Constitutive Activation of the DNA Damage Signaling Pathway in Acute Myeloid Leukemia with Complex Karyotype: Potential Importance for Checkpoint Targeting Therapy

Cindy Cavelier,1,2 Christine Didier,1,2 Nais Prade,3 Véronique Mansat-De Mas,3,4 Stéphane Manenti,3 Christian Recher,3,4 Cécile Demur,3,4 and Bernard Ducommun1,2,4

Abstract

Genomic instability in solid tumors participates in the oncogenic process and is associated with the activation of the DNA damage response pathway. Here, we report the activation of the constitutive DNA damage and checkpoint pathway associated with complex karyotypes in samples from patients with acute myeloid leukemia (AML). We show that antagonizing CHK1 kinase with a small inhibitory compound or by RNA interference strongly reduces the clonogenic properties of high-risk DNA damage level AML samples, particularly those with complex karyotypes. Moreover, we observe a beneficial effect of CHK1 inhibition in high–DNA damage level AML samples treated with 1-β-D-arabinofuranosylcytosine. In contrast, CHK1 inhibition has no effect on the clonogenic properties of normal hematopoietic progenitors. All together, our results indicate that CHK1 inhibition may represent an attractive therapeutic opportunity in AML with complex karyotype.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Experimental Therapeutics, Molecular Targets, and Chemical Biology

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Introduction

Acute myeloid leukemia (AML), the most frequent acute leukemia in adults, is a clonal disorder characterized by the accumulation of malignant hematopoietic progenitor cells (HPC) with an impaired differentiation program. Despite considerable progress in the therapy of AML and a high rate of complete remission after induction chemotherapy, most patients relapse and succumb to the disease (1). This clinical observation highlights the difficulties involved in eradicating the leukemic stem cells that are responsible for the emergence of new leukemic progenitor cells leading to relapse. AML is a heterogeneous disease and patients differ strongly in their response to therapy, in the occurrence of relapse and in overall survival, according to prognosis factors such as age and presence of molecular and/or cytogenetic abnormalities. Many different genetic defects are thought to underlie the heterogeneity of this disease (2). On the basis of cytogenetic analysis, the classification of Grimwade and Schlenk (3, 4) defines three prognosis groups (favorable, intermediate, and high risk). Complex karyotype AML, belonging to cytogenetic high-risk AML, is characterized by the presence of at least three unrelated cytogenetic abnormalities and accounts for 10% to 20% of AML cases. The percentage increases with age and this group is considered to have the worst prognosis, despite intensive therapy (5).

Our understanding of the molecular basis of the emergence of genomic instability in solid tumors has benefited considerably from recent work demonstrating the involvement of a replication stress activating the DNA damage response pathway (6). At an early stage of tumorigenesis, oncogene-induced DNA damage, associated with replicative and oxidative stresses, is responsible for creating double-strand breaks, thus leading to activation of the DNA damage signaling pathway, with phosphorylation of histone H2AX and ATM and activation of checkpoint kinases (CHK1 and CHK2; refs. 7, 8). Activation of the DNA signaling pathway in precancerous lesions has been suggested to constitute an anticancer barrier (7). A limited number of studies have addressed the question of the presence of DNA double-strand break in hematologic cancers. Constitutive DNA damage was observed in two myeloid leukemia cell lines (K562 and HL60; ref. 9) and, more recently, an increase in detectable DNA damage was reported in a mouse model of progression from myelodysplasia to leukemia (10). However, as concern samples from AML patients, the status of the DNA damage signaling pathway has not been documented.

The activation of the DNA damage pathway, through checkpoint kinase phosphorylation, leads to cell cycle arrest in S-phase or at the G2-M checkpoint (11, 12). CDC25 phosphatases have been shown to be the main effectors of active CHK1 (13), and the abrogation of the activity of this kinase using small inhibitory compounds or small interfering RNA (siRNA) knockdown strategies has been reported to result in checkpoint bypass and in sensitization of the cells to DNA-damaging agents (14–17). For instance, the use of 7-hydroxy staurosporine (UCN-01), a CHK1 inhibitor, induces sensitization of AML progenitors to the nucleoside analogue 1-β-D-arabinofuranosylcytosine (ara-C; ref. 18). Moreover, several phase-1 and -2 trials in solid tumors have been reported, for UCN-01, either alone (19–21) or in combination with established cytotoxic agents (22, 23). These observations have led to the recent development of checkpoint kinase-inhibitory compounds, some of which, including UCN-01, are currently being evaluated in early clinical trials.

In the study reported here, we have investigated the existence of constitutive DNA damage and activation of the DNA damage pathway in a large panel of AML patient samples. Our results reveal high levels of DNA damage in complex karyotype AML and show the high sensitivity of these cells to UCN-01. Together with

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the demonstration that CHK1 inhibition has no negative effect on the growth of normal hematopoietic progenitors, our results highlight the interest of this kinase for checkpoint targeting therapy in hematologic malignancies.

Materials and Methods

Cells. Fresh AML samples were obtained, after informed consent, from patients diagnosed at the Hematology Department of Toulouse University Medical Centre (France). AML cells were isolated from bone marrow by Ficoll-Hypaque density-gradient centrifugation and were cryopreserved in Iscove’s modified Dulbecco medium (IMDM) with DMSO (10% final concentration) and FCS (50% final concentration). All samples were kept frozen in liquid nitrogen by the HIMIP collection of the U563 Institut National de la Sante et de la Recherche Medicale department (n°DC-2008-307-CPTP1 HIMIP). Frozen cells were thawed in IMDM medium and immediately processed for clonogenic assays or cultured in IMDM, 10% FCS for 72 h for viability testing. For the DNA damaging treatment, cells were cultured in IMDM, 10% BIT (StemCell Technologies) supplemented with SCF (100 ng/mL), IL3 (5 ng/mL), FLT3 (100 ng/mL) and granulocyte macrophage colony-stimulating factor (10 ng/mL) for 24 h, then treated with etoposide (VP-16; 17 μmol/L) for 30 min, released in drug-free media, and finally analyzed for their H2AX phosphorylation profile. Normal bone marrow CD34+ HPCs were obtained after informed consent from discarded fragments from hematologically healthy patients who had undergone hip surgery. Mononuclear cells from bone marrow were obtained by Ficoll-Hypaque density-gradient centrifugation, after which isolation of HPCs was performed by positive selection of CD34-expressing cells using magnetic separation and EasySep procedure (StemCell Technologies).

Pharmacologic inhibitors and antibodies. UCN-01 was provided by the National Cancer Institute. Ara-C and other products used were purchased from Sigma-Aldrich. Antibodies were monoclonal and polyclonal and were obtained by the National Cancer Institute. Ara-C and other products used were obtained by Ficoll-Hypaque density-gradient centrifugation, after which cells had undergone hip surgery. Mononuclear cells from bone marrow were then treated with etoposide (VP-16; 17 μmol/L) for 30 min, released in drug-free media, and finally analyzed for their H2AX phosphorylation profile. Normal bone marrow CD34+ HPCs were obtained after informed consent from discarded fragments from hematologically healthy patients who had undergone hip surgery. Mononuclear cells from bone marrow were obtained by Ficoll-Hypaque density-gradient centrifugation, after which isolation of HPCs was performed by positive selection of CD34-expressing cells using magnetic separation and EasySep procedure (StemCell Technologies).

Flow cytometry. Immunostaining was performed according to the previously described protocol (24). Samples were analyzed in an FC500 flow cytometer (Beckman Coulter) and subsequently processed using the CXP software. Flow cytometry. Immunostaining was performed according to the previously described protocol (25). Samples were analyzed with LSRII flow cytometer (BD), followed by data treatment using the BD FACSDiva software. All flow cytometry results are expressed as the ratio between mean fluorescence intensity of the stained sample and that of its control isotypic antibody.

Immunofluorescence microscopy. Immunofluorescence studies were performed as previously described (26). Cellular DNA was counterstained with 4’,6-diamidino-2-phenylindole. Images were acquired using a DM6000 microscope (Leica microsystems) fitted with a Roper COOLsnap ES CCD camera and subsequently processed using the MetaMorph and ImageJ softwares.

Clonogenic assays. The percentage of leukemic cells present in patients’ bone marrow is reported in Table 1. Clonogenic assays on AML cells and on fresh human bone marrow CD34+ HPC cells were made according to a method previously described (27).

siRNA experiments. AML cells were transfected using the Amaxa nucleofection apparatus (Amamaxa). Leukemic cells (5 × 10^5) were suspended in 100 μL of either kit V or human monocyte or human CD34+ cell nucleofector solution mixed with 30 pmol control and CHK1 siRNA oligonucleotide sequences. These experiments were performed either with single control and CHK1 siRNA (Quigen and Dharmacon, as described in ref. 28) or with a pool of siRNA (control and CHK1) as described (Dharmacon control and CHK1 SMART pool reagents #001820 and #003255, respectively; ref. 29). Cells were immediately nucleofected with an Amaxa Nucleofector apparatus (program U-15 or Y-001 or U-008). They were then transferred to culture prewarmed medium and divided into two samples. One was used immediately for clonogenic assay and the second was cultured in IMDM 10% FCS for 24 h to evaluate the CHK1 knockdown efficiency by Western blot.

Viability test. AML cells (1 × 10^5/well) were incubated in 100 μL of IMDM 10% FCS with increasing UCN-01 concentrations for 72 h. To determine the percentage viability, 10 μL of Proliferation Reagent WST-1 (Roche) were added and the cells were incubated for 3 h. The absorbance at 420 nm (Mithras LB 940, Berthold technologies) of samples corrected for a background control allowed us to calculate the percentage viability of AML samples and to determine the UCN-01 IC50.

Statistics. Results are expressed as means ± SEM. Statistical analyses were performed by the unpaired Student’s t test and Pearson correlation test with Prism 4 software. Differences were considered as significant for P values of ≤0.05.

Results

High levels of constitutive DNA damage in complex karyotype AML patient samples. We first examined the existence of detectable constitutive DNA damage in samples of blasts from AML patient, using flow cytometry to quantify H2AX phosphorylation (30). As indicated in Fig. 1A, obtained from two AML blast cell samples (patients #20 and #44, Table 1 and Supplementary Data 1) and one normal hematopoietic progenitor CD34-positive cell (HPC) sample, flow cytometry histograms reveal a variable level of phospho-H2AX labeling in malignant samples and confirm the absence of detectable labeling in the normal HPC sample. A series of 49 patient samples (see Table 1) was then examined using the same assay and the phospho-H2AX level of each was calculated (see Materials and Methods). As depicted on the left side of Fig. 1B, normal HPC did not display any detectable DNA damage and their phospho-H2AX level was about 1 (mean, 1.31 ± 0.13; n = 7). In contrast, AML blasts from patient samples expressed a higher and very variable level of phospho-H2AX (mean, 3.90 ± 0.36; n = 49; **, \( P = 0.007 \)), indicating a variable level of constitutive DNA damage. On the right side of Fig. 1B, the phospho-H2AX level of the same patients is divided into two groups according to their cytogenetic profiles (see Table 1). The high-risk group (mean, 5.07 ± 0.86; n = 16, including 11 complex karyotype samples) displayed a significantly higher level of phospho-H2AX than the intermediate one (mean, 3.08 ± 0.21; n = 31; **, \( P = 0.007 \)).

We next tested the variability of these AML samples with respect to constitutive phospho-H2AX labeling as a physiologic response to DNA injury and examined their capacity to induce an additional level of phospho-H2AX after treatment with a genotoxic agent. To this end, we evaluated phospho-H2AX levels in two AML samples, before and 1 h after treatment with the DNA-damaging agent VP-16. We observed in both samples a rapid increase of phospho-H2AX level (data not shown), reaching a peak, 1 hour after treatment (Fig. 1C), in the same range as the constitutive damage levels observed in some AML samples (Fig. 1B). Thus, AML blasts samples with a certain level of constitutive DNA damage retained the ability to increase this level in response to a DNA-damaging agent.

Damage was not induced by freezing and thawing of the patient material, as we found similar phospho-H2AX levels in three samples of fresh and thawed AML cells and in one sample of normal HPC (Supplementary Data 2).

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Finally, we used immunofluorescence staining to look for phospho-H2AX–labeled cells in normal HPC and AML patient samples (Fig. 1D). As shown, phospho-H2AX labeling of cells was only detected in a fresh AML blast sample (patient #49), whereas fresh normal CD34+ HPC were unlabeled.

All together, these results indicate that AML blast cells from patients exhibit variable levels of constitutive DNA damage that are in the range of the physiologic response to DNA injury; they also show that samples from complex karyotype patients express a relatively higher level of constitutive DNA damage.

Table 1. Characteristics of AML patients

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<th>Patient no.</th>
<th>Age</th>
<th>FAB</th>
<th>Prognosis/karyotype</th>
<th>Major abnormality</th>
<th>WBC, *10^9/L</th>
<th>BM blasts (%)</th>
<th>FLT3-ITD</th>
<th>p-H2AX</th>
<th>p-CHK1/CHK1</th>
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<td>Nd</td>
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NOTE: Prognosis factor groups were established according the classification of Grimwade and Schlenk on the basis of the cytogenetics analysis (3, 4). Abbreviations: FAB, French-American-British classification; WBC, white blood cell count; FLT3-ITD, FLT3-internal tandem duplication; FP, favorable prognosis; IP, intermediate prognosis; HP, high-risk prognosis; ab, abnormalities; Nd, not done.
Checkpoint signaling pathway activation in AML patient samples. To evaluate the consequence of constitutive DNA damage on the downstream signaling pathway, we tested the activation of CHK1 protein kinase. To this end, we quantified CHK1 phosphorylation on the same AML sample cohort (Table 1). As reported in Fig. 2A, the level of CHK1 activation, expressed as the ratio between phosphorylated and total CHK1 levels, was quite variable and distributed between 0.2 and 2.2. However, as in the case of the phospho-H2AX level, we found a significantly higher activation of CHK1 in cytogenetically based high-risk AML (mean, 0.96 ± 0.13; n = 13, including 10 complex karyotype samples) than in intermediate AML (mean, 0.52 ± 0.06; n = 20; **, P = 0.002). We next evaluated the presence of constitutive DNA damage and activation of the damage signaling pathway in different mature and immature hematopoietic subpopulations. As shown in Fig. 2B, H2AX phosphorylation could be detected in all immature and mature leukemic subpopulations, independently of the CD38 status. Analogous results were obtained for the activated form of CHK1, and for downstream targets of ATM/ATR proteins. These results provide evidence that DNA damage appears at an early stage of leukemic hematopoietic differentiation. Moreover, it is relevant to use phospho-H2AX detection in the global leukemic population from AML patients, because DNA damage is uniformly present in all leukemic subpopulations. Our data indicate that the checkpoint signaling pathway is constitutively activated early in complex karyotype samples. AML blasts with high levels of DNA damage are more sensitive to CHK1 inhibition. The above results prompted us to evaluate the effect of CHK1 inhibition on AML blast samples. We tested the sensitivity to a CHK1 inhibitor, UCN-01, first, of the global leukemic population, using a viability test in liquid culture, then of AML progenitors, using a clonogenic assay. The IC50 for UCN-01 was calculated according the proliferation dose-response curves (Supplementary Data 3) obtained for a set of patient samples and is presented in Fig. 3A and B, together with the phospho-H2AX level previously determined. As shown in Fig. 3A, the sensitivity to UCN-01 was inversely
proportional to the DNA damage level. Moreover, the same differential effect was observed on AML progenitors (Fig. 3B). To confirm that CHK1 inhibition was responsible for the inhibitory effect observed on AML progenitors, we analyzed the effect of a CHK1 knockdown induced by RNA interference using either a smartpool (Fig. 3C, top) or a single siRNA (Fig. 3C, bottom; refs. 28, 29, 31). The clonogenic activity was determined in control and CHK1 siRNA–transfected samples after 7 days (Fig. 3C, right). CHK1 knockdown in these four AML samples (#9, #44, #46, and #50), although producing only a modest decrease in CHK1 level (Fig. 3C, left), resulted in an inhibition of clonogenicity. Together, these results indicate that progenitors and AML cells predominantly with a high-DNA-damage level are more sensitive to CHK1 inhibition.

**CHK1 inhibition sensitizes AML progenitors with high-DNA damage level to ara-C treatment.** As previously noted, inhibition of checkpoint kinases potentiates the effect of chemotherapy and radiotherapy. In the light of our observations, we examined the effect of CHK1 inhibition on the sensitization of AML blasts to the chemotherapeutic agent, ara-C, in relation to their levels of

![Figure 2](image-url). Checkpoint signaling pathway activation in AML patient samples. **A**, constitutive activation of CHK1 evaluated in AML blast samples (n = 34) was tested by flow cytometry using the anti-phospho and total CHK1 antibodies (p-CHK1/total CHK1 ratio). Results represent the ratio between fluorescence intensity of phospho-CHK1 and total CHK1. Left, CHK1 activation profile tested in 34 AML blast samples. Right, CHK1 activation profile as a function of the AML cytogenetic risk: intermediate and high risk. Statistical analyses were performed using a nonparametric unpaired t test (**, P = 0.002). **B**, flow cytometry detection of phospho-H2AX, phospho-CHK1, and phosphorylated substrates of ATM/ATR proteins (p-ATM/ATR s.) in the leukemic population (CD123+) as a function of CD38 status in CD34+ (top) or CD34– (bottom) cells, using four-color immunostaining.
constitutive DNA damage. We calculated the IC_{50} for ara-C, alone and in association with UCN-01, from the proliferation dose-response curves (Supplementary Data 4) after clonogenic assays. These data are shown in Fig. 4A, together with the phospho-H2AX levels and the sensitization factor (SF), which is a ratio between the IC_{50} for ara-C alone and in combination with UCN-01 (31). They confirm that, as observed in a clinical setting, complex karyotype samples are more resistant to ara-C treatment than normal ones (mean IC_{50}, 9.8 nmol/L ± 0.6; n = 5 versus 5.9 nmol/L ± 0.4; n = 4; * , P = 0.05). Moreover, high-DNA damage blast samples are also more resistant to ara-C alone (mean IC_{50}, 10.7 nmol/L ± 0.6; n = 5 versus 6.3 nmol/L ± 0.3; n = 5; *, P = 0.02). We found, as in a previous study (18), that UCN-01 treatment induced a strong sensitization of ara-C, with a decrease of IC_{50} in all cotreated

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Figure 3. Sensitivity of AML blast cells to CHK1 inhibition. A, AML blast cells (#10, 11, 17, 41, and 44) were grown in liquid culture in the presence of increasing concentrations of UCN-01. The respective IC_{50} were then calculated after 72 h of incubation. *, complex karyotype samples. B, AML blast cells (#2, 10, 17, 26, 34, 40, and 44) were grown in clonogenic assays in continuous exposure to increasing concentrations of UCN-01. The clonogenic survival was assessed after 7 d and the IC_{50} was then calculated. C, four AML blast samples (#9, 44, 46, and 50) were electroporated with control (gray columns) and CHK1 siRNA (white columns). Smartpool (top) or single siRNA (bottom) were used in these experiments (see Materials and Methods). CHK1 expression was evaluated 24 h after electroporation. The clonogenic properties were analyzed by scoring the CFU-L numbers at day 7. Results are expressed as absolute number of leukemic colonies; columns, means of duplicate assays; bars, SEM.
AML samples. As expected, the highest sensitization was observed in the samples of highest DNA damage level. Figure 4B reveals a strong correlation between the SF and the DNA damage level for the 10 AML samples tested (***, $P = 0.0005$). Importantly, the CHK1 knockdown performed on two patient samples had a similar sensitizing effect and highly increased the sensitivity to ara-C, with a considerable reduction of the IC$_{50}$ (Fig. 4C).

On the basis of these experiments, we conclude that CHK1 inhibition potentiates the effect of ara-C, as previously reported by Sampath and colleagues (18), but, in addition, this effect is significantly greater in patient samples with a high constitutive DNA damage level, such as those with complex karyotypes.

**UCN-01 has no effect on normal granulomonocytic progenitors.** To extrapolate the use of checkpoint kinase inhibitors to a potential therapeutic strategy, it was essential to evaluate the specificity of this approach for AML cells and confirm the lack of effect on normal progenitors. To address this issue, we examined the effect of UCN-01 treatment on the capacity of normal HPC to generate granulomonocytic (colony-forming unit-granulocyte macrophage, CFU-GM) colonies. As shown in Fig. 5A, no significant change in the number of colony-forming CFU-GM was observed after ara-C treatment in combination with UCN-01 (10 nmol/L) in five different normal HPC samples (similar results were obtained with monocytic colonies; data not shown). The average of SF in patient samples was significantly higher than in normal HPC samples (1.66 ± 0.14; $n = 10$ versus 1.05 ± 0.06; $n = 5$; *, $P = 0.01$; Fig. 5B). Moreover, we observed that normal HPC were relatively insensitive to the CHK1 inhibitor UCN-01 in clonogenic assay (Fig. 5C). The average of IC$_{50}$ for UCN-01 in AML samples was highly significantly lower than in the value obtained with normal HPC [77.9 ± 16.7 ($n = 7$) versus 378.3 ± 40.2 ($n = 5$); ***, $P < 0.0001$; Fig. 5D]. Proliferation dose-response curves are reported as Supplementary Data (Supplementary Data 5).

![Figure 4](https://example.com/figure4.png)

**Figure 4.** CHK1 inhibition sensitizes AML blast cells to ara-C treatment. A, AML blast cells (#7, 8, 11, 14, 15, 26, 34, 40, 43, and 44) were grown in clonogenic assays in the presence of ara-C, alone, or together with UCN-01 (10 nmol/L). The clonogenic survival was assessed after 7 d and the IC$_{50}$ for ara-C alone and in combination with UCN-01 were then calculated. The SF is the ratio between the IC$_{50}$ for ara-C alone and in combination with UCN-01. B, correlation between SF (A) and phospho-H2AX level (rMFI). Statistical analyses were performed using a Pearson test ($r = 0.89$; ***, $P = 0.0005$). C, AML blast samples (#1, 17) were electroporated with control (gray columns) and CHK1 siRNA (white columns), then incubated with increasing concentrations of ara-C. Western blot evaluation of the efficiency of the invalidation of CHK1 expression in sample #1 and 17 was performed 24 h after electroporation. CFU-L numbers were scored at day 7 and IC$_{50}$ was calculated.
These results indicate that UCN-01 treatment specifically inhibits AML progenitors and has no effect on normal granulocytic precursors.

Discussion

This study shows for the first time that cells from patients with AML exhibit a high spontaneous level of constitutive DNA damage, concomitant with DNA damage checkpoint pathway activation. This activation occurs early in hematopoietic differentiation, as it is detected in immature leukemic blast cells with a CD34+ CD38− CD123+ phenotype, which are enriched in severe combined immunodeficient leukemia–initiating cells (32). Furthermore, activation of the checkpoint pathway is closely associated with complex karyotype samples belonging to the high-risk cytogenetic group. This finding could have major pharmacologic consequences, because it opens a therapeutic window for new compounds targeting the checkpoint machinery. Indeed, in our work, we show that samples from complex karyotype AML patients who are classically resistant to conventional chemotherapy (33) respond to the combined use of ara-C with a CHK1 kinase inhibitor; in contrast, growth of normal hematopoietic progenitors is not impaired by such treatment.

Bartkova and colleagues (7) have proposed a model in which the activation of checkpoint signaling pathways in response to DNA damage first constitutes a barrier to the transformation process, preceding p53 mutations, and subsequently becomes a hallmark of the genetic instability of the tumor. It is not clear whether a similar process occurs in hematologic malignancies. Evidence for an increasing activation of the nonhomologous end-joining DNA repair machinery due to the presence of constitutive DNA damage in two AML leukemia cell lines was previously reported (9). In this latter study, the authors highlighted the possible role of the repair machinery in increasing genomic instability. More recently, a study performed in a mouse model of progression from myelodysplasia to leukemia showed that tumor progression is associated with an increase of spontaneous DNA damage involving reactive oxygen species (34).

Our results show that AML patient samples are also characterized by constitutive DNA damage and activation of the DNA damage checkpoint signaling pathway. Moreover, similarly to solid tumors, complex karyotype samples, which display major genomic instability, show the highest level of constitutive DNA damage and checkpoint activation. Indeed, we have shown that, for AML patient samples with complex karyotype, the level of DNA damage detected by phospho-H2AX is higher than in samples with normal karyotype. We also report that complex karyotype samples exhibit a significantly higher level of activated CHK1 kinase than those with normal karyotypes.

This CHK1 activation is consistent with a scenario in which various activated oncogenes, such as NRAS or KRAS, or multiple chromosomal rearrangements, result in loss, gain, or amplification of chromosomal material frequently observed in complex karyotype AML (2). All these events contribute to the establishment of genomic instability. The origin of the higher genomic instability in complex karyotype patients has not been fully elucidated. Schoch and colleagues (35, 36) have shown a specific transcriptional pattern with an upregulation of DNA repair and checkpoint signaling genes. Moreover, 78% of complex karyotype samples present p53 alterations, compared with 14% in other cytogenetic subtypes (37). Haferlach and colleagues’ study (37) correlated the presence of genetic abnormalities on chromosome 17 affecting the p53 pathway with a greater resistance to all conventional drugs used in chemotherapy, highlighting the p53 role in cell cycle checkpoint response. However, although p53 is mutated in complex karyotype AML cells, it is difficult to know if p53 mutation contributes to the great genomic instability observed in complex karyotype AML cells or if p53 mutation is a consequence of the preexisting genomic instability of complex karyotype AML cells. Additional studies specifically dedicated to the investigation of cell cycle checkpoint...
response and AML p53 mutation will be necessary to evaluate precisely its impact.

In Bartkova’s model, activated oncogenes, associated with repli- cative and oxidative stresses, are responsible for DNA injury, lead- ing to the activation of the DNA damage signaling pathway (7). Considering the high genetic instability of complex karyotype samples, it is more than likely that similar mechanisms occur in AML. Among various mechanisms of resistance to chemotherapy, it has been shown that complex karyotype AML displays a com- bined activity both of multidrug resistance proteins P-gp and MDR-related protein 1, which contributes to the chemoresistance (38). In this study, we confirm that complex karyotype AML samples display the highest resistance to chemotherapy, even that involving the widely used antimetabolite, ara-C, which is not a P-gp substrate. Moreover, this poor sensitivity is also likely to be correlated with their lower proliferative activity in comparison with the favorable and intermediate prognosis group (39). The weak proliferative activity explains the low incorporation of ara-C into DNA and, consequently, contributes to the poor effect of this treatment, which precludes complete clinical remission. In view of the global resistance mechanisms to chemotherapy used by complex karyotype cells, the use of checkpoint inhibitors may represent an attractive pharmacologic strategy to increase the efficacy of ara-C treatment in the group of complex karyotype AML patients. Our findings strongly support this idea. First, we show that complex karyotype samples are particularly sensitive to CHK1 inhibition by UCN-01 and by RNA interference. More importantly, we have shown that low concentrations of UCN-01 induce a sensitization to ara-C treatment that is particularly marked in the high–DNA damage sample. Similar results were obtained when UCN-01 and VP-16 were used together (data not shown). Moreover, Sampath and colleagues (18) have shown that UCN-01, in combination with ara-C, induces a rapid decrease of the constitutive activation CHK1 in 8 AML samples.

Above all, we have clearly shown that the same concentration UCN-01 has no effect on the growth and clonogenic capacity of normal HPCs, thus indicating that such an association between a checkpoint targeting agent and an antimetabolite has a specific effect on abnormal hematopoesis.

Finally, our study shows that evaluation of the DNA damage level by flow cytometry detection of histone H2AX phosphorylation is a very easy and direct assay to routinely select the patients for whom such a combinative treatment may be beneficial. The use of this simple technology could be of real interest to identify AML patients with complex karyotype AML and high DNA damage and to constitute the large cohort of such patients that are now required to evaluate in detail the therapeutic potential of checkpoint inhibitors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References


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