, suggesting that these cells have higher levels of endogenous DNA damaging agents and/or a more pronounced DNA repair defect. Treatment of the cells with the DNA repair inhibitor combination increased the number of unrepaired DSBs with the effect being the greatest in the cells expressing BCR-ABL1 ($p < 0.05$; Figure 3A–C). Since both PARP1 and DNA ligase III α participate in the repair of single strand breaks (SSB)s as well as in ALT NHEJ (29–35), inhibition of these enzymes may increase the levels of unrepaired DSBs by inhibiting the repair of DSBs by ALT NHEJ, in addition to increasing the number of replication-induced DSBs as a consequence of reduced SSB repair.

To measure the repair of DSBs by NHEJ and determine the effect of the DNA repair inhibitor combination, we used a plasmid-based repair assay with an EcoR1-linearized plasmid substrate (21). The overall level of plasmid repair was significantly higher in both K562 cells and its IMR derivative compared with the NC10 cells with increases in both accurate (blue colonies) and, to an even greater extent, inaccurate (white colonies) repair (Figure 4A). Similar results were obtained in the IMS and IMR derivatives of the hematopoietic cell lines, Mo7e and Baf3 that express BCR-ABL1 although the increase in inaccurate repair was less in the Mo7e derivatives (Figure 4A).

Since the white colonies may be a result of either small insertions or deletions generated by DNA PK-dependent NHEJ or larger deletions that are characteristic of ALT NHEJ, the plasmids from the white colonies were sequenced to detect the molecular signatures, microhomologies and deletion size at the repair site, that distinguish ALT from DNAPK-dependent NHEJ. As expected, the average size of DNA deletions (Figure 4B) and frequency of microhomologies (> 2 bp, Figure 4C) in repaired plasmids was higher in the K562 cells compared to NC10, indicating increased ALT NHEJ activity (29). There was no significant difference in the average size of deletions generated by the IMS and IMR derivatives of K562 (Figure 4B) but there was an increase in the frequency of microhomologies at the repair site in the IMR derivative (Figure 4C). It is possible that the increase in microhomology-mediated repair events is due to the reduced levels of Ku70 in the IMR derivative of K562 (Figure 1A–B). In similar experiments with the BCR-ABL1-transfected hematopoietic cell lines, the average size of deletions and the frequency of

microhomology-mediated repair events was higher in the IMS lines compared with the parental cells and even higher in the IMR cell lines (Figure 4D–E). Thus, the contribution of ALT NHEJ to DSB repair correlates with the extent of PARP1 and DNA ligase III α overexpression in these cell lines. Treatment with the DNA repair inhibitor combination reduced the abnormalities in DNA repair observed in IMS and IMR cells so that deletion size and the frequency of microhomology-mediated repair resembled that of normal cells (Figure 4B–E).

Taken together, our results indicate that cell lines expressing BCR-ABL1 are more dependent on ALT NHEJ for DSB repair than comparable normal cells and that the dependence upon ALT NHEJ increases during the acquisition of resistance to IM. Since the repair of DSBs by ALT NHEJ is error-prone, resulting in large deletions and chromosomal translocations (28), there should be increased genomic instability in IMS cells and to an even greater extent in IMR cells. Thus, we analyzed genomic deletions and insertions in Mo7e-P210 IMR1, Mo7e-P210 and Mo7e cells, using High-Resolution Discovery 1M CGH human microarrays. Using this approach we detected 6 deleted regions, equivalent to approximately 320 Mb of DNA, Mo7e-P210 cells compared to Mo7e cells (Figure 5A and C). The Mo7e-P210 IMR1 cells had acquired 7 additional deletions, equivalent to approximately 420 Mb of DNA, compared with the Mo7e-P210 cells (Figure 5B and C). Thus, 15 large deletion events occurred, resulting in the loss of 720 Mb of DNA, during the transition from BCR-ABL1 negative Mo7e cells to an IMR derivative expressing BCR-ABL1. In addition, our CGH analysis also showed amplification events: Two regions (equivalent approximately to 40 Mb) were amplified in Mo7e-P210 compared to Mo7e. In contrast, the transition from Mo7e-P210 to Mo7e-P210 IMR1 involved an additional 2 amplifications (equivalent approximately to 30 Mb). Thus, in transitioning from BCR-ABL1 negative cells (Mo7e) to Mo7e-P210 IMR1 there was a gain of DNA in 4 regions (equivalent to 70 Mb).

Overexpression of DNA ligase III α and PARP1 in primary cells from BCR-ABL1 CML patients correlates with sensitivity to the DNA repair inhibitor combination

Our cell culture studies suggest that the expression levels of DNA ligase III α and PARP1 can be used as biomarkers to identify leukemia cells from CML patients that will be specifically hypersensitive to the combination of L67 and NU1025. To test this hypothesis, we examined BM mononuclear cells (BMMNC) from 8 IMS and 11 IMR CML patients (Table 1, Figure S3A) and found increased expression of both DNA ligase III α and PARP1 mRNAs in 10/19 (53%) BMMNC (IMS: PT11, 12, 18, 10A and IMR: PT9, 10B, 2, 14, 17 and 19) compared to NBM ($p < 0.05$; Table 1, Figure 6A). Moreover, 4/19 (21%) BMMNC (IMS: PT1, 13, 15 and IMR: PT8) expressed elevated levels of either DNA ligase III α or PARP1 ($p < 0.05$; Table 1, Figure 6A). The remaining 5/19 (26%) BMMNC (IMS: PT3 and IMR: PT16, 4, 6, 7) expressed levels of DNA ligase III α and PARP1 comparable to NBM (Table 1, Figure 6A). We next determined the sensitivity of the BMMNC from the CML patients to the combination of L67 and PARP inhibitors in colony survival assays using NBM as control (Table 1, Figure 6B, **S3B**). Based on their sensitivity to L67 and PARP inhibitors, the leukemia cells can be divided into 3 groups: BMMNC that were; (i) hypersensitive to the combination of L67 and NU1025 with a significant reduction in colony formation compared to either inhibitor alone (PT2, 10A, 10B, 11, 12, 14, 17, 18, 19; $p < 0.005$); (ii) partially sensitive to the inhibitor combination due to inhibition of colony formation by either the DNA ligase or PARP inhibitor (PT1, 8, 9, 13, 15; $p < 0.05$) and (iii) insensitive to the combination (PT3, 4, 6, 7, 16). Notably, 90% of the BMMNC samples that were hypersensitive to the DNA repair inhibitor combination had increased levels of both DNA ligase III α and PARP1 ($p < 0.05$, Table 1, Figure 6A–B, **S3B**) and two patient samples (PT2 and 19) within this subgroup expressed the T315I version of BCR-ABL1 (Table 1) that

is resistant to all current TKIs (13, 14). BMMNC samples that exhibited partial sensitivity to the DNA repair inhibitor combination had increased expression of either DNA ligase III α or PARP1 mRNA in 80% of the samples ($p < 0.05$, Table 1, Figure 6A–B, **S3B**) whereas all insensitive BMMNC samples had levels of DNA ligase III α and PARP1 comparable to those of NBM (Table 1, Figure 6A–B, **S3B**). Hypersensitivity to the combination of DNA repair inhibitors was observed in all samples from patients in blast crisis (Table 1). Interestingly, BMMNC from PT10A, whose disease rapidly progressed from IMS chronic phase to IMR blast crisis (PT10B), exhibited similar sensitivity to the combination of DNA repair inhibitors at both stages of the disease (Table 1, Figure 6A–B, **S3B**).

Discussion

Alterations in the network of pathways that respond to DNA damage and maintain genome stability are presumed to underlie the genomic instability of cancer cells and their increased sensitivity to cytotoxic DNA damaging agents. Although abnormalities in the DNA damage response are poorly defined, particularly in sporadic cancers, they are potential targets for the development of therapeutics that either alone or in combination with cytotoxic DNA damaging agents, preferentially enhance killing of cancer cells. This rationale led to the development of PARP inhibitors that specifically kill cancer cells in inherited forms of breast cancer because cancer but not normal cells have a defect in the repair of DSBs (41).

There is compelling evidence that the repair of DSBs in BCR-ABL1-positive CML cells is abnormal (17, 21, 29). We have shown previously that these cells preferentially utilize a highly error-prone ALT NHEJ pathway that likely contributes to disease progression by causing increased genome instability (29). The increased contribution of the ALT NHEJ pathway to DSB repair in the BCR-ABL1-positive CML cells is due, at least in part, to increased steady state levels of the ALT NHEJ factors, DNA ligase III α and WRN (29). Although IM and other related TKIs are an effective frontline therapy for BCR-ABL1-positive CML, there is a lack of effective treatment options for patients whose disease has become resistant to TKIs (13, 14). This prompted us to examine the DNA repair properties of 4 BCR-ABL1-positive cell lines that were selected for IMR by long-term culture in the presence of IM. In accord with what is observed in patients with IMR CML (6, 9) two of the IMR cell lines had acquired mutations in BCR-ABL1 whereas two had not. Notably, the mutations in BCR-ABL1 resulted in amino acid changes, D276G and T315I, that have been observed in IMR CML patients (6, 9). Using a plasmid-based NHEJ assay, we found that the contribution of ALT NHEJ to DSB repair was even higher in the IMR cell lines than previously observed in IMS cell lines (29) and correlated with increased expression of the ALT NHEJ factors, PARP1 and DNA ligase III α in the 3 IMR hematopoietic cell lines transfected with BCR-ABL1. The increased steady state level of endogenous DSBs in BCR-ABL1-positive cells is due, at least in part, to increased levels of ROS (15–20). It is also likely that inefficient DSB repair by ALT NHEJ contributes to the increased number of unrepaired DSBs (15, 21, 29). In the IMR cell lines, there were even higher levels of endogenous DSBs, presumably reflecting the larger role of the inefficient error-prone ALT NHEJ pathway in DSB repair. The increased dependence of BCR-ABL1-positive cells and, in particular, the IMR cells on ALT NHEJ for the repair of DSBs makes this pathway an attractive potential cancer cell-specific therapeutic target.

Since PARP1 participates both in the repair of SSBs and ALT NHEJ (29–35), we postulated that PARP inhibitors would sensitize cells with increased dependence on ALT NHEJ because they concomitantly cause replication-associated DSBs by blocking SSB repair (36, 37) and inhibit PARP1-dependent ALT NHEJ. Despite the elevated steady state levels of PARP1 in the IMR BCR-ABL1-positive cell lines, the PARP inhibitor did not preferentially target either the IMR or the IMS cells. Similar results were obtained with a DNA ligase

inhibitor, L67, which inhibits DNA ligase I and III α . Notably, a combination of the DNA ligase and PARP inhibitors did preferentially kill all the IMR BCR-ABL1-positive cell lines, including the cell line expressing the T315I version of BCR-ABL1, that is refractory to all current TKIs (13, 14). Since treatment with the repair inhibitor combination, whose activity is dependent upon DNA ligase III α inhibition, also increased the level of DSBs and inhibited ALT NHEJ, it appears that the hypersensitivity of the IMR cell lines is due, at least in part, to the targeting of the ALT NHEJ pathway by the repair inhibitors. Like PARP1, DNA ligase III α participates in both SSB repair and ALT NHEJ (29–35). Thus, it is possible that partial inhibition of two components in the same pathway has an additive effect in terms of inhibition of the overall repair pathways of ALT NHEJ and SSB repair. Alternatively, the efficacy of the repair inhibitor combination may also be due to the targeting of other cellular transactions in addition to ALT NHEJ and SSB repair. For example, the PARP inhibitor may target cellular functions involving other members of the PARP family (43) in addition to PARP1 whereas base excision repair and mitochondrial DNA metabolism will also be impacted by inhibition of DNA ligase III α (44, 45).

Although detectable, the contribution of ALT NHEJ to DSB repair is normally minor in cells with a functional DNA PK-dependent NHEJ pathway (28) with Ku playing a major role in suppressing ALT NHEJ(46). Except for the IMR derivative of the K562 leukemia cell line, the levels of Ku in cell lines expressing BCR-ABL1 were not significantly reduced. It seems unlikely that the increased contribution of ALT NHEJ to DSB repair is due solely to the increased steady state levels of DNA ligase III α and PARP1, suggesting that, during the acquisition of IMR, there are other changes that reduce the activity of DNA PK-dependent NHEJ. Since the DNA end-binding activity of Ku is inhibited by oxidative stress(47), it is conceivable that the reduced activity of DNA PK-dependent NHEJ in IMS and IMR cells expressing BCR-ABL1 may be due to the increased levels of ROS (15–20). Alternatively, DNA PK-dependent NHEJ activity may be reduced in IMS and IMR cells expressing BCR-ABL1 because of increased end resection, a common step in both homologous recombination and ALT NHEJ that inhibits DNA end-binding by Ku (48–50).

Irrespective of the exact mechanism, the results of our cell line studies demonstrate that IMR cells expressing BCR-ABL1 are more dependent upon DNA ligase III α -dependent ALT NHEJ for the repair of DSBs and that PARP1 and DNA ligase III α expression levels may serve as biomarkers to identify patients with TKI-resistant CML whose disease will respond to therapies that target ALT NHEJ. Our analysis of primary samples from CML patients confirmed that overexpression of both PARP1 and DNA ligase III α correlated with hypersensitivity to the combination of DNA ligase and PARP inhibitors in 90% patients with both IMS and IMR disease. Since we observed elevated steady state levels of DNA ligase III α and PARP1 in the absence of BCR-ABL1 mutations in our cell line studies and in BMMNC from IMS and IMR CML patients, these changes are not absolutely dependent on BCR-ABL1 mutations. Among the 9 BMMNC samples from patients with IMR disease, three had acquired mutations in BCR-ABL1 with two of these encoding the T315I version of BCR-ABL1 that is resistant to all current TKIs. In accord with our cell culture studies, the BMMNC samples expressing BCR-ABL1 T315I had elevated steady state levels of both DNA ligase III α and PARP1 and were sensitive to the combination of DNA repair inhibitors. Other mechanisms of resistance, including BCR-ABL1 amplification and activation of parallel signaling pathways that have been described in about 50% of CML patients with TKI-resistant disease (6, 7, 9, 40) presumably also contribute to the elevated levels of DNA ligase III α and PARP1. Importantly, 50% of BMMNC from patients with IMR disease and all patients in blast crisis had elevated steady state levels of DNA ligase III α and PARP1 and were hypersensitive to the DNA repair inhibitor combination. Taken together, these results provide strong evidence that a DNA repair abnormality, increased dependence upon ALT NHEJ, can be identified and targeted in a significant fraction of

CML patients, who have acquired resistance to the frontline therapy and for whom there are currently no good treatment options. There is emerging evidence that this abnormality in DSB repair may also occur in a significant fraction of cell lines derived from different solid tumors(38)and in forms of breast cancer with acquired or intrinsic resistance to anti-estrogens (51). Thus, the strategy of targeting ALT NHEJ may also be applicable to a wide range of solid tumors.

Materials and methods

Cell Culture

The BCR-ABL1-positive human CML cell line, K562, was from ATCC (Manassas, VA). NC10, a BCR-ABL1-negative human lymphoblastoid cell line established from normal lymphocytes was obtained from Dr. Gazdar (University of Texas Southwestern, Dallas, TX). Mo7e, a BCR-ABL1-negative human myeloid leukemia cell line, and Mo7e stably expressing BCR-ABL1 (Mo7e-P210), were obtained from Dr Van Etten (Tufts University, Boston, MA). Baf3, a BCR-ABL1-negative murine hematopoietic progenitor cell line and Baf3 stably expressing BCR-ABL1 (Baf3-P210) were obtained from Dr Deininger (Oregon Health and Science University, Portland, OR). IMR derivatives were generated by growing IM-sensitive (IMS) cell lines in 2 μ M IM. Different clones (K562 IMR, Mo7e-P210 IMR1, Mo7e-P210 IMR2 and Baf3-P210 IMR) were selected by serial dilution under IM selection (Figure S1A–C and Table S1). All cells were cultured in RPMI 1640 (Sigma-Aldrich, St Louis, MO) with 4 mM L-glutamine (Cellgro, Manassas, VA), 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (FBS; Sigma-Aldrich) at 37°C in 5% CO₂, supplemented with 10 ng/mL GM-CSF and IL-3 for Mo7e and Baf3, respectively (Millipore, Temecula, CA). Media for IMR cell lines included 2 μ M IM. Normal human bone marrow (NBM) samples and bone marrow mononuclear cells (BMMNC) from IMS and IMR CML patients (Figure S3A, Table 1) were cultured in HPGM (Lonza, Walkersville, MD) supplemented with 1 ng/mL G-CSF (Calbiochem, Merck, Gibbstown, NJ), 25 ng/mL SCF (Calbiochem) and 10 ng/mL GM-CSF, IL-3, and IL-6 (Millipore).

Colony Survival Assays

Cells were seeded at a density of 700 cells/well in methylcellulose-based medium in the presence of the DNA ligases I and III inhibitor, L67 (0.3 μ M), the PARP inhibitor, NU1025 (50 μ M); L67 and NU1025, or IM (1–4 μ M) for approximately 10 days. Colonies were stained overnight with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (1 mg/ml, Sigma-Aldrich) before counting using an automated image analysis system (Omincon FAS IV, BIOSYS GmbH, Karben, Germany).

siRNA

ON-TARGET plus SMART pool human DNA ligase III α (L0009227) or non targeting control (D001810) siRNAs from Dharmacon RNA Technologies (Thermo Scientific, Chicago, IL) were transiently transfected into cells (0.1 nmolsiRNA/10⁶ cells) using Amaxa Nucleofector Kit V (VCA-1003) in a Nucleofector II Amaxa biosystems (Lonza, Allendale, NJ) according to manufacturer's instructions. For colony survival assays, NU1025 (50 μ M) was added 24 hours after transfection. Cells were harvested 72 hours after transfection for immunoblotting.

Immunofluorescence Staining

Cells (200,000) were treated for 72 hours with L67 (0.3 μ M) and/or NU1025 (50 μ M), washed with PBS, cytopun, fixed in 1% paraformaldehyde (P-6148; Sigma-Aldrich) for 10 minutes, permeabilized in 70% EtOH for 10 minutes and then blocked for 1 hour in 10%

FBS-TBS-Tween 20 (0.2%). After washing, slides were incubated for 1 hour with anti-phospho-histone H2AX (S139; 1:100; Millipore) and then with DyLight 594 anti-mouse secondary antibodies for 1 hour (1:200; KPL, Gaithersburg, MD). Slides were washed and dried prior to counter staining with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) and then examined using a Nikon fluorescent microscope Eclipse 80i (100X/1.4 oil, Melville, NY). Images of at least 50 cells/slide were captured using a CCD (charge-coupled device) camera and the imaging software NIMS Elements (BR 3.00, Nikon).

RNA Isolation

Total RNA was extracted from cultured cells ($2-5 \times 10^6$) according to the Illustra RNA spin Mini RNA Isolation Kit (GE Healthcare, Pittsburgh, PA).

Real-Time RT-PCR

Quantitect Primer Assays for DNA ligase III α (hsLIG3-1-SG), PARP1 (hsPARP1-1-SG), and GAPDH (hsGAPDH-2-SG, Qiagen, Valencia, CA) were used to perform real-time RT-PCR on 20 ng of total RNA in a 25 μ l reaction volume with QuantiTect SYBR Green RT-PCR Kit in a Mastercycler ep *realplex*² thermal cycler (Eppendorf, Hauppauge, NY) according to the manufacturer's protocol. The expression levels of DNA ligase III α and PARP1 were normalized to that of GAPDH.

cDNA Sequencing

Using procedures described previously (52) a direct sequencing approach encompassing the entire ABL kinase and ATP-loop domain (corresponding to amino acids 242–395) was performed on cDNA products from RT-PCR using forward primer (5' - CATCACCATGAAGCACAAGC-3') and the reverse (5' - GCTGTGTAGGTGTCCCCTGT-3') primers.

Immunoblotting

Protein extractions were performed without the use of a detergent using the CellLytic NuClear Extraction Kit (Sigma-Aldrich) according to the manufacturer's protocol. Proteins were separated by SDS-PAGE through 4 to 10% gradient gels and then transferred to PVDF membranes. After blocking, membranes were incubated with primary; rabbit DNA ligase III α (1:1000, Sigma-Aldrich), mouse PARP1 (1:1000, eBioscience, San Diego, CA), DNA Ligase IV, Ku70 (1:1000, Santa Cruz) or β -Actin (1:5000, Abcam, Cambridge, MA), followed by secondary antibodies; HRP goat anti-rabbit (1:2000) or anti-mouse (1:5000, Santa Cruz). Antigen-antibody complexes were detected by enhanced chemiluminescence and quantified by scanning nonsaturated luminograms using Quantity One software (version 4.6., Biorad).

Plasmid-based NHEJ repair assay

*Eco*R1-linearized pUC18 plasmids (ThermoScientific, Glen Burnie, MD) were transfected into cells using Amaxa Nucleofector Kit V. Plasmid DNA was extracted (Qiagen Plasmid Mini Kit) and transform *E. coli* strain DH5 α (Invitrogen). After plating on agar plates containing X-gal and IPTG, the number of white (misrepaired) and blue (correctly repaired) colonies were counted. Plasmid DNA from the white (misrepaired) colonies was characterized by PCR amplification of the breakpoint region using forward (5' - CGGCATCAGAGCAGATTGTA-3') and reverse (5' - TGGATAACCGTATTACCGCC-3') primers followed by DNA sequencing (Genomics core facility, University of Maryland School of Medicine, Baltimore).

Comparative Genomic Hybridization (CGH)

Genomic DNA was isolated from frozen cell pellets using DNeasy tissue mini kit (Qiagen) following the manufacturer's protocol. Sample labeling was performed following Agilent's recommendation for 244K array CGH. Agilent Human High-Resolution Discovery 1x 1M CGH microarrays containing probes representing 963,000+ human genomic sequences were used. Hybridization mixtures were denatured at 95°C for 3 min and then immediately transferred to 37°C for 30 min. The mixtures were hybridized to microarrays for 40 hours at 65°C in a rotating oven. Hybridized microarrays were washed and dried according to the manufacturer's protocols and then imaged with an Agilent G2565BA microarray scanner. Data were extracted using Feature Extraction Software v9.5.3.1 (Agilent Technologies) and analyzed using Agilent's Genomic Workbench v 5.0. Noise was estimated for each sample array by calculating the spread of the log ratio differences between consecutive probes (DLRsd) along all chromosomes, and dividing by $\sqrt{1}$ to counteract the effect of noise averaging. Aberrant regions (gains or losses) were then identified based on hidden Markov model (HMM) algorithm provided in the software (53).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Professor Stephen Baylin (JHU) for insightful comments and careful reading of our manuscript. CML patient samples were collected under IMRB # H25314. These studies were supported by the Cigarette Restitution Funds of Maryland (FR and LT), the Leukemia Lymphoma Society (FR, CR and LT), the V Foundation (FR, LT and AET) and NIH grants ES 012512 and CA92584 (AET).

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Figure 1. Steady state levels of NHEJ proteins in IMS and IMR cell lines
(A) Representative immunoblot and **(B–C)** relative steady state levels of DNA ligase III α (LIG3, white bars), PARP1 (dark grey bars), DNA ligase IV (LIG4, light grey bars) and Ku70 (KU70, black bars) and β -actin (ACTIN) as loading control, in **(B)** IMS(K562) and IMR derivatives (K562 IMR) of a BCR-ABL1-positive human CML cell line, K562, compared to a lymphoblastoid cell line established from normal lymphocytes (NC10) and **(C)** IMS (Mo7e-P210 and Baf3-P210) and IMR derivatives (Mo7e-P210 IMR1, Mo7e-P210 IMR2 and Baf3-P210 IMR) of hematopoietic cell lines, Mo7e and Baf3 that had been engineered to stably express BCR-ABL1 compared to the parental cells Mo7e and Baf3, respectively.

Figure 2. Effect of DNA ligase and PARP inhibitors on the survival of IMS and IMR cell lines (A–B) Colony survival after a 10-day treatment with L67 (0.3 μ M, dark grey Bars), NU1025 (50 μ M, light grey bars), L67+NU1025 (black bars) compared to vehicle control (CTRL, white bars) of (A) K562, K562 IMR compared to NC10, (B) Mo7e-P210, Baf3-P210, Mo7e-P210 IMR1, Mo7e-P210 IMR2 and Baf3-P210 IMR compared to Mo7e and Baf3 respectively. (C) Immunoblotting for DNA ligase III α (LIG3) and β -actin (ACTIN) in extracts from Mo7e, Mo7e-P210 and Mo7e-P210 IMR1 cells after treatment with either control (–) or DNA ligase III α siRNA.(+). (D) Colony survival after siRNA knockdown of DNA ligase III α (dark grey bars) and/or a 10-day treatment with NU1025 (50 μ M, black bars) compared to siRNA CTRL (white bars) and/or a 10-day treatment with NU1025 (light grey bars). Results are representative of three independent experiments $\pm$ SEM, * p <0.05 based on TTEST from IMS versus control or IMR versus IMS cells.

Figure 3. Effect of DNA ligase and PARP inhibitors on the steady state levels of DSBs in IMS and IMR cell lines

(A) Representative nuclei (blue, DAPI) from K562, K562 IMR and NC10 cells immunostained for γ H2AX (red). (B and C). Percentage of cells with more than 3 endogenous γ H2AX foci/cell following a 24-hour treatment with L67 (0.3 μ M) and NU1025 (50 μ M, black bars) or CTRL (white bars); (B) K562, K562 IMR and NC10 cells; (C) Mo7e-P210, Baf3-P210, Mo7e-P210 IMR1, Mo7e-P210 IMR2, Baf3-P210 IMR, Mo7e and Baf3 cells. Results are representative of three independent experiments $\pm$ SEM, * p <0.05 based on TTEST from IMS versus control or IMR versus IMS cells.

Figure 4. Repair of DSBs by NHEJ in IMS and IMR: Effect of DNA ligase and PARP inhibitors
(A) Plasmid-based NHEJ repair assay was carried out as described in Materials of methods. The number of blue (accurate repair, dark bar) and white (inaccurate repair, white bars) recovered from the NC10, K562, K562IMR, Mo7e-P210, Mo7e-P210IMR1, Mo7e-P210IMR2, Baf3, Baf3-P210 and Baf3P210IMR cell lines. The results of 3 independent experiments are shown. **(B–E)**NHEJ repair assays following a 24-hour treatment without (white diamonds or –) or with L67 and NU1025 (0.3 μ M and 50 μ M, black diamonds or + in **(B–C)** K562, K562 IMR and NC10 cells; **(D–E)** Mo7e-P210, Baf3-P210, Mo7e-P210 IMR1, Mo7e-P210 IMR2, Baf3-P210 IMR, Mo7e and Baf3 cells. **(B and D)**Size of DNA deletions within the DSB region of repaired plasmids in white colonies. Average (AVG) size of DNA deletions is represented by black bars. **(C and E)** Ratio of the mean number of microhomologies ($\geq$ 2 bp, black bars) versus no microhomology (white bars) within repaired plasmids in white colonies.

Figure 5. Genome-wide view of DNA copy number variation on Agilent 1M array CGH in IMS and IMR derivatives of Mo7e
(A) Log2 ratio of Mo7e-P210 (Cy5) versus Mo7e (Cy3) and (B) log2 ratio of Mo7e-P210 IMR1 (Cy5) versus Mo7e-P210 (Cy3). The numbers labeled on the horizontal axis indicate different chromosomes. Log2 ratios of signal intensities are plotted with horizontal central line equal to zero. Alterations above the line (dotted black arrows) indicate amplifications and below the line indicate deletions (black arrows). Because of the way the genome view figure is drawn, some of the genomic alterations encompassing regions at the ends of chromosomes, are not visible. Nevertheless, we have indicated these genomic alterations with arrows. The copy number variations are shown as trend lines with 2MB base pair moving average. The log2 ratio on the vertical axis ranges from +4 (top) to -4 (bottom). (C) Summary of the deletion and amplifications found in Mo7e-P210 compared to Mo7e and in Mo7e-P210 IMR1 compared to Mo7e-P210.

Figure 6. Steady state levels of DNA ligase III α and PARP1 in IMS and IMR samples from BCR-ABL1-positive CML patients: effect of DNA ligase and PARP inhibitors on the survival of IMS and IMR samples from BCR-ABL1-positive CML patients

(A) Relative steady state levels of DNA ligase III α (LIG3, White bars) and PARP1 (Black bars) mRNA transcripts in BCR-ABL1-positive CML patient samples compared to normal bone marrow (NBM). Results are presented graphically with the patient samples shown in the following groups; LIG3 and PARP1, significant ($*p < 0.05$ based on TTEST) increases in both DNA ligase III α and PARP1 mRNA transcripts; LIG3 or PARP1, significant ($*p < 0.05$ based on TTEST) increases in either DNA ligase III α or PARP1 mRNA transcript; Neither, no significant change in either DNA ligase III α or PARP1 mRNA transcript. (B) Colony survival assays after a 10-day growth of BCR-ABL1-positive CML patient samples and NBM in the absence (white bars) or presence of L67 and NU1025 (0.3 μ M and 50 μ M, black bars). Results are presented graphically with the patient samples grouped according to response to the combination of DNA repair inhibitors; L67 + NU1025 Sensitive, patient samples that were sensitive ($**p < 0.005$ based on TTEST); Partially sensitive patient samples that showed partial sensitivity ($*p < 0.05$ based on TTEST); Insensitive, patient samples that were insensitive.

Table 1

Clinical and molecular features of BCR-ABL1 CML patients.

		↑LIG3	↑PARP1	Imatinib	Mutation	Phase
Sensitive	PT11	Y	Y	IMS	None	Chronic
	PT12	Y	Y	IMS	None	Chronic
	PT18	Y	Y	IMS	None	Blast
	PT10A	Y	Y	IMS	None	Chronic
	PT10B	Y	Y	IMR	None	Blast
	PT2	Y	Y	IMR	T315I	Blast
	PT14	Y	Y	IMR	ND	Chronic
	PT17	Y	Y	IMR	None	Blast
	PT19	Y	Y	IMR	T315I	Blast
Partially Sensitive	PT1	Y	N	IMS	None	Chronic
	PT13	Y	N	IMS	None	Chronic
	PT15	Y	N	IMS	None	Chronic
	PT8	Y	N	IMR	None	Chronic
	PT9	Y	Y	IMR	None	Chronic
Insensitive	PT3	N	N	IMS	None	Chronic
	PT16	N	N	IMR	None	Chronic
	PT4	N	N	IMR	G250E	Accelerated
	PT6	N	N	IMR	ND	Accelerated
	PT7	N	N	IMR	None	Accelerated

Y=yes, N=no, ND=not determined based on p<0.05 by TTEST

IMR=Imatinib Resistant

IMS=Imatinib Sensitive