Enhanced cytarabine-induced killing in OGG1-deficient acute myeloid leukemia cells

Nichole Owen, Irina G. Minko, Samantha A. Moellmer, Sydney K. Cammann, R. Stephen Lloyd, and Amanda K. McCullough

*Oregon Institute of Occupational Health Sciences, Oregon Health & Science University, Portland, OR 97239; and +Department of Molecular and Medical Genetics, Oregon Health & Science University, Portland, OR 97239

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Human clinical trials suggest that inhibition of enzymes in the DNA base excision repair (BER) pathway, such as PARP1 and APE1, can be useful in anticancer strategies when combined with certain DNA-damaging agents or tumor-specific genetic deficiencies. There is also evidence suggesting that inhibition of the BER enzyme 8-oxoguanine DNA glycosylase-1 (OGG1), which initiates repair of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-dG), could be useful in treating certain cancers. Specifically, in acute myeloid leukemia (AML), both the RUNX1-RUNX1T1 fusion and the CBFB-MYH11 subtypes have lower levels of OGG1 expression, which correlate with increased therapeutic-induced cell cytotoxicity and good prognosis for improved, relapse-free survival compared with other AML patients. Here we present data demonstrating that AML cell lines deficient in OGG1 have enhanced sensitivity to cytarabine (cytosine arabinoside [Ara-C]) relative to OGG1-proficient cells. This enhanced cytotoxicity correlated with endogenous oxidatively-induced DNA damage and Ara-C-induced DNA strand breaks, with a large proportion of these breaks occurring at common fragile sites. This lethality was highly specific for Ara-C treatment of AML cells deficient in OGG1, with no other replication stress-inducing agents showing a correlation between cell killing and low OGG1 levels. The mechanism for this preferential toxicity was addressed using in vitro replication assays in which DNA polymerase δ was shown to insert Ara-C opposite 8-oxo-dG, resulting in termination of DNA synthesis. Overall, these data suggest that incorporation of Ara-C opposite unrepaired 8-oxo-dG may be the fundamental mechanism conferring selective toxicity and therapeutic effectiveness in OGG1-deficient AML cells.

DNA repair | DNA replication | fragile site | DNA polymerase delta | AML therapy

Each year, there are ~21,000 new cases of acute myeloid leukemia (AML) diagnosed in the United States, with nearly 11,000 AML-associated deaths (1). Based on published data and statistics collected through the National Cancer Institute, survivorship for newly diagnosed AML patients has slowly improved over the past 5 decades with a current 5-y survival of ~28% (1, 2). These data highlight the critical need to identify and validate new targets for AML therapeutics. AML is a heterogeneous disease that exhibits different gene mutations or chromosomal alterations, which define specific AML subtypes. Although the genetic basis underlying AML varies, most patients with newly diagnosed AML are treated uniformly, with a combination of cytarabine (cytosine arabinoside [Ara-C]) and an anthracycline (either daunorubicin or idarubicin) as the standard of care. The standard protocol, 7+3 therapy, involves 7 d of cytarabine (cytosine arabinoside [Ara-C]) relative to OGG1-proficient cells. This enhanced cytotoxicity correlated with endogenous oxidatively-induced DNA damage and Ara-C-induced DNA strand breaks, with a large proportion of these breaks occurring at common fragile sites. This lethality was highly specific for Ara-C treatment of AML cells deficient in OGG1, with no other replication stress-inducing agents showing a correlation between cell killing and low OGG1 levels. The mechanism for this preferential toxicity was addressed using in vitro replication assays in which DNA polymerase δ was shown to insert Ara-C opposite 8-oxo-dG, resulting in termination of DNA synthesis. Overall, these data suggest that incorporation of Ara-C opposite unrepaired 8-oxo-dG may be the fundamental mechanism conferring selective toxicity and therapeutic effectiveness in OGG1-deficient AML cells.

Significance

Acute myeloid leukemia (AML) patients whose cancers are classified as either the RUNX1-RUNX1T1 fusion subtype or the CBFB-MYH11 fusion subtype share the common characteristic of significantly decreased levels of mRNA transcripts encoding the DNA repair enzyme OGG1. While these two subtypes consistently correlate with the best prognosis for improved relapse-free and overall survival, the molecular basis for relating enhanced survival to low OGG1 has not been established. To identify the basis for this subtype-specific therapeutic efficacy, we demonstrate the mechanism through which the unique combination of the diminution in OGG1 activity and cytarabine treatment confers preferential cell killing, thereby identifying OGG1 as a novel therapeutic target for AML patients with normal or increased levels of OGG1.


The authors declare no competing interest.

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1Present address: Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030.

2To whom correspondence may be addressed. Email: mcculloa@ohsu.edu.

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Results

Low Levels of OGG1 messenger RNA Uniquely Correlate with RUNX1-RUNX1T1 AML Subtype. Previous investigations have proposed that decreased levels of OGG1 messenger RNA (mRNA) commonly observed for RUNX1-RUNX1T1 and CBFB-MYH11 subtypes are a prognostic indicator of AML therapeutic responsiveness (18). Our analyses of The Cancer Genome Atlas (TCGA) (26) compared relative OGG1 expression across different cytogenetic subtypes of AML and also demonstrated that patient samples of these subtypes have low-level expression of OGG1 relative to any of the other subtypes (Fig. 1A). Because all these patients have AML, these samples represent the same hematopoietic cell lineage, and thus any consistent differences in gene expression are likely related to underlying mutations or translocations within each subtype. Since the major mechanisms of action of both Ara-C and anthracyclines are DNA replication stress and DNA adduct formation, we hypothesized that additional potential candidate genes that could increase the sensitivity to DNA damaging agents may be associated with dysregulation of DNA replication, repair, recombination, and cell cycle regulation. Thus, using the TCGA database of AML samples, we queried an additional 404 genes for correlation between expression levels and AML cytogenetic subtype. These genes included the 318 DNA damage response genes previously reported in our siRNA screen for formaldehyde response (27) and 87 additional genes related to DNA replication, cell cycle, apoptosis, and Ara-C import and metabolism (SI Appendix, Table S1). Strikingly, no other gene in our query demonstrated any significant differences in expression as a function of AML subtype, suggesting a potentially unique role for OGG1 in modulating responsiveness of AML cells to chemotherapy.

In addition to our analyses of the TCGA database, we also investigated whether the reduced OGG1 mRNA levels could be detected in patient cell samples from the Oregon Health & Science University (OHSU) Beat AML cohort (Fig. 1B) (28). Analyses of this dataset in Vizome revealed that the median OGG1 expression levels were lower in the RUNX1-RUNX1T1 and CBFB-MYH11 samples relative to other subtypes and healthy controls.

Transcripts for OGG1 are differentially spliced to yield both nuclear- and mitochondrial-targeted forms of the enzyme, with isoform 1A the predominant nuclear isoform (29). Since the TCGA and Vizome data captured multiple OGG1 splice variants, we designed isoform-specific DNA primers to assess the relative abundance of these splice variants in a subset of the Beat AML patient samples and selected cell lines representing several AML subtypes (SI Appendix, Table S2). Specifically, primers that quantify isoform 1A versus isoform 1B and other alternatively spliced isoforms were used (Fig. 1C), with the left panel showing the relative amount of PCR products generated from a forward primer located within OGG1 exon 6 and a reverse primer that spanned the junction of exons 6 and 7 that is unique to isoform 1A. The center and right panels show data derived from patient samples for isoforms 1A (spliced) and 1B (intron retained), respectively. Subsets of samples were chosen, including all Beat AML patient samples with a confirmed RUNX1-RUNX1T1 translocation [[(8, 21)] and cross-sections of “control” patient samples representing other AML subtypes including MLL rearrangements, inv(16) (PML-RARA), and RUNX1-RUNX1T1 and CBFB-MYH11 samples relative to other subtypes and healthy controls.

To conduct mechanistic studies regarding the potential relationship between levels of OGG1 and chemotherapy responsiveness, it was necessary to determine whether AML cell lines also displayed the isoform 1A-specific profile. Thus, isoform-specific PCR analyses were carried out for AML cell lines harboring RUNX1-RUNX1T1 translocations (Kasumi-1 and SKNO-1), wild-type OGG1 (MOLM-14), and cells harboring a biallelic R229Q inactivating OGG1 mutation (KG-1). These analyses revealed a specific decrease in the expression level of isoform 1A, but not of isoform 1B, in the Kasumi-1 and SKNO-1 cells (Fig. 1D) validating the choice of these cell lines in ongoing mechanistic experimental designs. The KG-1 cells showed no reduction in isoform 1A relative to control MOLM-14 cells. This result was anticipated, since the OGG1 deficiency in KG-1 is known to be the result of protein inactivation under the physiological temperature conditions and not decreased expression levels (30).

RUNX1-RUNX1T1 AML Cells Have Reduced Capacity to Repair Nuclear 8-o xo-dG. Based on the transcription profile data, we hypothesized that reduced levels of mRNA encoding nuclear-targeted OGG1 (isoform 1A) would result in less efficient repair of 8-o xo-dG in the nuclear compartment and increased steady-state

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Fig. 1. OGG1 expression as a function of AML cytogenetic subtype. (A) Relative levels of OGG1 transcript in cytogenetic subtype-specific AML patient samples from the TCGA database. (B) Relative levels of OGG1 transcript in subtype-specific AML patient samples from the Beat AML Vizome database. The red brackets in A and B indicate the RUNX1-RUNX1T1 samples. RPKM, reads per kilobase million. (C) Isoform-specific PCR identifies differential levels of OGG1 mRNA nuclear isoform 1A in cell lines and patients with RUNX1-RUNX1T1 [8, 21]. mRNA from patient samples (control samples from selected non-RUNX1-RUNX1T1 and all RUNX1-RUNX1T1) derived from the Beat AML cohort were converted to cDNA and analyzed using isoform-specific PCR primers. (Left) Primer design specific to isoform 1A using a primer that spans the exon 6/7 boundary unique to isoform 1A. (Center and Right) Primer design amplified between exons 6 and 7, producing a shorter product (fully spliced intron) for isoform 1A and a larger product (portion of intron retained) for isoform 1B. Isoform sequences and expected sizes of product were determined using OGG1 isoform sequences from NCBI RefSeq. The mean values with corresponding SDs are given; each circle corresponds to a unique patient sample. (D) Quantification of isoform-specific mRNAs in AML cell lines, with the primer design identical to that shown in C. The mean values with corresponding SEs were obtained from three independent experiments. Significance is calculated versus MOLM-14. *P < 0.05; **P < 0.01.
levels of 8-oxo-dG in genomic DNA. To test this hypothesis, we isolated nuclear proteins from OGG1-proficient MOLM-14, OGG1-reduced RUNX1-RUNXIT1 (Kasumi-1 and SKNO-1), and OGG1-mutated (KG-1) AML cells and compared the abilities of these nuclear extracts to incise synthetic oligodeoxynucleotides containing an 8-oxo-dG lesion. These in vitro assays were carried out using fluorescent-labeled oligodeoxynucleotides in which a 5′ carboxyfluorescein-labeled oligodeoxynucleotide (Tamra)-conjugated 17-mer containing a site-specific 8-oxo-dG lesion was annealed with a complementary strand containing Black Hole Quencher 2 (BHQ2) on its 3′ terminus (SI Appendix, Fig. S1A). The control reactions were initiated by the addition of OGG1 and monitored at 37 °C in a plate reader. OGG1-catalyzed DNA incision at the lesion site and subsequent dissociation of the TAMRA-conjugated product from the complementary strand resulted in enhancement of the fluorescent signal (SI Appendix, Fig. S1B).

When nuclear extracts were tested under identical conditions, products were also formed, with the highest level observed in the presence of nuclear proteins isolated from MOLM-14 cells (SI Appendix, Fig. S1C). The area under the curve was integrated for each reaction and relative levels of OGG1-dependent DNA incision were calculated for Kasumi-1, SKNO-1, and KG-1 cell extracts using the corresponding data for MOLM-14 as the reference (Fig. 2A). These analyses demonstrated that compared with MOLM-14, nuclear extracts isolated from the RUNX1-RUNXIT1 or OGG1-mutated cells were less competent in incisions of 8-oxo-dG-containing DNA; the differences in incision levels observed for SKNO-1 and KG-1 versus MOLM-14 cells were statistically significant. The result obtained for KG-1 suggested that the glycosylase activity of the thermosensitive R229Q OGG1 mutant was partially restored during isolation of nuclear extracts at the permissive 4 °C temperature, consistent with the literature (30).

In addition to measuring relative levels of OGG1 incision activity, we also used an alternative approach for further validation of reduced abilities of RUNX1-RUNXIT1 or OGG1-mutated cells to incise nuclear 8-oxo-dG. Specifically, we used a modified comet assay designed to detect unrepaired OGG1 substrates in genomic DNA. MOLM-14, Kasumi-1, and SKNO-1 cells were harvested, permeabilized, treated with exogenous OGG1 or left untreated (±OGG1), and processed for quantification of comet tails (Fig. 2B). The KG-1 cell line, which at 37 °C is essentially a null mutant with regard to OGG1 activity, was also used (30). RUNX1-RUNXIT1 cell lines had significantly more 8-oxo-dG lesions than the OGG1-proficient cell line, with levels of OGG1-specific base damage, as measured by the percent DNA in the tail and the tail moment, comparable to those detected in the KG-1 cells (Fig. 2 C and D). These data suggest that the decreased levels of nuclear OGG1 result in a BER-deficient cellular phenotype that may underlie any potential synergism with chemotherapeutic agents.

OGG1-Deficient AML Cells Are Differentially Sensitive to Ara-C Treatment but Not to General Replication Stressors. To determine whether decreased expression of nuclear OGG1 translated to a functional loss of OGG1, cell viability was measured following exposure to either Ara-C or daunorubicin (Fig. 3 A and B). OGG1-mutated KG-1 cells were highly sensitive to Ara-C, while Kasumi-1 and SKNO-1 cells had an intermediate sensitivity and MOLM-14 cells were highly resistant, suggesting possible OGG1-dependent sensitivity (Fig. 3A). In contrast, treatment with increasing concentrations of daunorubicin showed no correlation between OGG1 level and survival (Fig. 3B).

Given the apparent OGG1-dependent sensitivity to Ara-C, we investigated whether this response was Ara-C-specific or a generalized response to replication stress. Two different known replication stressors, hydroxyurea (Fig. 3C) and aphidicolin (Fig. 3D), were used. Similar to the results of daunorubicin treatment, neither compound showed an OGG1-dependent cytotoxicity response. To further explore the specificity of the Ara-C-mediated, OGG1-dependent cytotoxicity, we tested whether a related arabinose-based replication inhibitor, Ara-G, would yield comparable survival. This nucleoside contains the same modified sugar as Ara-C but has a guanine instead of a cytosine base (Fig. 3G). These data show that Ara-G did not enhance cytotoxicity in OGG1-deficient cells, indicating that the observed response to Ara-C was not indicative of a more generalized inability to cope with arabinose-based nucleoside analogs (Fig. 3E).

We also tested whether another cytosine-based nucleoside analog, gemcitabine (Fig. 3G), would confer toxicity comparable to those of Ara-C; however, substitution of the arabinose sugar with difluororibose did not show any evidence of OGG1-dependent sensitivity (Fig. 3F).

Fig. 2. Differential OGG1 activity and 8-oxoG levels in AML cells. (A) Relative incision activity in nuclear extracts from AML cell lines on 8-oxo-dG-containing DNAs. The mean relative activities with corresponding SEs were calculated from four independent nuclear extract preparations. (B) Representative images of the modified comet + OGG1 enzyme incubation. (C and D) Percent DNA in the tail (C) and tail moment (D) to quantify differences in the modified comet assay. Values are normalized to the control (−OGG1) for each condition. For each, 100 cells were scored, and each condition was tested in biological triplicate. The mean values with corresponding SEs were obtained from three independent experiments. For all panels, significance is calculated versus OGG1-proficient MOLM-14. *P < 0.05; **P < 0.01; ***P < 0.001.
Collectively, these data demonstrate a specific effect of Ara-C in OGG1-deficient cells that cannot be attributed to a general inability to cope with replication stress, or even highly similar molecules such as Ara-G or gemcitabine. These data also suggest that the observed cytotoxicity was not simply the result of impaired replication due to Ara-C incorporation, but rather to the specific combination of Ara-C and elevated levels of 8-oxo-dG damage.

Ara-C Induces Fragile Site Expression in an OGG1-dependent Manner. Since the mechanism of action of Ara-C has been primarily associated with DNA fragmentation and chain termination (31), we examined chromosome instability following treatment. As shown in Fig. 4A, modest levels of breaks were observed in the SKNO-1 and KG-1 cells as early as 24 h after Ara-C treatment compared to the MOLM-14 cells. All the OGG1-deficient AML cells had significantly elevated levels of chromosomal breaks after 48 h and 72 h. There were no statistical differences in the number of breaks observed among any of the OGG1-deficient cell lines at the 48 h or 72 h time points (Fig. 4A). Interestingly, these breaks did not follow the traditional chromosomal breakage pattern typified by homologous recombination-deficient cells (i.e., BRCA or Fanconi anemia) in response to DNA crosslinking agents, with notably low levels of radial formation given the large numbers of breaks observed (Fig. 4B). Ara-C–induced breaks were mapped using G-banding, and the data revealed that the sites of breakage were not random but were correlated with common fragile sites (Fig. 4C). Of the 450 breaks scored, 72% mapped to 5 of the 86 known common fragile sites, with the most
prevalent being FRA3B (3p14.2; 20%), FRAXB (Xp22; 18%), and FRA16D (16q23; 13%) (Fig. 4 D and E). Although these are common fragile sites, this percentage far exceeds the expected frequency calculated for random occurrence at any fragile site (19% for all 86 fragile sites combined given 450 breaks analyzed).

These common fragile sites are chromosomal locations that commonly break when cells are stressed with aphidicolin or other replication stressors and are thought to be due to a combination of factors, including delayed and prolonged replication, a paucity of origins, AT-rich sequences, and secondary DNA structures. These
data suggest that there is no Ara-C-dependent bias of fragile site expression toward any unique specific regions, but rather exacerbation of chromosomal fragility associated with generally difficult-to-replicate regions of the genome.

To move from correlation to causation, we used the KG-1 cells to switch OGG1 on and off by changing the temperature conditions before treatment with Ara-C. Prior characterization of the KG-1 cells had determined that a 6-h incubation at 25 °C was sufficient time for restoration of OGG1 activity and repair of accumulated 8-oxo-dG lesions to wild-type levels (30). To test whether elevated 8-oxo-dG levels were directly related to the observed Ara-C-dependent chromosome breaks, KG-1 and Kasumi-1 cells were incubated at 25 °C for 6 h, then treated with Ara-C and switched back to 37 °C for 48 h. The Kasumi-1 cells were used as a positive control for temperature-insensitive breaks and for any effects of the temperature shift on the breakage assay results. As shown in Fig. 4F, when pretreated for 6 h at 25 °C to allow sufficient time for the KG-1 cells to clear 8-oxo-dG lesions, KG-1 cells had significantly fewer breaks than Kasumi-1 cells, with a 66% reduction in breaks relative to those with no preincubation. These data demonstrate that the repair of 8-oxo-dG lesions before treatment with Ara-C directly resulted with no preincubation. These data demonstrate that the repair of 8-oxo-dG lesions associated with generally difficult-to-replicate regions of the genome.

Considering the data presented above, it was important to elucidate molecular interactions between Ara-C and 8-oxo-dG. Thus, in vitro replication assays were conducted to determine whether the Ara-C opposite 8-oxo-dG pair can be formed and how it is different from the Ara-C opposite dG or dC opposite 8-oxo-dG pairs. Several DNA polymerases have been previously identified that are capable of inserting a dC or dA opposite the lesion, with the efficiencies of products beyond the target site being 22- and 23-mer insertion products. To evaluate activity of pol δ on an 8-oxo-dG-containing template in the presence of Ara-C, reactions were conducted with the exonuclease-proficient form of enzyme (Fig. 5 B and C and SI Appendix, Fig. S24). The data showed that on nondamaged dG template, substitution of dCTP by Ara-CTP had no significant effect on DNA synthesis, with the incorporation of Ara-C resulting in a slight shift in electrophoretic mobility of the product bands relative to reactions containing dCTP. The relative amounts of 21-mer insertion products were indistinguishable (lanes 2 and 4). In reactions containing Ara-CTP and dGTP, very minor pausing was observed opposite dC (lane 5), with the major extension products being 22-mers, suggesting incorporation of Ara-C at both position 21 and position 23. The total amount of products beyond the target site was slightly higher in the presence of dCTP than in the presence of Ara-CTP (lanes 3 and 5), but the difference was not statistically significant. Consistent with previous analyses (40), exonuclease-proficient pol δ was also able to insert dC opposite 8-oxo-dG (lane 7) and in the presence of dGTP, extended this primer to position 23 (lane 8). In contrast, there was a significant decrease in the accumulation of insertion products on the 8-oxo-dG-containing template in the presence of Ara-CTP.
(lane 9), such that relative to the reaction supplemented with dCTP (lane 7), the amount of this product was reduced by ∼3.4-fold ($P < 0.001$). Additionally, the products beyond the 8-oxo-dG site were barely detectable (lane 10). The latter result suggests that following incorporation of Ara-C opposite 8-oxo-dG, either further primer extension was strongly inhibited or nascent Ara-C could be subjected to exonucleolytic removal by pol δ.

Further investigations examined whether the presence of Ara-C opposite template dG or 8-oxo-dG would affect the exonuclease function of pol δ. The polymerase was incubated with various primer-template combinations in the absence of dNTPs (SI Appendix, Fig. S3). These data revealed that the exonuclease processing of a 3′-Ara-C nucleotide was considerably slower than that observed for the control 3′-dC.

To gain additional insights into the incorporation and extension properties of pol δ when synthesizing past 8-oxo-dG sites, we also examined the exonuclease-deficient form of pol δ (DS20V) (Fig. 5 D and E and SI Appendix, Fig. S2B). The patterns of product formation using nondamaged template with either dCTP (lanes 2 and 3) or Ara-C (lanes 4 and 5) or using 8-oxo-dG template with dCTP (lanes 7 and 8) were very similar to the pattern observed for its exonuclease-proficient counterpart (Fig. 5 B and C). The level of incorporation of Ara-C opposite of 8-oxo-dG by exonuclease-deficient pol δ was relatively high. The amount of product was only slightly lower in the presence of Ara-CTP (lane 9) than in the presence of dCTP (lane 7), and the difference was not statistically significant. However, the extension step of reaction was strongly inhibited (lane 10). Collectively, these data reveal that pol δ can insert Ara-C opposite 8-oxo-dG, generating a replication intermediate that is inhibitory to both forward (polymerase) and reverse (exonuclease) reactions.

Discussion

The overall responsiveness of the aggregate of AML patients treated by 7+3 therapy varies widely, with patients with the two subtypes RUNX1-RUNX1T1 and CBFB-MYH11 having significantly more favorable long-term prognoses. We hypothesized that analyses of differential gene expression in favorably-responding patient subtypes may hold the key to the design of therapies for poor responders. Thus, AML survivorship is particularly instructive in establishing OGG1 as the critical DNA damage even in the absence of exogenous stressors (Fig. 2).

Based on this relationship, we tested whether this decreased expression correlated with functional biological endpoints relative to the standard of care chemotherapeutic drug, Ara-C. As shown in Figs. 3A and 4, we observed correlations between increased cytotoxicity and chromosomal breaks induced by Ara-C treatment. Furthermore, use of the tsOGG1 KG-1 cells was particularly instructive in establishing OGG1 as the critical DNA repair enzyme associated with resistance to Ara-C treatment. Specifically, our analyses demonstrated that KG-1 cells at a nonpermissive temperature (37 °C) showed significant increases in chromosomal breaks (Fig. 4F) relative to permissive growth conditions (25 °C). At nonpermissive temperatures, KG-1 cells also showed high levels of endogenous oxidatively-induced DNA damage (Fig. 2B and C). Although these data correlating OGG1 deficiency with sensitivity to Ara-C suggested that other replication-blocking agents might exert comparable genotoxicity via its effects on replication of nuclear DNA, AraC damage. It is worth noting that in addition to AraC-induced genotoxicity via its effects on replication of nuclear DNA, AraC has also been implicated in exerting mitochondrial-associated cellular toxicities in postmitotic neurons (44).

These data suggest a model for cell death that is dependent on elevated 8-oxo-dG in DNA and incorporation of Ara-C opposite this lesion. Using in vitro polymerase reactions, we demonstrated that formation of pairs between Ara-C and 8-oxo-dG is possible, even by action of a high-fidelity DNA polymerase (Fig. 5). We hypothesize that a subset of low-fidelity DNA polymerases which are specialized in translesion synthesis also would be able to insert Ara-C opposite 8-oxo-dG and likely continue polymerization beyond that site. Formation of such a noncanonical pair would be expected to have a severe impact on genomic integrity. It was apparent that the structure of this pair at the primer terminus differed from either Ara-C opposite dG or dC opposite 8-oxo-dG. In both of these cases, subsequent extension by pol δ was minimally affected. In contrast, incorporation of Ara-C opposite 8-oxo-dG essentially prohibited the forward and reverse reactions. We further hypothesize that the presence of Ara-C opposite 8-oxo-dG in genomic DNA would perturb the biochemistry of DNA repair, recombination, and modification, which would translate into reduced rates of replication, induced DNA strand breaks, and DNA fragmentation.

Collectively, our data provide a solid foundation for validating OGG1 as a target in AML therapeutics for patients with subtypes with normal or elevated OGG1 levels. It would be
anticipated that concomitant administration of Ara-C with OGG1 inhibitors could significantly improve the overall survival of non-RUNX1-RUNX1T1 patients.

Materials and Methods

Description of Patient Samples. Deidentified patient samples were provided by Jeffrey Tyner, OHSU, from the collection of the Beat AML cohort (28). All patients provided informed consent to participate in this study on Institutional Review Board-approved protocols, as described previously (28). The 30 RNA samples included in our study represented 26 patients, 11 with the RUNX1-RUNX1T1 translocation and 15 with other AML subtypes (two patients with serial samples). The details of each sample are presented in the SI Appendix, Table S2. Samples from the same patient are identified by color coding in the table.

Cell Lines. The human leukemia cell lines MOLM-14 (OGG1 proficient), Kasumi-1 and SKNO-1 (both RUNX1-RUNX1T1 fusion; OGG1 reduced), and KG-1 (tsOGG1) were kindly provided by Jeffrey Tyner, OHSU. All cells were grown and maintained in RPMI-1640 (HyClone) with 1% antibiotic-antimycotic ( Gibco). MOLM-14 and KG-1 cells were supplemented with 10% FBS, Kasumi-1 cells were supplemented with 20% FBS, and SKNO-1 cells were supplemented with 15% FBS and human granulocyte macrophage colony-stimulating factor (Peprotech) at a final concentration of 10 ng/mL. Cells were maintained in T25 flasks (VWR) in a humidified ambient oxygen incubator at 37 °C with 5% CO2. All cell lines routinely tested negative for the presence of mycoplasma using the MycoAlert Mycoplasma Detection Kit (Lonza). Cell lines were authenticated in our laboratory via karyotyping. All cell suspensions were resuspended at −20 °C to avoid genetic drift.

Measurement of OGG1 Transcript Levels in Patient Samples and Cell Lines. RNA that had been previously isolated from patient samples collected through the OHSU Beat AML project were diluted to <100 ng/μl prior to cDNA production. Cells from culture were collected by centrifugation for 5 min at 180 × g, washed in cold PBS, and recentrifuged before being flash frozen at −80 °C. RNA isolation was performed using the Direct-zol RNA MiniPrep Plus Kit (Zymo Research). cDNA was constructed from both patient RNA samples and cell line RNA using the iScript cDNA Synthesis Kit (Bio-Rad). PCR was performed using normalized cDNA concentrations (NanoDrop), DreamTaq cell line RNA using the iScript cDNA Synthesis Kit (Bio-Rad). PCR was performed using normalized cDNA concentrations (NanoDrop), DreamTaq Master Mix (Thermo Fisher Scientific), and the following primers. The forward primer for isoform 1 (5′-GACTACAGTGCCGACCTACACC-3′) is based in exon 6 and was used to anchor all OGG1-specific PCR amplifications. To amplify OGG1 isoform 1A, a reverse primer (5′-GACCTAGCCAGGACCATG-TGG-3′) was constructed that spanned the junction between OGG1 exons 6 and 7. This 131 bp PCR product is referred to as the isoform 1A-specific product. Another reverse primer (5′-GGAGTGCGGACCTGAAACGC-3′) was based in exon 7 and when used with the forward primer generated two PCR products, 139 bp (isoform 1A) and 383 bp (isoform 1B). The PCR primers for the GAPDH control reactions were 5′-ACTGCTATCTGGTGTCAT-3′ (forward) and 5′-CTCTCCCTTGGTCTGCTTC-3′ (reverse). The PCR conditions were Tm = 59 °C and a 30-s extension for OGG1 primers and Tm = 53 °C and a 30-s extension for GAPDH primers, for a total of 30 cycles. Reactions were performed in triplicate, and samples were run on a 5% polyacrylamide gel in 0.5% TBE for 3 h at 120 V and then stained for 1 h in GelRed. All patient samples were processed in parallel and randomized on the PCR block. To negate gel-to-gel variation in staining intensity, samples were randomized on the gels. PCR products were visualized with the FluorChem M system (Protein Simple). Analysis of band intensity was performed using ImageJ (45), and all measurements were normalized to respective GAPDH intensity.

Cytotoxicity Induced by DNA Replication Blocking Agents. For all drug treatments, cells were seeded in 96-well plates at 10,000 cells per well in technical triplicate for each drug dose. Increasing amounts of Ara-C (Tocris Biosciences), daunorubicin (Sigma-Aldrich), Ara-G (Fisher Scientific), or gemcitabine (VWR) were added to cells for a total of 5 d. On day 5, cell survival was analyzed using AlamarBlue (Bio-Rad) and a TECAN plate reader. The data were plotted using Graph Pad Prism software.

Preparation of Nuclear Extracts. For preparation of nuclear extracts, cells were grown to a density of ~2 to 4 × 106 cells/100 μl, collected by centrifugation at 180 × g, and fractionated using an Abcam kit (ab113474). The nuclear proteins were obtained by incubation of nuclei with Abcam extraction buffer (5 μL per 106 cells), followed by centrifugation at 12,000 × g for 10 min. The supernatant fraction was collected, kept on ice, and used in the OGG1 activity assays within 2 to 2 h. The protein concentrations were measured using Bradford reagent (Bio-Rad) and a TECAN plate reader.

DNA substrate for the OGG1 activity assays was a 17-mer double-stranded oligodeoxynucleotide containing a site-specific 8-oxo-dG, the TAMRA moiety on the 5′ terminus of the lesion-containing strand, and BHQ2 on the 3′ terminus of the complement strand (5′-TAMRA-TCACCC-3′) (28). An additional control reaction was conducted in the presence of both NEIL1 and OGG1 to account for the presence of 50 mM NEIL1. The reaction was conducted in the presence of mycoplasma using the MycoAlert Mycoplasma Detection Kit (Lonza). The data were collected from four independent protein extract preparations and treated as follows. The area under the curve was calculated for each experiment, and following subtraction of the corresponding value from the parallel NEIL1 reaction, relative activities were calculated. The OGG1 activity of MOLM-14 nuclear extracts measured in the same experiment as a reference. The area under curve values, average relative activities with corresponding SEs, and P values were calculated using KaleidaGraph software (Synergy Software).

Comet Assay. A modified comet assay was performed with the OGG1-FLARE kit (Trevigen) with modifications. The positive control was a 3-h treatment with 20 μM KBrO3. Cells were collected by centrifugation at 180 × g for 5 min and resuspended in serum-free medium at a density of 106/mL, followed by gentle mixing in a 1:2 ratio with 37 °C low-melting agar. The suspension was quickly pipetted onto 37 °C FLARE slides. Once a ring of dried agar appeared around the edge of each well, parafilam was placed over the slide, and agar was set at 4 °C for 45 min. Parafilam was then removed prior to incubation in chilled lysis buffer overnight. Slides were processed according to the supplier’s instructions. In brief, slides were soaked in FLARE buffer and then incubated in either buffer alone (−OGG1) or buffer with OGG1 enzyme (+OGG1) under a parafilam coverslip at 37 °C in a preequilibrated humid chamber. Parafilam was then removed, and slides were soaked in alkaline electrophoresis buffer prior to running at 24 V for 30 min. Samples were neutralized in 0.4 M Tris-HCl, pH 7.4, and stained with 200 μg/mL SYBR Gold for 20 min at room temperature. Slides were imaged at 10× magnification with a Keyence BZ-X710 microscope. Each condition was tested in biological triplicate. For each well, 100 cells were scored using CometScore v1.5.

Chromosomal Breakage Assay. Cells were plated at 8 × 105 cells/mL in a 6-well plate and treated with agents for indicated times. The cells were harvested by centrifugation at 180 × g for 5 min and the pellet was resuspended in warmed hypotonic solution (75 mM KCl, 5% FBS) for 10 min. A 3:1 methanol-acetic acid fixative was added to each tube, and the cell pellet was collected and resuspended in 3 mL of the fixative at room temperature for 15 min. The cells were pelleted and then stored at −20 °C until being dropped and stained for chromosomal breakage analyses.

Chromosome Fragile Site Analyses. Kasumi-1 cells were treated with 100 nM Ara-C for 48 h prior to harvest. Colcemid (0.05 μg/mL) was added for 3 h to arrest cells at metaphase. Cells were harvested as described above, fixed on slides, and baked at 90 °C for 20 min. After cooling, cells were trypsinized for 45 s, stained with Wright's stain for 80 s, rinsed with H2O2, and dried. Chromosome breaks were imaged using bright field microscopy at 100× magnification and analyzed using CytoVision software (Applied Imaging). Breakpoints were identified according to standard ISCN nomenclature (47) and the TAMRA fluorescent signal was recorded in a TECAN plate reader at 37 °C for 2 h using a 525/9-nm excitation filter and a 598/20-nm emission filter. The reactions were conducted in 20 mM Tris-HCl pH 7.4, 100 mM KCl, and 0.01% (vol/vol) Tween 20 with 50 or 250 nM DNA substrate and 0.1 or 0.2 μg/μL nuclear proteins. The reaction volume was 20 μL. Each experiment included the positive control reaction with 50 nM OGG1 to ensure that the amount of DNA substrate did not limit the product formation in reactions with nuclear proteins, as well as the negative control reaction containing Abcam extraction buffer (Fig. S1). An additional control reaction was conducted in the presence of both NEIL1 and OGG1 to account for the presence of 50 mM NEIL1. The reaction contained an unidentified lesion that was a substrate for NEIL1 but not for OGG1. The fraction of this contaminating species constituted ~5% of total DNA and was regarded as background. The data were collected from at least four independent protein extract preparations and treated as follows. The area under the curve was calculated for each experiment, and following subtraction of the corresponding value from the parallel NEIL1 reaction, relative activities were calculated. The OGG1 activity of MOLM-14 nuclear extracts measured in the same experiment as a reference. The area under curve values, average relative activities with corresponding SEs, and P values were calculated using KaleidaGraph software (Synergy Software).

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Preparation of DNA Substrates for In Vitro Replication Assays. All unmodified oligodeoxynucleotides, a 12-mer oligodeoxynucleotide containing an internal 8-oxo-dG, and a 21-mer oligodeoxynucleotide containing a terminal Ara-C were synthesized and purified by Integrated DNA Technologies. The 59-mer oligodeoxynucleotides used as templates for in vitro polymerase reactions were constructed according to a published procedure (40) and were described previously (49). The sequence of the template was 5′-AGGCC-CAGTTGAGTACGGTCGTTCCCTGATCTGTTCTGGAATCA-3′, where X was either dG or 8-oxo-dG. The sequences of oligodeoxynucleotides used as primers were 5′-ACTACGGATGTGACC-3′ (dC primer), and 5′-ACTACGATGACC (Ara-C)-3′ (0-Ara-C primer). Primer oligodeoxynucleotides were radioactively labeled with 32P-α-UTP using T4 polynucleotide kinase (New England Biolabs) and annealed to templates as described previously (49).

Replication Assays In Vitro. Ara-C triphosphate (Ara-CTP) was purchased from Abcam (ab146731), Recombinant Saccharomyces cerevisiae pol α (Pol3-Pol31-Pol32) and its exonuclease-deficient DS20V variant, a generous gift from Peter Burgers, were overexpressed and isolated as reported previously (50, 51). Polymerase reactions contained 5 nM DNA substrate and were conducted at 37 °C in a buffer composed of 25 mM Tris HCl pH 7.5, 10 mM NaCl, 8 mM MgCl2, 10% glycerol, 1 μg/ml bovine serum albumin, and 5 mM dithiothreitol. Concentrations of polymerase were varied from 0.1 to 0.5 U/μl. The reaction mixtures were denatured for 15 min at 95 °C and reannealed on ice. Polymerase reactions were resolved by electrophoresis in a 15% denaturing polyacrylamide gel containing 8 M urea in Tris-borate-EDTA buffer and visualized using a Personal Molecular Imager (Bio-Rad). The gel images were calculated using Student t-tests. All data were analyzed using the main text and/or SI Appendix.

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