



## Stability of acetaldehyde-derived DNA adduct in vitro

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### ABSTRACT

Acetaldehyde (AA) derived from alcoholic beverages is a confirmed carcinogen for esophageal and head and neck cancers. AA forms various DNA adducts and is thought to play a crucial role in carcinogenesis. Transient DNA adducts are usually repaired, but the stability of AA-derived DNA adducts has not been elucidated. We investigated the stability of *N*<sup>2</sup>-ethylidene-2'-deoxyguanosine (*N*<sup>2</sup>-ethylidene-dG), a major AA-derived DNA adduct, in cultured cells. First, to determine the optimal concentration of AA for detecting *N*<sup>2</sup>-ethylidene-dG in cell culture, a dose–response study was performed using HL60 cells of the human promyelocytic leukemia cell line. An AA concentration  $\geq 0.01\%$  (1.8 mM) was required to detect *N*<sup>2</sup>-ethylidene-dG in vitro. We next examined the stability of *N*<sup>2</sup>-ethylidene-dG. After a 1 or 2 h exposure to 0.01% of AA in a tightly sealed bottle, *N*<sup>2</sup>-ethylidene-dG content was measured by sensitive liquid chromatography tandem mass spectrometry immediately, 24 h, and 48 h after exposure. After the 1 h exposure, the mean ( $\pm$  SD) *N*<sup>2</sup>-ethylidene-dG contents were  $12.1 \pm 1.28$ ,  $8.20 \pm 0.64$ , and  $6.70 \pm 0.52$  adducts per  $10^7$  bases at each postexposure time. After the 2 h exposure, *N*<sup>2</sup>-ethylidene-dG content increased to  $21.4 \pm 7.50$ ,  $10.5 \pm 3.61$ , and  $9.83 \pm 3.90$  adducts per  $10^7$  bases at each postexposure time. The half-life of this adduct was calculated as  $\sim 35$  h in independent experiments. These results indicate that AA exposure from daily alcohol consumption may cause DNA damage and may increase the risk of alcohol-related carcinogenesis.

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### 1. Introduction

In 2010, the International Agency for Research on Cancer reported that acetaldehyde (AA) derived from alcoholic beverages is carcinogenic and contributes to esophageal and head and neck cancers. The consumption of alcoholic beverages is related to an increased risk of cancer of the liver, colorectum, and female breast [1,2]. AA, an intermediate of ethanol, is genotoxic and causes mutations, sister chromatid exchanges, micronuclei, and aneuploidy in cultured mammalian cells. AA reacts with DNA to form a variety of adducts, of which deoxyguanosine (dG) is the most reactive. DNA adducts may cause polymerase errors and induce mutations in critical genes that activate proto-oncogenes and inactivate tumor suppressor genes [3,4].

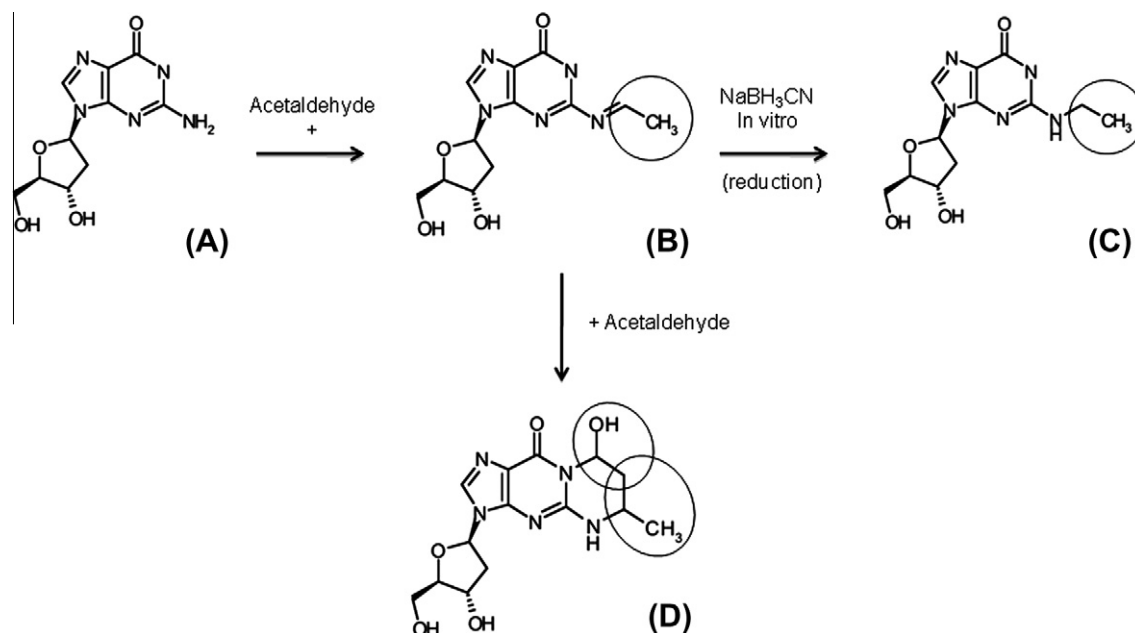
**Abbreviations:** AA, acetaldehyde; ALDH2, aldehyde dehydrogenase-2; LC/MS/MS, liquid chromatography–tandem mass spectrometry; *N*<sup>2</sup>-ethylidene-dG, *N*<sup>2</sup>-ethylidene-2'-deoxyguanosine; *N*<sup>2</sup>-Et-dG, *N*<sup>2</sup>-ethyl-2'-deoxyguanosine;  $\alpha$ -Me- $\gamma$ -OH-PdG,  $\alpha$ -methyl- $\gamma$ -hydroxy-1, *N*<sup>2</sup>-propano-2'-dG; *N*<sup>2</sup>-Dio-dG, 2-(2,6-dimethyl-1,3-dioxan-4-yl)-2'-dG; 8-oxo-dG, 8-oxo-7,8-dihydro-2'-dG.

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Quantification of AA-derived DNA adducts in human tissues is important for assessing their carcinogenic potential. The major DNA adduct of AA formed upon reaction with dG in vitro is *N*<sup>2</sup>-ethylidene-2'-deoxyguanosine (*N*<sup>2</sup>-ethylidene-dG), an unstable Schiff base at the *N*<sup>2</sup> position of dG. *N*<sup>2</sup>-ethylidene-dG is quite stable in DNA but decomposes quickly at the nucleoside level. Wang et al. showed that the content of *N*<sup>2</sup>-ethylidene-dG could be measured using liquid chromatography–tandem mass spectrometry (LC/MS/MS) by detection of *N*<sup>2</sup>-ethyl-2'-deoxyguanosine (*N*<sup>2</sup>-Et-dG) after reduction of DNA during isolation and enzymatic hydrolysis [5,6]. We purified DNA using several reagents containing a strong reducing agent, NaBH<sub>3</sub>CN. During the purification procedure, it was expected that *N*<sup>2</sup>-ethylidene-dG would be converted to stable *N*<sup>2</sup>-Et-dG (Fig. 1). *N*<sup>2</sup>-Et-dG in human liver could be detected a few hundredfold by undergoing this process [7]. This method was used to show that the content of hepatic and gastric *N*<sup>2</sup>-ethylidene-dG is much higher in ethanol-treated aldehyde dehydrogenase-2-knockout mice than in control mice [8,9].

However, the biochemical properties of *N*<sup>2</sup>-ethylidene-dG are not understood fully. DNA adducts are usually repaired by various pathways, but their stability has not been investigated. In this study, we analyzed the stability of *N*<sup>2</sup>-ethylidene-dG in vitro.



**Fig. 1.** Formation of acetaldehyde (AA) deoxyguanosine adducts. (A) Deoxyguanosine (dG). (B) *N*<sup>2</sup>-ethylidene-2-deoxyguanosine (*N*<sup>2</sup>-ethylidene-dG). (C) *N*<sup>2</sup>-ethyl-2-deoxyguanosine (*N*<sup>2</sup>-Et-dG). (D)  $\alpha$ -Methyl- $\gamma$ -hydroxy-1, *N*<sup>2</sup>-propano-2-deoxyguanosine ( $\alpha$ -Me- $\gamma$ -OH-PdG). *N*<sup>2</sup>-ethylidene-dG (B) is the major DNA adduct formed by AA and is too unstable in the nucleoside form to be detected directly. However, *N*<sup>2</sup>-ethylidene-dG (B) could be measured by detection of *N*<sup>2</sup>-Et-dG (C) after reduction of DNA with NaBH<sub>3</sub>CN during isolation and hydrolysis. The adduct parts are shown inside the circles.

## 2. Materials and methods

### 2.1. Cell line

HL60 cells, of the human promyelocytic leukemia cell line (American Type Culture Collection, Manassas, VA), were cultured in suspension in RPMI 1640 medium (Gibco Ltd., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) in a humidified incubator at 37 °C in 5% CO<sub>2</sub>. Tissue culture plastic ware was obtained from Corning Glass Works (Corning, NY).

### 2.2. DNA isolation

To quantify *N*<sup>2</sup>-ethylidene-dG, DNA was isolated from HL60 cells as described previously [9]. The Puregene DNA Purification System Cell and Tissue kit (Gentra Systems, Inc., Minneapolis, MN) was used. The protocol was basically as described according to the manufacturer's directions, except for the addition of NaBH<sub>3</sub>CN to all solutions (final concentration: 100 mM).

After the purification step, DNA was dissolved in 10 mM Tris-HCl/5 mM EDTA buffer (pH 7.0), extracted with chloroform, and precipitated with ethanol.

### 2.3. DNA digestion

DNA samples (20  $\mu$ g) were digested to their corresponding 2-deoxyribonucleoside-3-monophosphates by the addition of 15  $\mu$ l of 17 mM citrate plus 8 mM CaCl<sub>2</sub> buffer containing micrococcal nuclease (22.5 U) and spleen phosphodiesterase (0.075 U) plus internal standards. The solutions were mixed and incubated for 3 h at 37 °C, after which alkaline phosphatase (1 U), 10  $\mu$ l of 0.5 M Tris-HCl (pH 8.5), 5  $\mu$ l of 20 mM ZnSO<sub>4</sub>, and 67  $\mu$ l of distilled water were added, and the solution was incubated for 3 h at 37 °C. The digested sample was extracted twice with methanol. The

methanolic fractions were evaporated to dryness, resuspended in 50  $\mu$ l of distilled water, and subjected to sensitive LC/MS/MS.

### 2.4. Instrumentation

LC/MS/MS analyzes were performed using a Shimadzu LC system (Shimadzu, Kyoto, Japan) interfaced with a Quattro Ultima triple-stage quadrupole MS (Waters Micromass, Manchester, UK). The LC column was eluted over a gradient that began at a ratio of 5% methanol to 95% water; the ratio was changed to 40% methanol over a period of 30 min, to 80% methanol from 30 to 35 min, and finally returned to the original starting condition of 5–95% for the remaining 11 min. The total run time was 46 min. Sample injection volumes of 20  $\mu$ l each were separated on a Shim-pack XR-DOS column (3.0 mm  $\times$  75 mm; Shimadzu, Kyoto, Japan) and eluted at a flow rate of 0.2 ml/min. Mass spectral analyzes were performed in positive ion mode with nitrogen as the nebulizing gas. The ion source temperature was 130 °C, the desolvation gas temperature was 380 °C, and the cone voltage was operated at a constant 35 V. Nitrogen gas was also used as the desolvation gas (700 L/h), and cone gas (35 L/h) and argon were used as the collision gas at a collision cell pressure of  $1.5 \times 10^{-3}$  mbar. Positive ions were acquired in multiple-reaction monitoring (MRM) mode. The MRM transitions monitored were as follows: [<sup>15</sup>N<sub>5</sub>]-*N*<sup>2</sup>-Et-dG, *m/z* 301  $\rightarrow$  185 and *N*<sup>2</sup>-Et-dG, *m/z* 296  $\rightarrow$  180. The amount of DNA adduct was quantified by the ratio of the peak area of the target adduct to its stable isotope. QuanLynx (version 4.0) software (Waters Micromass, Milford, MA) was used to create a standard curve and to calculate the adduct concentration. The amount of dG was monitored by a SPD-10A UV-Vis detector (Shimadzu, Kyoto, Japan) placed before the tandem MS.

### 2.5. DNA adduct standard and its stable isotope

*N*<sup>2</sup>-Et-dG and its [<sup>15</sup>N<sub>5</sub>]-labeled standard were synthesized as described previously [10].

## 2.6. Exposure of HL60 cells to AA

Because AA is highly volatile (boiling point, 20.2 °C), a complete closed system was required to expose the cells to AA. Cells were transferred to PBS to remove any factors that might affect cell growth provided by the medium and FBS. A glass vial with a rubber lid was filled with 10 ml of fresh PBS containing  $40 \times 10^6$  cells without headspace. To ensure airtightness, the rubber lid was sealed tightly with Parafilm (Bemis Company Inc., Neenah, WI). The AA solution was prepared on ice and 0.1 ml of AA solution was added quickly to the vial with an injection needle through the rubber lid.

## 2.7. Determination of the optimal cultural concentration of AA to detect $N^2$ -ethylidene-dG

To determine the optimal concentration of AA to detect  $N^2$ -ethylidene-dG in vitro, the levels of adducts were measured in HL60 cells after a 1 h exposure to various concentrations of AA (1.8  $\mu$ M (0.00001%), 18  $\mu$ M (0.0001%), 180  $\mu$ M (0.001%), and 1.8 mM (0.01%).

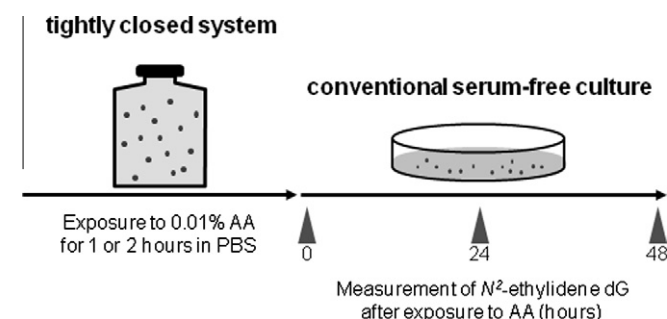
## 2.8. Calculation of the half-life ( $t_{1/2}$ ) of $N^2$ -ethylidene-dG in vitro

After the 1 or 2 h exposure to AA, cells were washed with fresh PBS, placed in serum-free medium, and cultured under conventional conditions. Cells were collected and DNA was isolated immediately, 24 h, and 48 h after exposure, as shown in Fig. 2. In the control group, the AA solution was replaced by 0.1 ml of PBS. The level of  $N^2$ -ethylidene-dG was measured at each postexposure time using LC/MS/MS and its  $t_{1/2}$  was calculated.

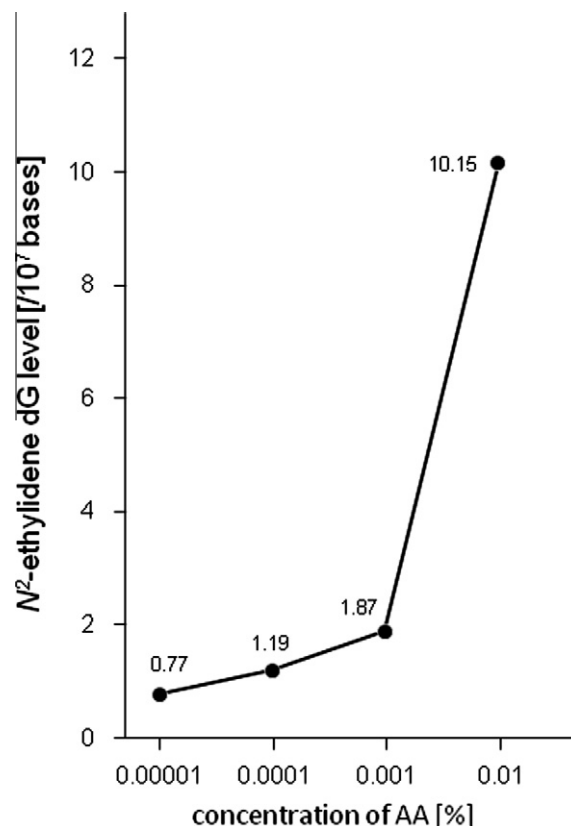
## 3. Results

### 3.1. Optimal cultural concentration of AA to detect $N^2$ -ethylidene-dG

We confirmed that an AA concentration up to 0.01% (1.8 mM) for 2 h did not lead to cell death but did cause arrest of cell growth (data, not shown). After the 1 h exposure to various concentrations of AA (0.00001% (1.8  $\mu$ M), 0.0001% (18  $\mu$ M), 0.001% (180  $\mu$ M), or 0.01% (1.8 mM)), the mean levels ( $n = 2$ ) of  $N^2$ -ethylidene-dG at each time were 0.77, 1.19, 1.87, and 10.15 per  $10^7$  bases, respectively. Based on these results, we decided that the optimal concentration of AA was 0.01% (1.8 mM) (Fig. 3).



**Fig. 2.** Experimental procedure. HL60 cells were exposed to acetaldehyde in a tightly closed system because of its high volatility. After exposure, cells were switched to a conventional serum-free culture system (37 °C, 5% CO<sub>2</sub>), DNA was collected immediately, 24 h, and 48 h after exposure, and the levels of  $N^2$ -ethylidene-dG were measured at each time ( $n = 4$ ). AA, acetaldehyde; PBS, phosphate-buffered saline.



**Fig. 3.** Determination of the optimal concentration of acetaldehyde (AA) to detect  $N^2$ -ethylidene-dG in vitro. HL60 cells were exposed for 1 h to various concentrations of AA. The levels of  $N^2$ -ethylidene-dG were measured, and the mean levels ( $n = 2$ ) are shown in the figure at each concentration. AA, acetaldehyde.

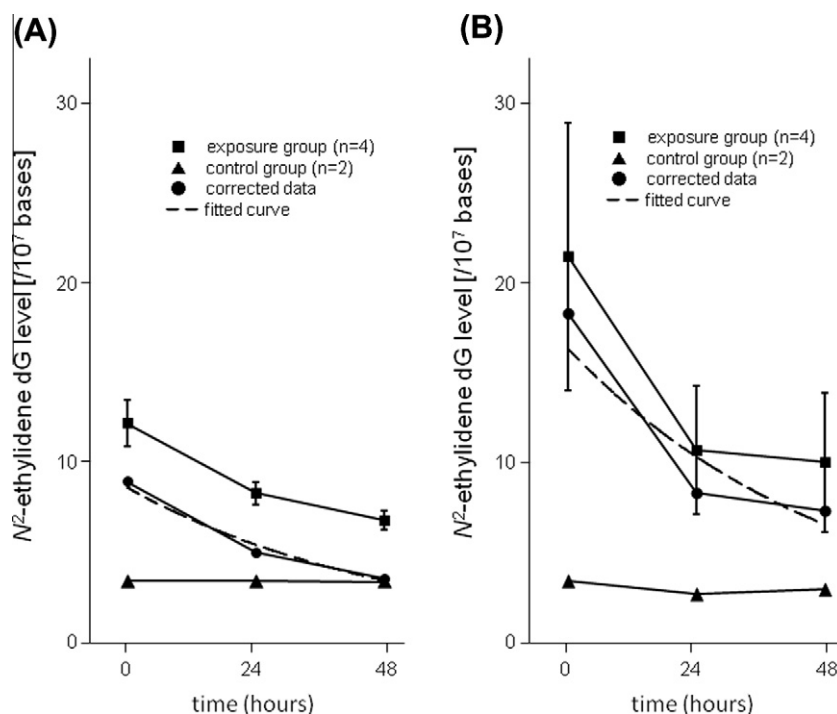
### 3.2. Half-life of $N^2$ -ethylidene-dG in vitro

In the cells exposed to AA for 1 h (0.01% (1.8 mM) AA,  $n = 4$ ), the mean ( $\pm$  SD) levels immediately, 24 h, and 48 h after exposure were  $12.1 \pm 1.28$ ,  $8.20 \pm 0.64$ , and  $6.70 \pm 0.52$  adducts per  $10^7$  bases, respectively. In the control group ( $n = 2$ ), the mean levels were 3.26, 3.31, and 3.26 adducts per  $10^7$  bases, respectively (Fig. 4A). In the cells exposed to AA for 2 h (0.01% (1.8 mM) AA,  $n = 4$ ), the levels were  $21.4 \pm 7.50$ ,  $10.5 \pm 3.61$ , and  $9.83 \pm 3.90$  adducts per  $10^7$  bases, respectively. In the control group ( $n = 2$ ), the mean levels were 3.19, 2.44, and 2.70 adducts per  $10^7$  bases, respectively (Fig. 4B).

To estimate the levels of  $N^2$ -ethylidene-dG formed from AA alone, the data were corrected by subtracting the control levels from the mean levels in the exposure group at each time. The corrected data were 8.85, 4.89, and 3.45 adducts per  $10^7$  bases in the 1 h exposure group and 18.2, 8.09, and 7.13 adducts per  $10^7$  bases in the 2 h exposure group for levels measured immediately, 24 h, and 48 h after exposure, respectively. Fitted curves were obtained from the corrected data. The two fitted curves could be presented as an exponential approximation and its mathematical expression was as follows:  $y = 8.50e^{-0.02x}$  ( $R^2 = 0.98$ ) in the 1 h exposure group (Fig. 4A) and  $y = 16.2e^{-0.02x}$  ( $R^2 = 0.85$ ) in the 2 h exposure group (Fig. 4B). The same  $t_{1/2}$  of approximately 35 h ( $t_{1/2} = \log_e 2 / 0.02 = 0.693 / 0.02 = 34.65$ ) was obtained in independent experiments.

## 4. Discussion

We show for the first time that the  $t_{1/2}$  of DNA adducts formed by AA is about 35 h in vitro. There are some limitations in our



**Fig. 4.** Half-life of  $N^2$ -ethylidene-dG in vitro. (A) Adduct levels after 1 h exposure to 0.01% (1.8 mM) acetaldehyde (AA). (B) Adduct levels after 2 h exposure to 0.01% (1.8 mM) AA. The mean levels are shown in the figure at each time. The data points represent the corrected data, which were calculated by subtracting the mean control level ( $n = 2$ ) from the mean level in the exposed cells ( $n = 4$ ) at each time. The two fitted curves (dashed lines) could be represented as an exponential approximation expressed mathematically as follows:  $y = 8.50e^{-0.02x}$  ( $R^2 = 0.98$ ) in the 1 h exposure group and  $y = 16.2e^{-0.02x}$  ( $R^2 = 0.85$ ) in the 2 h exposure group. The same half-life ( $t_{1/2}$ ) of about 35 h ( $t_{1/2} = \log_e 2 / 0.02 = 0.693 / 0.02 = 34.65$ ) was derived from these independent experiments. The error bars represent the standard deviation.

experiment. The first limitation is that the AA concentration of 0.01% (1.8 mM) used in our experiment is much higher than in the in vivo condition. In humans, the ability to metabolize alcohol differs considerably between individuals and is dependent on the activity of aldehyde dehydrogenase-2 (ALDH2). After ingestion of 0.4 g/kg ethanol, the blood AA level scarcely increases in people homozygous for ALDH2\*1, but it increases to 23.4  $\mu$ M in people heterozygous for ALDH2\*1/\*2 and further to 79.3  $\mu$ M in those homozygous for ALDH2\*2 [11]. We confirmed that HL60 cells have fully active ALDH2 activity (homozygous ALDH2\*1 genotype), and we could not avoid having to use a high concentration of AA to detect the DNA adduct in vitro. Further study using another cell line with the low-activity ALDH2\*2 allele is desirable. Alcohol-dependent increases in  $N^2$ -ethylidene-dG levels can be detected even in mice with the homozygous ALDH2\*1 genotype [8,9], suggesting that the stability of AA-derived DNA adducts is different in vivo and in vitro.

The second limitation of our study is that we detected only one kind of adduct,  $N^2$ -ethylidene-dG. To ensure accurate measurement, all AA-derived DNA adducts should be analyzed. Matsuda et al. developed a sensitive method using LC/MS/MS to quantitatively detect other AA-derived DNA adducts including  $\alpha$ -methyl-hydroxy-1,  $N^2$ -propano-2-dG ( $\alpha$ -Me- $\gamma$ -OH-PdG),  $N^2$ -(2,6-dimethyl-1,3-dioxan-4-yl)-2-dG ( $N^2$ -Dio-dG), and 8-oxo-7,8-dihydro-2-dG (8-oxo-dG) [8–10]. However, in the case of  $\alpha$ -Me- $\gamma$ -OH-PdG and 8-oxo-dG, neither alcohol-dependent nor ALDH2 genotype-dependent increases in adduct levels were observed [8,9].  $N^2$ -Dio-dG has not been detected in human DNA samples [10]. Therefore, it is reasonable to expect that AA-derived DNA adducts can be represented by  $N^2$ -ethylidene-dG.

The third limitation of our study is that only one exposure of HL60 cells to AA was performed in our experiments, and this experimental model does not mimic the condition of chronic alcohol consumption. Repeated or continuous exposure to AA may lead

to the extension of the  $t_{1/2}$  of AA-derived DNA adducts. Conversely, we were surprised by the long  $t_{1/2}$  of AA-derived DNA adducts (35 h) even under the conditions of rapid AA metabolism in this study (i.e., full ALDH2 activity and transient exposure to AA).

In conclusion, we examined the stability of an AA-derived DNA adduct in vitro. The long  $t_{1/2}$  of the AA-derived DNA adduct (35 h), even under the fully active ALDH2 condition, suggests that exposure to AA by daily alcohol consumption may cause DNA damage and may increase the risk of alcohol-related carcinogenesis.

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