Quantitative determination of decitabine incorporation into DNA and its effect on mutation rates in human cancer cells

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ABSTRACT
Decitabine (5-aza-2′-deoxycytidine) is a DNA methyltransferase inhibitor and an archetypal epigenetic drug for the therapy of myeloid leukemias. The mode of action of decitabine strictly depends on the incorporation of the drug into DNA. However, DNA incorporation and ensuing genotoxic effects of decitabine have not yet been investigated in human cancer cell lines or in models related to the approved indication of the drug. Here we describe a robust assay for the quantitative determination of decitabine incorporation rates into DNA from human cancer cells. Using a panel of human myeloid leukemia cell lines we show appreciable amounts of decitabine incorporation that closely correlated with cellular drug uptake. Decitabine incorporation was also detectable in primary cells from myeloid leukemia patients, indicating that the assay is suitable for biomarker analyses to predict drug responses in patients. Finally, we also used next-generation sequencing to comprehensively analyze the effects of decitabine incorporation on the DNA sequence level. Interestingly, this approach failed to reveal significant changes in the rates of point mutations and genome rearrangements in myeloid leukemia cell lines. These results indicate that standard rates of decitabine incorporation are not genotoxic in myeloid leukemia cells.

INTRODUCTION
The cytidine analog decitabine (5-aza-2′-deoxycytidine, DAC) represents a potent anticancer drug for the treatment of myelodysplastic syndromes (MDS) and acute myelogenous leukemia (AML) (1,2). After cellular uptake, decitabine becomes metabolically activated before it can be incorporated into DNA. The incorporation of azacytosine bases into DNA triggers a variety of cellular pathways and ultimately results in complex cytotoxic and epigenetic effects (3).

Most studies that investigated the mode of action of decitabine have focused on the drug’s ability to induce DNA hypomethylation and gene reactivation. This represents a unique feature that distinguishes decitabine (and the closely related drug azacytidine) from other nucleoside drugs (4). After incorporation into DNA, the modified base can function as a suicide substrate for DNA methyltransferases, which initiate the methylation reaction with azacytosine, but fail to resolve a covalent bond with the modified base (5). This covalent trapping triggers the proteasomal degradation of DNA methyltransferase enzymes and thereby depletes cells of their capacity to effectively maintain DNA methylation patterns (3). If cells synthesize DNA under conditions of reduced maintenance methylation, their genomic DNA becomes hypomethylated. As a consequence, aberrantly methylated genes can become demethylated and their expression can be restored. This represents the original principle of epigenetic cancer therapy, which aims at using drug-induced DNA hypomethylation to restore the expression of genes that are affected by hypermethylation-induced gene silencing (6).

Besides DNA hypomethylation, decitabine also induces significant cytostatic and cytotoxic effects. The underlying mechanisms have been linked to the formation of covalent adducts between incorporated azacytosine bases and DNA methyltransferase enzymes, which cause stalled replication forks and pronounced changes in the cell cycle of decitabine-treated cells (7). Another important feature of the cytotoxic response to decitabine is the induction of gamma-H2AX expression, a prominent marker for DNA damage (7–9). Indeed, it has also been shown that

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decitabine can induce point mutations and genome rearrangements (9,10). Both effects have been linked to the formation of covalent adducts between incorporated azacytosine bases and DNA methyltransferase enzymes (9,10). As such, the incorporation of decitabine into DNA represents a key factor in understanding the mode of action of the drug (11). This is also illustrated by the dose-dependent differences in the cellular responses to decitabine: at higher (micromolar) drug concentrations decitabine shows predominantly cytotoxicity, while lower (nanomolar) drug concentrations usually induce DNA hypomethylation without causing cytotoxicity (12).

Decitabine needs to be metabolized to decitabine triphosphate before it can be incorporated into DNA (11). Methods that allow the quantification of decitabine triphosphate have been developed recently and have indicated that higher levels of this metabolite may be associated with clinical responses (13,14). However, to our knowledge, drug incorporation into DNA has never been reported in human cancer cell lines, even though an initial study had shown that the incorporation of radioactively labeled decitabine can be quantitatively detected in a mouse embryonic fibroblast cell line (15). Similarly, the incorporation-dependent genotoxic effects of azacytosine have been analyzed in mouse fibroblasts (9,10), but not in models related to the approved indications of the drug. Here we present a method that allows the quantitative analysis of decitabine incorporation into genomic DNA from human cancer cells. Significant amounts of incorporation were detected in all experiments and correlated closely with cellular decitabine uptake and global DNA hypomethylation. Next-generation sequencing strongly suggested the absence of decitabine-induced point mutations and genome rearrangements, thus further confirming the safety of low-dose decitabine regimens for epigenetic therapy.

MATERIALS AND METHODS

Chemicals

[6-3H]-Decitabine and [14C]-deoxycytidine were obtained from Sigma–Aldrich and prepared by dissolving in distilled H2O. [42x301]Chemicals

MATERIALS AND METHODS

[42x342]genetic therapy.

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Chemicals

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Cell culture

Cancer cell lines: ML-2, K562, U937 and HL-60 were cultured in RPMI-1640 medium, KG-1 and KG-1a in Iscove’s Modified Dulbecco’s Medium (IMDM) medium and HCT116 in McCoy’s 5A medium. Normal cell lines: WI-38 were cultured in Minimum Essential Medium Eagle medium, hTERT RPE-1 in Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium and HS-5 in DMEM medium. All media were supplemented with 10% fetal bovine serum and 200 U/ml penicillin and 200 µg/ml streptomycin. IMDM medium was supplemented with 20% fetal bovine serum. All cell lines were re-authenticated after completion of the relevant experiments and periodically controlled for cell contamination (Multiplexion). Bone marrow or peripheral blood samples from nine newly diagnosed, untreated AML patients were obtained from the Department of Hematology, Oncology and Stem Cell Transplantation, University of Freiburg (Supplementary Table S1). All patients signed informed consent prior to participation, and the study was approved by the ethics committee of the University Medical Center Freiburg. Mononuclear cells were isolated by density gradient centrifugation. For patient E, CD34pos/CD117pos blast cells were isolated with magnetically labeled CD34 and CD117 MicroBeads using an autoMACS Separator (Miltenyi Biotec). AML samples were cultured in RPMI-1640 medium supplemented with 20% fetal bovine serum, 100 ng/ml GM-CSF (Gibco), 200 U/ml penicillin and 200 µg/ml streptomycin. All cells were cultured in 5% CO2 at 37°C.

Incorporation assay

Twenty-four hours before treatment, cells were seeded in triplicate in 12-well plates, at a density of 2 × 105 cells per well and then incubated with 100 nM [3H]-decitabine (or other concentrations, if indicated). After incubation for 24 h (or other time periods, if indicated), cells were washed with phosphate buffered saline (PBS). For incorporation measurements, DNA or RNA was purified using DNeasy Blood and Tissue kit (Qiagen) or RNeasy kit (Qiagen), respectively, and quantified by a ultraviolet (UV) photometer. The purified samples were mixed with liquid scintillation cocktail (Ultima Gold, Perkin Elmer) and their radioactivity was measured by liquid scintillation counting. Measurements were normalized to the amount of DNA (or RNA). The percentage of substitution of decitabine for cytosine was calculated as the amount of decitabine incorporated as a fraction of total cytosine, as described previously (15). For competition experiments, cells were either treated with 100 nM [3H]-decitabine in addition with increasing concentrations (100 nM, 500 nM, 1 µM, 2 µM) of [14C]-deoxycytidine or were treated with 100 nM [14C]-deoxycytidine in addition with increasing concentrations (100 nM, 500 nM, 1 µM, 2 µM) of [3H]-decitabine. After 24 h, cells were washed with PBS, DNA was extracted using DNeasy Blood and Tissue kit (Qiagen) and was measured using liquid scintillation counting.

Transport assay

Transport assays were conducted as described previously (16). Twenty-four hours before treatment, cells were seeded in triplicate in 12-well plates, at a density of 2 × 105 cells per well and then incubated with 100 nM [3H]-decitabine. After incubation for the indicated time periods, cells were washed with PBS and lysed with 0.2% sodium dodecyl sulphate (SDS). The isolated samples were mixed with liquid scintillation cocktail and radioactivity was measured by scintillation counting. In parallel, protein concentration was measured using a bicinchoninic acid (BCA) protein assay to normalize the total cellular uptake to total protein concentrations.

DNA methylation analysis

Genomic DNA was isolated from cells using the DNeasy Blood and Tissue kit (Qiagen). Global DNA methylation
levels were determined by capillary electrophoresis, as described previously (17).

**Cell cycle analysis**

Approximately $1 \times 10^6$ cells were treated with 100 nM decitabine. Cells were collected at time points indicated and fixed with ice-cold absolute ethanol. After fixation, cells were washed with PBS, centrifuged and resuspended in staining solution (0.1% Triton X-100, 0.2 mg/ml RNase A and 20 μg/ml propidium iodide) for 15 min at 37°C in the dark. For the flow cytometric analyses 10,000 cells were measured with a FACS Canto II (BD Biosciences) and data were analyzed using FlowJo software.

**Whole-genome sequencing**

Cells were seeded 24 h prior to the experiment. Cells were treated with 100 nM decitabine for 24 h and genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen). DNA was sheared into fragments of $\sim 300$ bp. Adapters were then ligated and fragments were size selected and purified. Cluster generation was performed on the Illumina cBot. The generated clusters from eight samples (four control, four treatment) were sequenced simultaneously on one lane in an Illumina HiSeq 2000 platform using 101 bp paired-end reads. Quality control of the generated sequences was performed using FastQC. Mapping was done by using Bowtie2 and hg19 as reference. For the analysis of point mutations SAMtools was used. The amount of genomic rearrangements was estimated by determining the proportion of discordantly aligning read pairs. All experiments were repeated with independent biological replicates. Mapping efficiencies and coverages are given in Supplementary Table S2. Sequencing data have been deposited in the SRA database under the accession number SRP040672.

**RESULTS**

**Establishment of an assay for the quantitative determination of decitabine incorporation rates**

To establish a cell-based assay for decitabine incorporation, we used the human ML-2 myeloid leukemia cell line as a model for the primary clinical indication of decitabine. Cells were grown in [3H]-decitabine containing media and DNA was purified after various time points. Levels of DNA-incorporated [3H]-decitabine were determined by scintillation counting and the DNA content of the sample was determined by a UV photometer (Figure 1A). Finally, incorporation rates were determined by quantifying the amount of radioactivity in purified DNA and then normalizing the results to the amount of DNA contained in the sample.

In a first series of experiments, we treated ML-2 cells with increasing concentrations of decitabine for 6 h. Following DNA isolation and radioactivity measurements, we observed concentration-dependent incorporation of decitabine into DNA (Figure 1B). Next, we treated ML-2 cells with 100 nM decitabine, and purified DNA and RNA after 3, 6 and 9 h of drug incubation, respectively (Figure 1C). A drug concentration of 100 nM was chosen because it is similar to the plasma concentration in myeloid leukemia patients that are treated with standard low-dose decitabine schedules (18,19). The results showed that significant levels of radioactivity were incorporated into DNA after 3 h of drug treatment, with further increases after 6 and 9 h of decitabine treatment, respectively (Figure 1C), reflecting progressive incorporation of decitabine. In contrast, RNA purified from the same cells did not contain significant levels of radioactivity (Figure 1C), which is in agreement with the known mode of action of decitabine (3).

To further confirm the incorporation of radioactively labeled decitabine into DNA, we performed a competition experiment. ML-2 cells were grown in media that contained 100 nM [3H]-decitabine and increasing concentrations of [14C]-labeled deoxycytidine. After 24 h, cells were harvested, DNA was purified and the two radioactive isotopes were separately quantified by scintillation counting. This revealed that deoxycytidine effectively competed with decitabine for DNA incorporation. At deoxycytidine concentrations of 500 nM and more, [3H]-decitabine incorporation was substantially suppressed, while [14C]-deoxycytidine incorporation became detectable (Figure 2A). We also cultured ML-2 cells with 100 nM concentrations of both [3H]-decitabine and [14C]-deoxycytidine to determine the frequency of cytosine to decitabine substitutions. This revealed that after 24 h of incubation, 0.45% of the genomic cytosine residues were substituted by decitabine, which is in good agreement with previous estimates (15). These results further confirm the incorporation of decitabine into DNA.

Finally, we also addressed the robustness of our assay by analyzing decitabine incorporation in three independent cultures of ML-2 cells (Figure 2B). The results confirmed the reliability of our assay and showed no significant differences between biological replicates.

**Decitabine incorporation in cancer cell lines and in patient samples**

To further explore the potential significance of decitabine incorporation for cellular drug responses, ML-2 cells were analyzed under various experimental conditions. When cells were grown in the presence of a single dose of 100 nM decitabine and harvested at various time points over 96 h, the results showed a steady accumulation of decitabine in DNA during the first 72 h of drug treatment (Figure 3A). After 96 h, incorporation rates became decreased (Figure 3A), which likely reflect the turnover of the drug in the cell culture media and in cellular DNA. In subsequent experiments, we also analyzed the total amount of intracellular decitabine from the same experiment and during the same time points. We define intracellular decitabine as the total amount of radioactivity measured within the cells, including mono-, di- and triphosphorylated decitabine. To this end, levels of [3H]-decitabine were determined in whole-cell extracts and normalized to the amount of protein contained in the respective samples. The observed pattern (Figure 3B) closely mirrored the time course of DNA incorporation, which suggests a close relationship between the cellular uptake of the drug and its DNA incorporation. Finally, we also determined the global DNA methylation level of ML-2 cells after a single dose of 100 nM decitabine. Capil-
Figure 1. Establishment of a quantitative decitabine incorporation assay. (A) Schematic explanation of the assay to measure intracellular decitabine levels. Radioactively labeled [3H]-decitabine is delivered into the cells where it is metabolically activated before becoming incorporated into DNA (black circles). DNA is then purified and the radioactivity is measured by liquid scintillation counting. (B) Concentration-dependent incorporation of [3H]-decitabine into DNA. ML cells were incubated with the indicated drug concentrations for 6 h. (C) Time-dependent incorporation of 100 nM [3H]-decitabine into DNA and RNA of ML-2 cells.

Figure 2. Specificity and robustness of the decitabine incorporation assay. (A) Competition experiment between [3H]-decitabine and [14C]-deoxycytidine for 24 h. The left panel shows the incorporation of [3H]-decitabine that was measured with the addition of increasing concentrations (100 nM, 500 nM, 1 μM, 2 μM) of [14C]-deoxycytidine. The right panel shows [14C]-deoxycytidine that was measured with the addition of increasing concentrations (100 nM, 500 nM, 1 μM, 2 μM) of [3H]-decitabine. (B) Incorporation of [3H]-decitabine (100 nM) levels in DNA of ML-2 cells in three independent experiments (biological replicates).

Lary electrophoretic analysis of DNA purified during various time points of the experiment revealed a baseline methylation level of 3.7% in untreated cells (Figure 3C). Following decitabine treatment, methylation levels steadily declined and reached a minimum of 1.9% after 48 h of drug incubation (Figure 3C). Longer incubation times resulted in somewhat higher methylation levels (Figure 3C), which may reflect DNA re-methylation following the turnover of the drug in the cell culture media and in cellular DNA. The specific drug treatment conditions used for this experiment did not cause any overt changes in the cell cycle profile of the ML-2 cells (Figure 3D). This indicates that the observed DNA demethylation is a consequence of DNA methylation inhibition and not influenced by the apoptosis-inducing properties of decitabine.

In following experiments, we further expanded the scope of our analysis by investigating decitabine incorporation in a panel of six leukemia cell lines (ML-2, K562, KG-1, KG-1a, U937 and HL-60) and a colon cancer cell line (HCT116). When these cell lines were treated with the same dose (100 nM, 24 h) of [3H]-decitabine, we did not observe any detectable drug-induced cytotoxicity (Supplementary Table S3). However, incorporation rates ranged from 0.1 pmol/μg DNA to 0.3 pmol/μg DNA (Figure 4A, upper
Figure 3. Decitabine incorporation correlates with DNA demethylation in ML-2 cells. (A) Incorporation rates of [3H]-decitabine (100 nM) in DNA of ML-2 cells at the indicated time points. (B) Total intracellular levels of [3H]-decitabine (100 nM) in ML-2 cells at the indicated time points. (C) After treatment with decitabine (100 nM), DNA was purified at the indicated time points and global DNA methylation levels were determined by capillary electrophoresis. (D) Cell cycle analysis of decitabine treated ML-2 cells, as determined by flow cytometry at the indicated time points. Data points and standard deviations (error bars) are derived from three or more replicates.

panel). Total intracellular decitabine levels appeared similar to the DNA-incorporated levels, with HL-60 and U937 cells showing the highest uptake (Figure 4A, lower panel). Drug-dependent differences in the cell cycle profiles were minor and not significant ($P > 0.5$, two-way ANOVA) for any of the seven cell lines (Figure 4B), which indicates that our results are a consequence of differential drug uptake and not influenced by differential drug toxicity. Interestingly, non-cancerous cell lines showed significantly ($P = 0.007$, $t$-test) lower levels of incorporated decitabine (Figure 4C). Similarly, total levels of intracellular decitabine were significantly ($P = 0.018$, $t$-test) lower in non-cancerous cells (Figure 4D). These findings are in agreement with the reduced cellular responses to low-dose decitabine in normal bone marrow cells (12).

To analyze decitabine incorporation in another model for the approved indication of decitabine, we cultured primary bone marrow aspirates from nine independent AML patients (Supplementary Table S1) with 100 nM [3H]-decitabine for 24 h. These cells showed variable, but clearly detectable levels of decitabine incorporation (Figure 4E). In parallel, intracellular uptake of decitabine was measured in the same samples. The results showed that samples with high amounts of intracellular decitabine also had high incorporation rates (Figure 4F), which again suggests a close relationship between drug uptake and DNA incorporation.

Clinically relevant doses of decitabine do not increase mutation rates in human cancer cells

Previous studies have suggested that decitabine incorporation causes significant genotoxicity, as evidenced by drug-induced point mutations and DNA structural rearrangements in various test systems (9,10). However, these studies used high (micromolar) drug concentrations and cell lines that are not related to the approved indication of the drug. In order to comprehensively analyze the effects of decitabine incorporation on the DNA sequence level, we treated a panel of four leukemia cell lines using our standard experimental conditions (100 nM decitabine for 24 h). We then used whole-genome sequencing to analyze the genome sequences of drug-treated cells in two independent biological replicates. This generated 415 million read pairs for further analysis (Supplementary Table S2), thus providing an adequate analytical power for our approach. Mapping of sequence reads to the human genome reference sequence uncovered a significant number of single nucleotide polymorphisms in all untreated cell lines, reflecting genetic variants and accumulated genetic mutations (Table 1). However, we did not observe any decitabine-dependent increases in mu-
Figure 4. Decitabine incorporation in various human cell lines. (A) Incorporation (upper panel) and total intracellular levels (lower panel) of [3H]-decitabine (100 nM) in DNA of various cancer cell lines after 24 h of treatment. (B) Cell cycle analysis of untreated (upper panel) and decitabine-treated (lower panel, 100 nM for 24 h) cancer cell lines by flow cytometry. (C) Box plot showing incorporation levels of [3H]-decitabine (100 nM, 24 h) for cancer cell lines and non-cancerous cell lines. (D) Box plot showing total intracellular levels of [3H]-decitabine (100 nM, 24 h) for cancer cell lines and non-cancerous cells. *P*-values were calculated by a Student’s *t*-test. (E) Incorporation levels of [3H]-decitabine (100 nM, 24 h) in DNA of various AML patient samples. (F) Total intracellular levels of [3H]-decitabine in the same AML patient samples.

Table 1. Sequencing-based detection of DAC-induced point mutations

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Experiment #1</th>
<th>Experiment #2</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>+DAC</td>
</tr>
<tr>
<td>HL-60</td>
<td>$5.3 \times 10^{-4}$</td>
<td>$5.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>K562</td>
<td>$5.6 \times 10^{-4}$</td>
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</tr>
<tr>
<td>KG-1a</td>
<td>$5.8 \times 10^{-4}$</td>
<td>$6.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>ML-2</td>
<td>$4.9 \times 10^{-4}$</td>
<td>$5.2 \times 10^{-4}$</td>
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Numbers represent single nucleotide polymorphisms per genomic base.
Decitabine metabolism. Our results suggest that decitabine into DNA, which represents the endpoint of intracellular decitabine metabolism. For this reason, recent studies also analyzed intracellular decitabine metabolites, including decitabine triphosphate by liquid chromatography-tandem mass-spectrometry quantification (13,14). Indeed, high levels of triphosphorylated decitabine were associated with clinical responses (14), suggesting that similar approaches might permit the identification of predictive biomarkers. We have now established a robust molecular assay for the quantitative determination of decitabine incorporation into DNA, which represents the endpoint of intracellular decitabine metabolism. Our results suggest that decitabine incorporation rates can vary considerably between patient cells. Further studies that address both the patient-specific pharmacokinetics of decitabine and the incorporation of the drug into patient cells will be required to investigate the potential of decitabine incorporation as a biomarker for predicting patient responses.

Previous studies have indicated major differences between normal and cancer cells in the cellular responses toward decitabine (4,12). Our results suggest that DNA incorporation rates of non-cancerous cell lines were significantly lower compared with the myeloid leukemia cell lines. This can be explained by lower amounts of decitabine uptake in normal cell lines. In agreement with this notion, WI-38 fibroblasts have been shown to express only very low levels of hENT1, a transport protein responsible for the cellular uptake of azanucleosides (24). These results further illustrate the link between drug uptake, DNA incorporation and demethylation and suggest that DNA incorporation might adequately reflect the epigenetic responses toward decitabine.

DISCUSSION

Decitabine represents an archetypal epigenetic drug that has been approved for the treatment of MDS and AML. However, the molecular mode of action of decitabine is complex and the establishment of molecular assays that accurately reflect cellular drug responses represents a major challenge in the field. Attempts to systematically correlate decitabine responsiveness with the demethylation and reactivation of specific genes have not been successful (22,23). For this reason, recent studies also analyzed intracellular decitabine metabolites, including decitabine triphosphate by liquid chromatography-tandem mass-spectrometry quantification (13,14). Indeed, high levels of triphosphorylated decitabine were associated with clinical responses (14), suggesting that similar approaches might permit the identification of predictive biomarkers. We have now established a robust molecular assay for the quantitative determination of decitabine incorporation into DNA, which represents the endpoint of intracellular decitabine metabolism. Our results suggest that decitabine incorporation rates can vary considerably between patient cells. Further studies that address both the patient-specific...
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