

Quantification of global DNA methylation levels in acute leukemia cell lines before and after decitabine treatment using a rapid, specific and sensitive LC-MS/MS method

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### Abstract

**Purpose:** Global DNA methylation (GDM) relates to genomic stability and is an important epigenetic event in malignant transformation of human cancers. As DNA hypermethylation is being explored as a therapeutic target in cancer, its changes might serve as a relevant endpoint for the pharmacodynamic effects of hypomethylating agents. Herein, we report quantification of GDM in 5 leukemia cell lines before and after exposure to the DNA hypomethylating agent decitabine or the histone deacetylase inhibitor FK-228, using a rapid, specific and sensitive electrospray (ESI) LC-MS/MS method.

**Methods:** Kasumi 1, K562, Jurkat, Eo1-1, THP-1 K562 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. Each cell line was treated with 2.5 μM decitabine or 5 nM FK-228 for 48 hr. DNA was extracted from 106 cells using DImP DNA blood midi Kit. A 1 μg aliquot of the extracted DNA from each cell line was sequentially hydrolyzed using nuclease P1 for 1 hr, venom phosphodiesterase I for 2 hr, and alkaline phosphatase for 1 hr. The genomic DNA methylation levels of these cell lines before and following drug treatment were measured using a modified ESI LC-MS/MS method. The method utilized the precursor/product ion pair at m/z 242/127 for 5-methyl-2-deoxycytidine (5mdC) and at m/z 268/152 for 2-deoxyguanosine (2dG) as the internal standard. Separation of 5mdC from 2dG and other nucleosides was accomplished on a C18 Aquasil column eluting with 30% methanol in 10 mM HCOONH<sub>4</sub>, with post-column addition of methanol.

**Results:** The LC-MS/MS method was validated in mobile phase with a linear range from 1 to 500 ng/mL. The within-day precision values ranged from 2.8 % to 9.9 % and the between-day ranged from 1.1 % to 17.6 % (n=6). The accuracy values of the assay varied from 96.7 % to 109.5 %. The baseline DNA methylation mean values of Kasumi 1, K562, Jurkat, Eol-1, THP-1 were 4.98, 2.43, 4.25, 4.45, and 4.79%, respectively. K562 cell was also used as the low quality control with a 7.0 %CV (n=6) and Kasumi 1 as the high quality control with a 6.8 %CV (n=6). Following treatment with decitabine, the GDM levels of Kasumi 1, K562, Jurkat, Eol-1, THP-1 were reduced to 57.1, 62.9, 63.7, 59.8, and 55.1% of those pre-treatment, respectively. Consistently, no alteration of the DNA methylation level was found in these cell lines following treatment with FK-228.

**Conclusion:** A significant hypomethylating effect of decitabine for 5 tumor cell lines was found. As a specificity control, we demonstrated that no hypomethylation occurred following treatment with FK-228. The modified ESI LC-MS/MS method described here provides an unambiguous measurement of GDM with high reproducibility and high sensitivity and can be adapted for high-throughput rapid screening of DNA methylation effects by DNA hypomethylating agents. Supported by NIH-NCI-RO1-CA102031.

## Introduction

- Decitabine, following activation to its triphosphate and incorporation into DNA, causes covalent linking with DNA methyltransferases leading to DNA hypomethylation and to reactivation of tumor-suppressive gene.
- Decitabine alone and in combination with other chemotherapeutic agents have been evaluated in clinical trials of patients with myelodysplastic syndrome, acute and chronic myeloid leukemia with favorable results.
- The *in-vivo* hypomethylating effect of decitabine in patients remains elusive. The alteration of global DNA methylation may serve as a relevant endpoint for the pharmacodynamic effects of decitabine and other hypomethylating agents.
- Herein, we developed and validated a highly sensitive and specific LC-MS/MS method for measurement of global DNA methylation and apply it to characterize the *in-vitro* and *in-vivo* hypomethylating effect of decitabine.

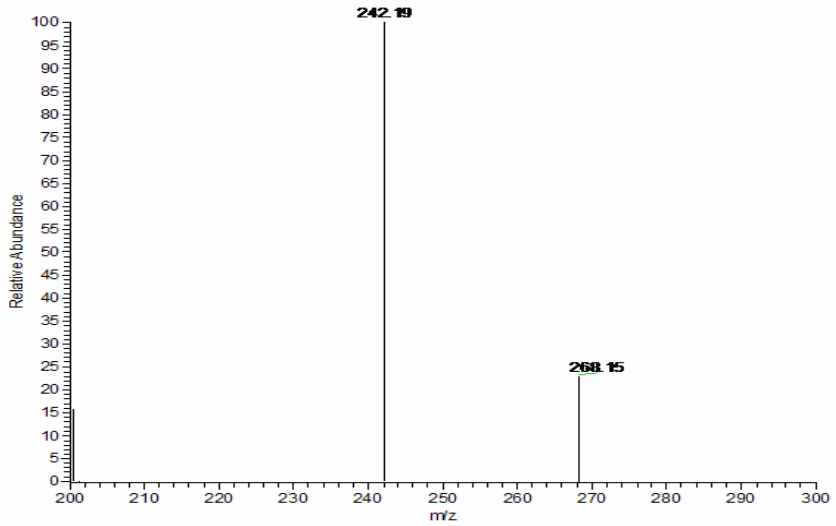
### Method

- HPLC: Shimadzu LC-10AD pumps with SIL-10A auto-sampler.
- Column: Aquasil C18, 250 × 2.1 mm.
- Mobile phase: 0.2 mL/min 30% methanol with 10 mM ammonium formate, plus 0.2 mL/min post-column addition of acetonitrile.
- MS: PE Sciex API 300 with Electrospray Ionization (ESI).
- MRM channels of 5mdC, 242.2/126.2, and 2dG (I.S.), 268.2/152.1.
- *In-Vitro* experiment: Kasumi 1, K562, Jurkat, Eo1-1, THP-1 K562 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. Each cell line was treated with 2.5 μM decitabine or 5 nM FK-228 for 48 hr and Kasumi 1 cell line was treated with various concentrations of decitabine (0.01 to 5 μM) for 48 hr and 72 hr.

### Method (cont'd)

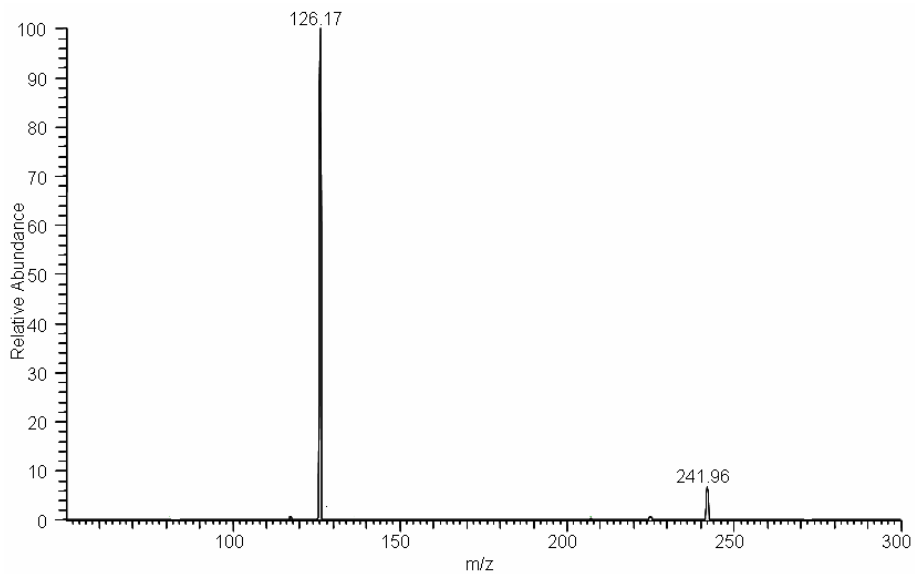
- Fourteen patients (pts) with either relapsed/refractory AML (n=8), ineligible/refused standard induction therapy (n=6) were evaluated. Pts included were from 57-83 years old and had received ≥ 2 prior induction (n=6), 1 prior induction therapy (2), or untreated (4). 8 pts received decitabine at 15 mg/m<sup>2</sup>/IV over 1 hr daily (d), and 6 received 20 mg/m<sup>2</sup>/d on the same schedule, q 28 d.
- Bone marrow aspirates were collected at the following time points: prior to beginning decitabine therapy, Day 4 and Day 11 of the first cycle of decitabine therapy, prior to beginning cycle 2 of decitabine therapy.
- DNA was extracted from 10<sup>6</sup> cells using DIAmp DNA blood midi Kit.
- Aliquots of 1 μg of the extracted DNA from each cell line or bone marrow was sequentially hydrolyzed using nuclease P1 for 1 hr, venom phosphodiesterase I for 2 hr, and alkaline phosphatase for 1 hr.

### Result



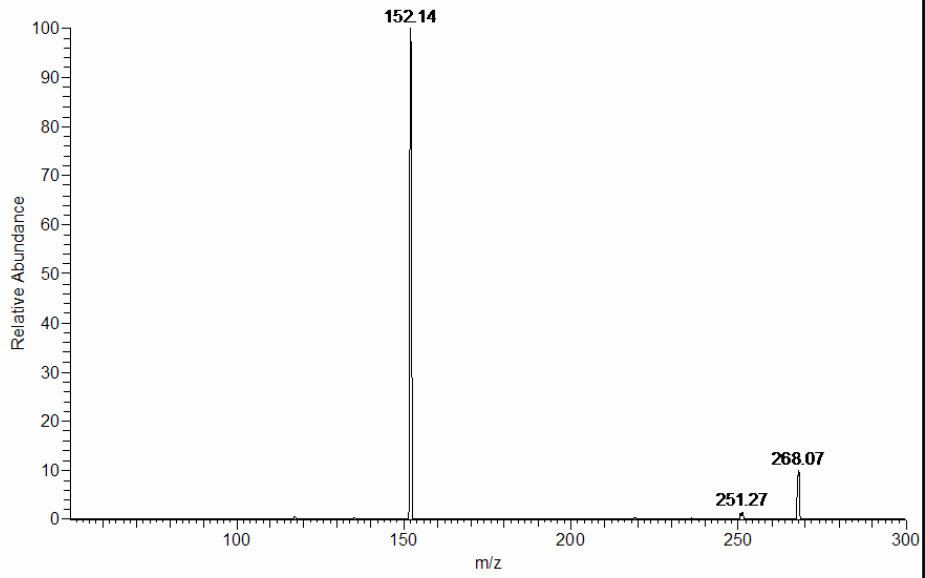
The average mass spectrum of 1 min infusion of 2-deoxyguanosine and 5-methyl-2'-deoxycytidine solution in 50% methanol and 50% 10 mM ammonium formate (1  $\mu\text{g/mL}$ )

### Result (cont'd)



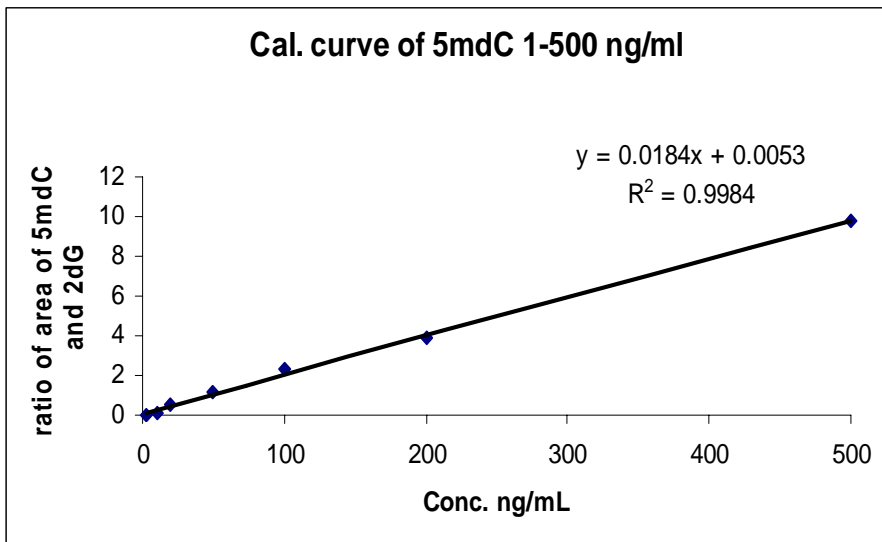
The CAD spectrum of the ion at m/z 242.2

**Result (cont'd)**



**The CAD spectrum of the ion at m/z 268.2**

**Result (cont'd)**



**Result (cont'd)**

**Intra-day and Inter-day Validation Characteristics for Measurement of GMD**

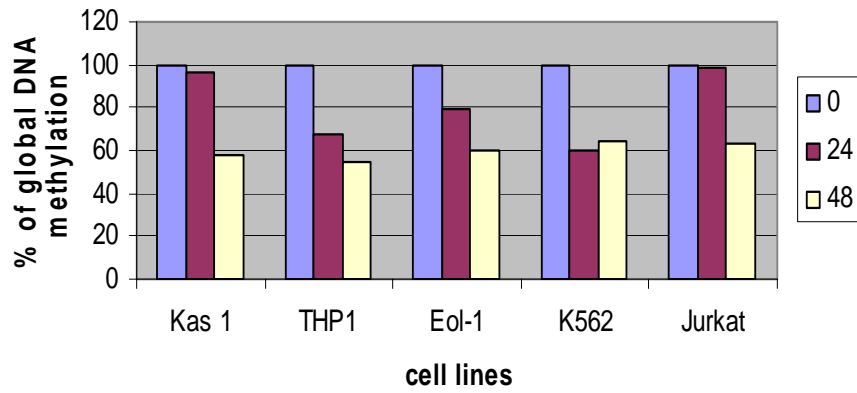
% 5mdC to 2dG	Intra-day						Av±SD n=6	% CV
	0.2	2.0	10.0	0.2	2.0	10.0		
0.2	0.176	0.220	0.229	0.229	0.229	0.233	0.219±0.022	9.86
2.0	2.13	2.11	1.98	2.10	1.99	2.01	2.05±0.07	3.30
10.0	9.46	9.47	9.44	9.99	9.73	10.0	9.67±0.27	2.78
% 5mdC to 2dG	Inter-day						Av±SD n=6	% CV
	0.2	2.0	10.0	0.2	2.0	10.0		
0.2	0.17	0.23	0.22	0.14	0.22	0.20	0.20±0.03	15.0
2.0	2.07	1.79	1.95	2.10	2.08	2.06	2.01±0.12	6.0
10.0	9.96	9.87	10.01	10.02	9.75	10.00	9.94±0.11	1.1
Kasumi1 (1)	5.25	4.69	5.05	4.85	4.80	4.58	4.87±0.24	5.02
Kasumi1 (2)	5.74	5.40	5.27	5.09	4.97	5.38	5.31±0.27	5.08
K562 (1)	2.39	2.54	2.37	2.65	2.49	2.50	2.49±0.10	4.08
K562 (2)	2.33	2.69	2.53	2.47	2.84	2.52	2.56±0.18	6.95

**Result (cont'd)**

- A highly sensitive and specific LC-MS/MS method for determination of global DNA methylation has been developed.
- Post column addition of an appropriate amount of methanol enhances the ionization efficiency and decreases interference of endogenous compounds.
- The assay was linear from 1 to 500 ng/ml using 1000 ng/mL 2-deoxyguanosine as the internal standard. The intra-day precision ranged from 2.78% to 9.86% and the inter-day precision ranged from 1.1 % to 15.0 %. For Kasumi-1 leukemia cell as HQC (High Quality Control) and K562 leukemia cell as LQC (Low Quality Control), the inter-day precision ranged from 4.08 to 6.95%. The accuracy values of the assay varied from 96.7 % to 109.5 %.

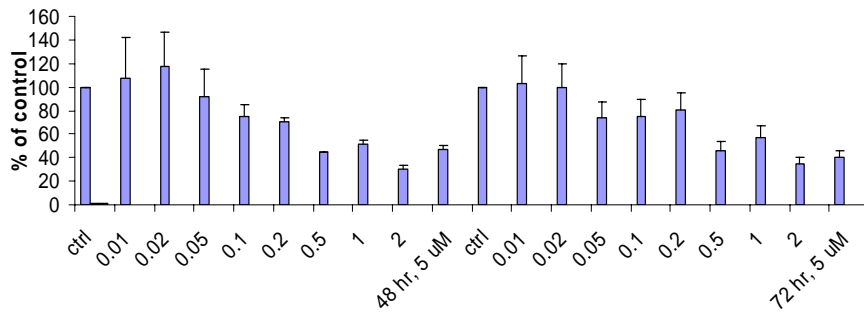
**Result (cont'd)**

**Global DNA methylation alteration of leukemia cell lines treated with decitabine**



**Result (cont'd)**

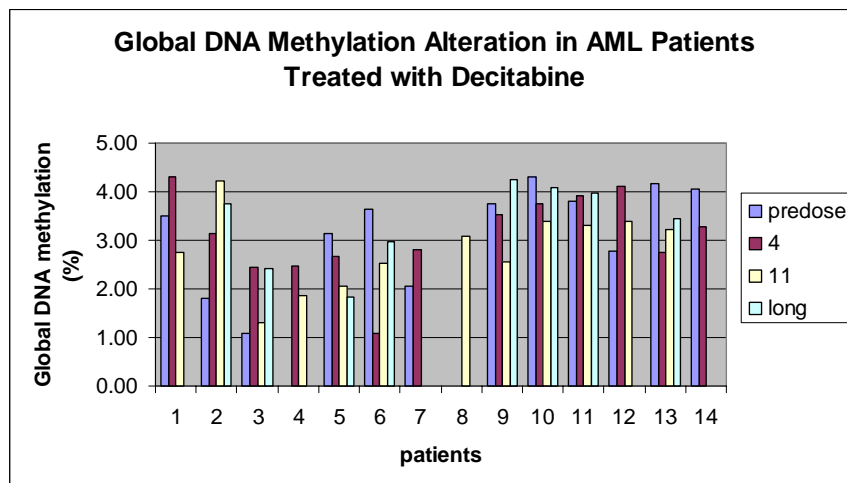
**Dose-dependent hypomethylation effect of decitabine on Kasumi-1 cell line**



### Result (cont'd)

- The mean baseline DNA methylation values for Kasumi 1, K562, Jurkat, Eol-1, THP-1 were found to be 4.98, 2.43, 4.25, 4.45, and 4.79%, respectively.
- Following treatment with decitabine, the GDM levels of Kasumi 1, K562, Jurkat, Eol-1, THP-1 were reduced to 57.1, 62.9, 63.7, 59.8, and 55.1% of those pre-treatment, respectively.
- The hypomethylating effect of decitabine on these cell lines appears to be cell cycle dependent as shown in Kasumi 1 and Jurkat cells; no alteration in global DNA methylation was observed in 24 hr but in 48 hr, which is consistent to its slow growth, two day cell cycle.
- The hypomethylating effect of decitabine on Kasumi-1 cell line is dose-dependent below 500 nM and reaches steady-state above 500 nM.

### Result (cont'd)



### **Result (cont'd)**

- DNA from pt's bone marrow was obtained before treatment (1 hr i.v. infusion, daily x 10), Day 4 during treatment, Day 11 and day 24-42, post treatment.
- 6/12 pts showed hypomethylation at Day 4, while at Day 11, 8/11 patients showed hypomethylation.
- 24-42 days post treatment, 6/8 patient returned to their normal DNA methylation level.
- 4/12 pts showed a base line DNA methylation level below 3.0, lower than normal global DNA methylation level in healthy people. All showed increased DNA methylation level on Day 4 after decitabine treatment.

### **Conclusion**

- A significant hypomethylating effect of decitabine for 5 tumor cell lines and bone marrow of AML patients was found. The hypomethylating effect is dose- and cell-cycle dependent. As a specificity control, no hypomethylation effect occurred following treatment with FK-228.
- The ESI LC-MS/MS method described here provides an unambiguous measurement of GDM with high reproducibility and high sensitivity and can be adapted for high-throughput rapid screening of DNA methylation effects by DNA hypomethylating agents.

# Acknowledgement

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