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DNA methylation by dimethyl sulfoxide and methionine sulfoxide triggered by hydroxyl radical and implications for epigenetic modifications

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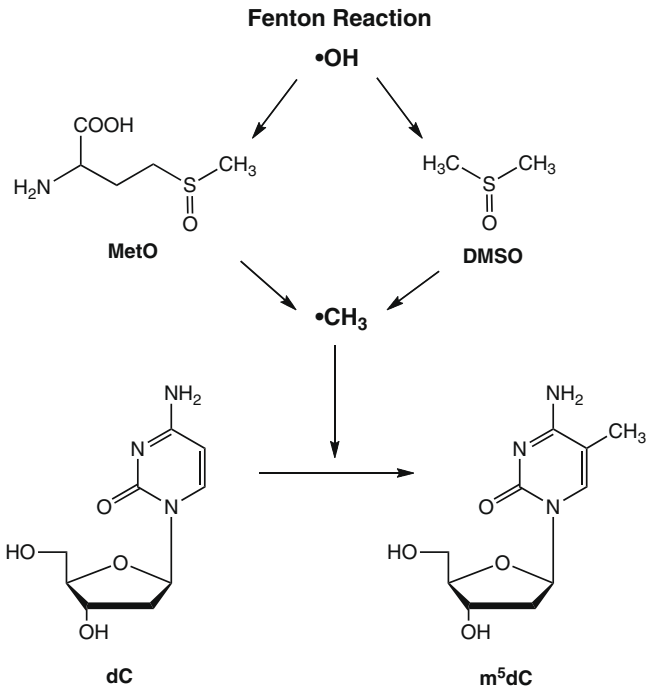
ABSTRACT

In this Letter, we demonstrate the formation of m^5dC from dC or in DNA by dimethylsulfoxide (DMSO) and methionine sulfoxide (MetO), under physiological conditions in the presence of the Fenton reagent in vitro. DMSO reportedly affects the cellular epigenetic profile, and enhances the metastatic potential of cultured epithelial cells. The methionine sulfoxide reductase (Msr) gene was suggested to be a metastatis suppressor gene, and the accumulation of MetO in proteins may induce metastatic cancer. Our findings are compatible with these biological data and support the hypothesis that chemical cytosine methylation via methyl radicals is one of the mechanisms of DNA hypermethylation during carcinogenesis. In addition to m^5dC, the formation of 8-methyldeoxyguanosine (m^8dG) was also detected in DNA under the same reaction conditions. The m^8dG level in human DNA may be a useful indicator of DNA methylation by radical mechanisms.

Alkyl radicals, including methyl radicals, are generated from the tumor promoters, cumene hydroperoxide and *t*-butyl hydroperoxide in mouse keratinocytes, based on ESR experiments, suggesting that carbon radicals are involved in cancer induction.[1] In addition to these chemicals, the generation of methyl radicals from various carcinogens has been observed in vitro and in vivo. The metabolism of 1,2-dimethylhydrazine[2] and procarbazine[3] produces methyl radicals. Acetaldehyde generates methyl radicals by treatments with xanthine oxidase,[4] peroxynitrite,[5] and Fe^{2+}/H_2O_2.[5] The formation of a methyl radical - deoxyguanosine adduct, 8-methyl-2′-deoxyguanosine (m^8dG), has been detected in DNA after a treatment with *t*-butyl hydroperoxide and ferrous ion in vitro,[6] and after the administration of 1,2-dimethylhydrazine to rats.[7] Methylation of RNA purine bases at the C-8 position by methyl radicals has been also observed.[8] We recently reported that cytosine C-5 methylation in the monomer dC and DNA occurred via methyl radicals generated by the tumor promoters, cumene hydroperoxide and *t*-butyl hydroperoxide, in the presence of ferrous ion.[9] This discovery suggested new mechanisms of aberrant DNA hypermethylation in the epigenetic process during chemical carcinogenesis. Enzymatic DNA methylation due to the increased expression of DNA methyltransferases (DNMTs) is a widely accepted mechanism of DNA hypermethylation.[10,11] However, controversial data showing no clear association between gene hypermethylation in cancer



Scheme 1. Formation of m^5dC via methyl radicals by DMSO and MetO, triggered by Fenton reaction.

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and high DNMT expression were also reported.[12,13] Therefore, it would be interesting to examine whether cytosine C-5 methylation via methyl radicals occurs by other biological/chemical systems.

Methyl radicals are reportedly generated by the reaction of OH radicals with dimethylsulfoxide (DMSO),[14] from the amino acid methionine (Met) upon gamma irradiation,[15] and by the treatment of methionine sulfoxide (MetO) with peroxynitrite.[16] Particularly, endogenous MetO formation in proteins is implicated as a pathological biomarker in relation to aging, inflammation and smoking.[17–20] It is worth mentioning that DNA hypermethylation during carcinogenesis is correlated to aging, inflammation and smoking.[21–23] DMSO also reportedly has an effect on the cellular epigenetic profile, by inducing DNA hypermethylation.[24] In this study, we examined the biological relevance of m^5dC formation in dC and DNA by methyl radicals produced from DMSO and MetO treated with a Fenton system (Scheme 1).

When dC was reacted with DMSO in the presence of Fenton reagent[8] at pH 7.3, the formation of m^5dC was clearly identified by HPLC equipped with a photodiode array UV detector (Fig. 1). The retention time and the UV spectrum of the reaction product were the same as those of the authentic m^5dC. Its formation was dependent on the concentration of DMSO (Fig. 2), and the reaction was rather rapid, due to its radical character. The reaction was approx-

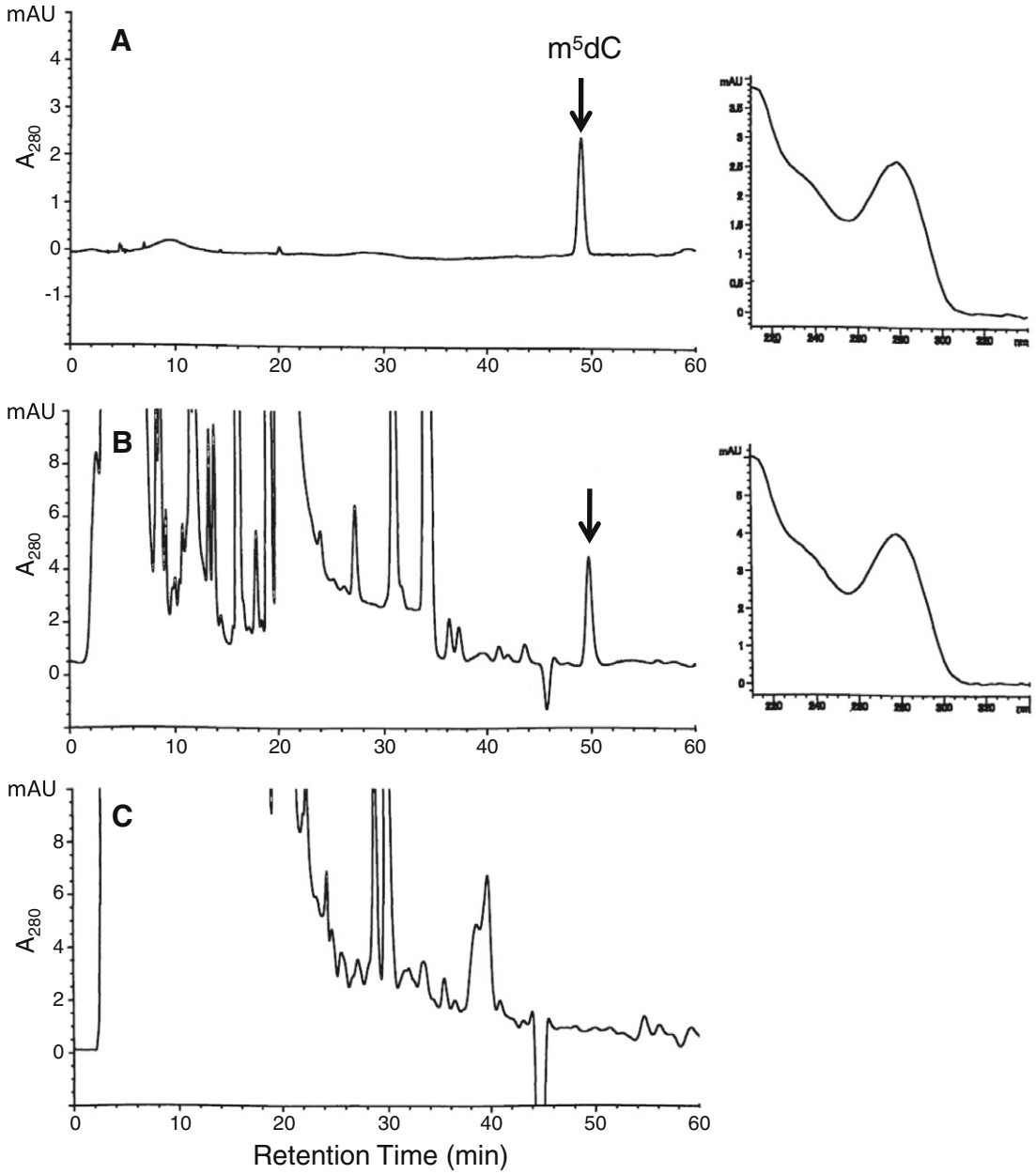

Figure 1. Detection of m^5dC in the reaction mixture of dC, DMSO and Fenton reagent by HPLC. The reaction mixture[8] (mixed under N_2 atmosphere, final volume, 0.225 ml), containing dC (final concentration, 5.46 mM), DMSO (100 mM), L-ascorbic acid (10.7 mM), EDTA·2Na (3.1 mM), $FeSO_4$ (5.3 mM), and H_2O_2 (19.6 mM) in 110 mM phosphate buffer (pH 7.3), was reacted in a sealed plastic tube (tube volume, 2 ml) by vigorous shaking at 37 °C. After a 3 h reaction, the solution was centrifuged and an aliquot of the supernatant was injected into the HPLC apparatus. (A) Chromatogram of the m^5dC standard (left) and its UV spectrum (right); (B) chromatogram of the reaction mixture (left) and UV spectrum of the peak at 50 min (right); (C) chromatogram of the control reaction mixture without DMSO.

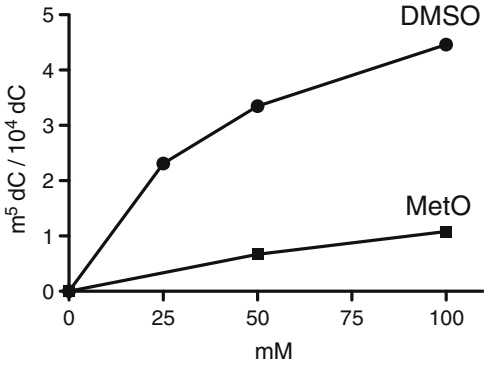

Figure 2. Dose-dependency of m^5dC formation in the dC/DMSO/Fenton reaction and the dC/MetO/Fenton reaction. The reaction conditions were the same as those in Figure 1, except that different concentrations of DMSO (25, 50 and 100 mM) and MetO (50 and 100 mM) were used. Mean values of duplicate experiments are plotted.

imately 65% complete within 5 min (data not shown). This reaction may proceed via a free radical mechanism, probably via a methyl radical. The dose-dependent formation of m^5dC from dC was also observed after a reaction with MetO plus Fenton reagent (Fig. 2).

In addition, after a double-stranded alternating copolymer, ds poly(dG-dC), was reacted with DMSO or MetO in the presence of Fenton reagent at pH 7.3, the formation of m^5dC was clearly detected in the poly(dG-dC) after the treatment, by an immuno-dot blot analysis[9,25] (Fig. 3). As a positive control, we analyzed 0.02–0.2 ng of calf thymus DNA, which contained 1.39 mol % m^5dC per P atoms. The chemiluminescence intensity increased depending upon the calf thymus DNA concentration. The control poly(dG-dC) without treatment also showed weak chemiluminescence. This means that commercial poly(dG-dC) contains a small amount of m^5dC. Since an exact quantitation was difficult with the immuno-dot blot analysis, the amount of m^5dC in the reaction mixture was further analyzed by the LC/MS/MS.

In the LC/MS analysis, the standard m^5dC exhibited an MH^+ ion at m/z 242, and product ion analysis from m/z 242 with

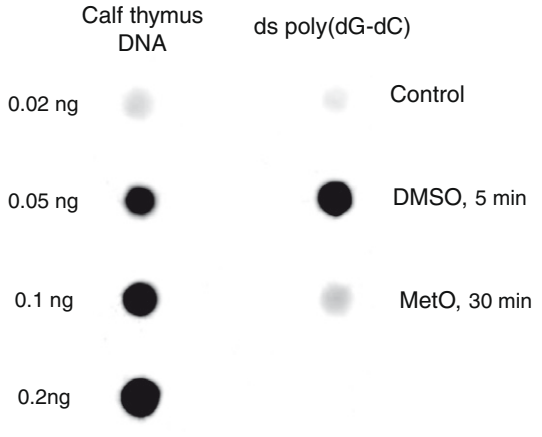

Figure 3. Detection of m^5dC in ds poly(dG-dC) by an immuno-dot blot analysis.[9,25] The reaction mixture (mixed under N_2 atmosphere, final volume, 0.45 ml) contained ds poly(dG-dC) (final concentration, 8.9 A_{260} OD units/ml), DMSO or MetO (100 mM), L-ascorbic acid (10.7 mM), EDTA·2Na (3.1 mM), $FeSO_4$ (5.3 mM), and H_2O_2 (19.6 mM), in 110 mM phosphate buffer (pH 7.3), and was reacted in a sealed plastic tube (tube volume, 2 mL) by vigorous shaking at 37 °C. After 5 min or 30 min, the ds poly(dG-dC) was recovered from the reaction mixture and was used for the analysis. As positive controls, m^5dC in various amounts of calf thymus DNA was visualized. As negative controls, untreated ds poly(dG-dC) was analyzed.

11 eV revealed a fragment BH_2^+ ion at m/z 126, formed by the loss of 2′-deoxyribose.[9] Therefore, the m^5dC in the reaction mixture was analyzed by LC/MS/MS, by monitoring the m/z 242→126 transition. In Figure 4, chromatograms of the LC/MS/MS analysis of standard m^5dC (A), poly(dG-dC)–DMSO–Fenton reagent (B), poly(dG-dC)–MetO–Fenton reagent (C), and control poly(dG-dC) without treatment (D) are shown. In both of the poly(dG-dC) samples treated with DMSO and MetO in the presence of Fenton reagent, a 242→126 transition peak appeared at 7.64 min, which is the same retention time as that of authentic m^5dC. The control poly(dG-dC) without treatment also showed a small peak (Fig. 4D). This means that the commercial poly(dG-dC) contains a small amount of m^5dC.

Based on the LC/MS/MS analysis, the yields of m^5dC in the DNA–DMSO–Fenton and DNA–MetO–Fenton reactions were calculated to be $5.00/10^4$ dC and $1.64/10^4$ dC, respectively, which are in the same range ($2–4/10^4$ dC) as the yield produced by the monomer reactions for 30 min with the DMSO- and MetO–Fenton reagents (data not shown). The high yield in DNA is unexpected, as compared to the previous result that showing the yield of m^5dC formation by a cumene hydroperoxide/Fe^{2+} system is 10-fold lower in DNA than in the dC monomer.[9] The following reasons are possible. (i) The Fenton system used in this study has higher affinity to DNA than to the monomer dC, thus generating higher levels of ·OH radicals in DNA;[26] (ii) long-range electron transfer along the DNA chain from the metal binding sites to the dC residues enhanced the yield of m^5dC in DNA.[27] Further studies with precise measurements of m^5dC using a stable isotope internal standard are required for the final conclusion.

We also analyzed the m^8dG in these reaction products by LC/MS/MS. The standard m^8dG[28] exhibited an MH^+ ion at m/z 282, and product ion analysis from m/z 282 revealed a fragment BH_2^+ ion at m/z 166, formed by the loss of 2′-deoxyribose. Therefore, the m^8dG was analyzed by monitoring the m/z 282→166 transition. In Figure 5, chromatograms of the LC/MS/MS analysis of standard m^8dG (A), poly(dG-dC)–DMSO–Fenton reagent (B), poly(dG-dC)–MetO–Fenton reagent (C), and control poly(dG-dC) without treatment (D) are shown. In the poly(dG-dC) treated with DMSO and MetO in the presence of Fenton reagent, a 282→166 transition peak appeared at 18.6 min (Fig. 5B and C), which is the same retention time as that of authentic m^8dG (Fig. 5A). The control poly(dG-dC) without treatment also displayed a small peak of m^8dG (Fig. 5D), which shows that the commercial poly(dG-dC) contains a small amount of m^8dG in addition to m^5dC. The formation of m^5dC and m^8dG in DNA polymers is summarized in Table 1. The yield of m^5dC formation in DNA was found to be 15–30-fold higher than that of m^8dG by the reaction with DMSO and MetO in the presence of Fenton reagent.

DMSO is known to induce hypermethylation of various genetic loci and affects the epigenetic profile in mouse embryoid bodies. Although those authors ascribed DNA methylation to the increase of DNMT3a activity,[24] other mechanisms of DNA methylation cannot be ruled out. DMSO also enhanced the invasiveness and metastatic potential of cultured epithelial cells, with an epigenetic effect.[29]

DNA hypermethylation is increased with age, inflammation and smoking.[21–23] The MetO/Met ratio in proteins also reportedly increased with age, inflammation and smoking.[17–20] MetO in proteins is repaired by methionine sulfoxide reductase (Msr). Three reports have suggested that the Msr gene is a tumor suppressor.[30–32]

The chemical methylation of cytosine C-5 may occur in vivo. A Met residue in a chromatin protein reportedly interacts with DNA by the intercalation of the Met side chain into a GC pair, based on NMR studies.[33] If the Met residue is oxidized to MetO, then the cytosine residues in the vicinity could undergo C-5 methylation

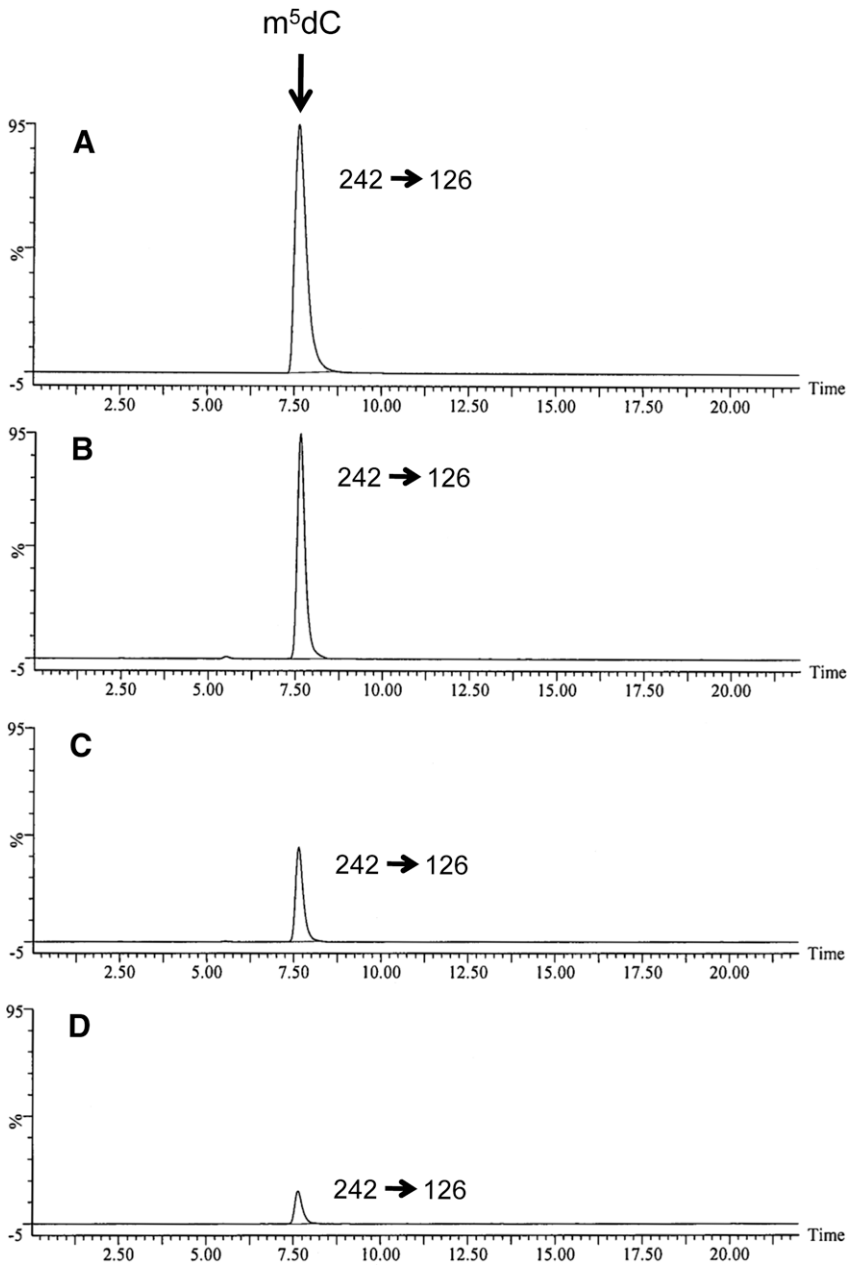

Figure 4. LC/MS/MS analysis of m^5dC in the reaction products. (A) Standard m^5dC (1.78 mg/mL); (B) hydrolysate of poly(dG-dC)/DMSO/Fenton reaction product (reaction conditions were the same as those in Figure 3, with 30 min reaction time); (C) hydrolysate of poly(dG-dC)/MetO/Fenton reaction product (reaction conditions were the same as those in Figure 3, with 30 min reaction time); and (D) control ds poly(dG-dC) without treatment. A four mL portion of each sample was injected. The transition *m*/*z* 242→126 was monitored. Chromatograms B, C and D are shown on the same scale.

when triggered by ·OH radicals. Various oxidized proteins and their degradation products may accumulate in the cytosol during aging and inflammation and may react with dCTP to form m^5dCTP, which could become incorporated into DNA and induce gene silencing.[34]

In the present study, in addition to m^5dC, the formation of a small amount of m^8dG was detected in the poly(dG-dC) after the treatment. The higher level of m^5dC formation than that of m^8dG may be due to a steric effect. Namely, the cytosine C-5 position may be present in a more open structure in the DNA, while the guanine C-8 is sterically hindered by the phosphate backbone.

If chemical methylation via methyl radicals is one of the mechanisms of epigenetic change during carcinogenesis, and m^8dG is formed in a specific ratio to m^5dC, then m^8dG formation analyzed by LC/MS/MS would be a good marker of chemical DNA methylation, because the background level of m^8dG would be very low, as compared to that of m^5dC, in mammalian cell DNA. Therefore, epi-mutagens inducing DNA hypermethylation can be assayed by in vitro experiments using cultured cells. The analysis of the accumulation status of m^8dG in human DNA may also be a good indicator of the increase of m^5dC in DNA by radical mechanisms, and would be useful for human cancer risk assessment.

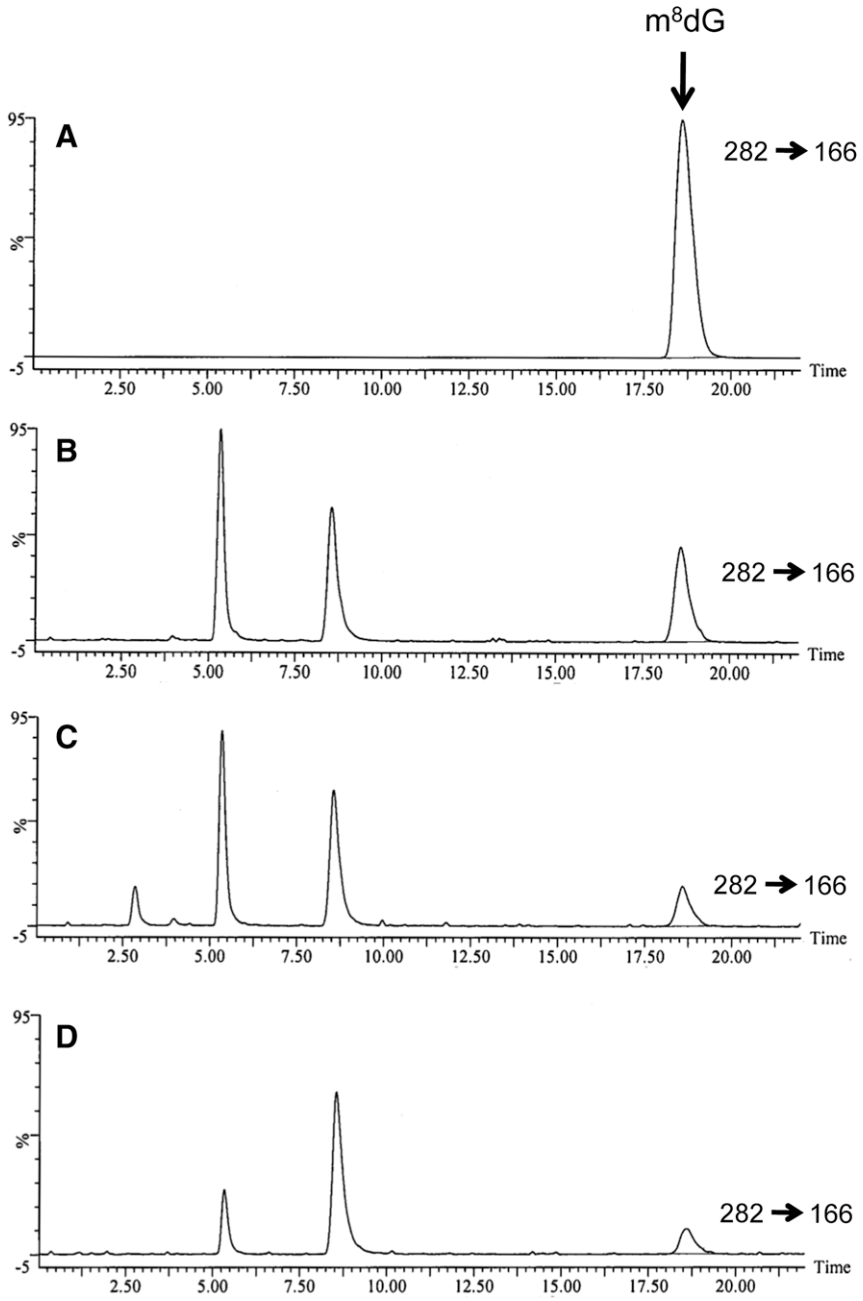

Figure 5. LC/MS/MS analysis of m^8dG in the reaction products. (A) Standard m^8dG[28] (2.08 μg/mL); (B, C, D) the same samples as B, C, D in Figure 4.

Table 1
Formation of m^5dC and m^8dG in ds poly(dG-dC) by DMSO and MetO in the presence of Fenton reagent

Reaction	$m^5dC/10^4$ dC	$m^8dG/10^4$ dG
DMSO + Fenton reagent	5.00	0.27
MetO + Fenton reagent	1.64	0.06

Acknowledgments

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References and notes

1. Taffe, B. G.; Takahashi, N.; Kensler, T. W.; Mason, R. P. *J. Biol. Chem.* **1987**, *262*, 12143.
2. Augusto, O.; Du Plessis, L. R.; Weingrill, C. L. *Biochem. Biophys. Res. Commun.* **1985**, *126*, 853.
3. Goria-Gatti, L.; Iannone, A.; Tomasi, A.; Poli, G.; Albano, E. *Carcinogenesis* **1992**, *13*, 799.
4. Nakao, L. S.; Kadiiska, M. B.; Mason, R. P.; Grijalba, M. T.; Augusto, O. *Free Radical Biol. Med.* **2000**, *29*, 721.
5. Nakao, L. S.; Ouchi, D.; Augusto, O. *Chem. Res. Toxicol.* **1999**, *12*, 1010.
6. Hix, S.; Morais Mda, S.; Augusto, O. *Free Radical Biol. Med.* **1995**, *19*, 293.
7. Netto, L. E.; RamaKrishna, N. V.; Kolar, C.; Cavalieri, E. L.; Rogan, E. G.; Lawson, T. A.; Augusto, O. *J. Biol. Chem.* **1992**, *267*, 21524.
8. Kang, J. O.; Gallagher, K. S.; Cohen, G. *Arch Biochem. Biophys.* **1993**, *306*, 178.
9. Kasai, H.; Kawai, K. *Chem. Res. Toxicol.* **2009**, *22*, 984.
10. Benbrahim-Tallaa, L.; Waterland, R. A.; Dill, A. L.; Webber, M. M.; Waalkes, M. P. *Environ. Health Perspect.* **2007**, *115*, 1454.

11. Robertson, K. D.; Uzvolgyi, E.; Liang, G.; Talmadge, C.; Sumegi, J.; Gonzales, F. A.; Jones, P. A. *Nucleic Acids Res.* **1999**, *27*, 2291.
12. Eads, C. A.; Danenberg, K. D.; Kawakami, K.; Saltz, L. B.; Danenberg, P. V.; Laird, P. W. *Cancer Res.* **1999**, *59*, 2302.
13. Ehrlich, M.; Woods, C. B.; Yu, M. C.; Dubeau, L.; Yang, F.; Campan, M.; Weisenberger, D. J.; Long, T.; Youn, B.; Fiala, E. S.; Laird, P. W. *Oncogene* **2006**, *25*, 2636.
14. Eberhardt, M. K.; Colina, R. *J. Org. Chem.* **1988**, *53*, 1071.
15. Makino, K. *J. Phys. Chem.* **1979**, *83*, 2520.
16. Nakao, L. S.; Iwai, L. K.; Kalil, J.; Augusto, O. *FEBS Lett.* **2003**, *547*, 87.
17. Wells-Knecht, M. C.; Lyons, T. J.; McCance, D. R.; Thorpe, S. R.; Baynes, J. W. *J. Clin. Invest.* **1997**, *100*, 839.
18. Onorato, J. M.; Thorpe, S. R.; Baynes, J. W. *Ann. N.Y. Acad. Sci.* **1998**, *854*, 277.
19. Hosako, M.; Ogino, T.; Omori, M.; Okada, S. *Free Radical Biol. Med.* **2004**, *36*, 112.
20. Carp, H.; Miller, F.; Hoidal, J. R.; Janoff, A. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 2041.
21. Kim, S. K.; Jang, H. R.; Kim, J. H.; Kim, M.; Noh, S. M.; Song, K. S.; Kang, G. H.; Kim, H. J.; Kim, S. Y.; Yoo, H. S.; Kim, Y. S. *Carcinogenesis* **2008**, *29*, 1623.
22. Suzuki, H.; Toyota, M.; Kondo, Y.; Shinomura, Y. *Methods Mol. Biol.* **2009**, *512*, 55.
23. Kim, J. S.; Kim, H.; Shim, Y. M.; Han, J.; Park, J.; Kim, D. H. *Carcinogenesis* **2004**, *25*, 2165.
24. Iwatani, M.; Ikegami, K.; Kremenska, Y.; Hattori, N.; Tanaka, S.; Yagi, S.; Shiota, K. *Stem Cells* **2006**, *24*, 2549.
25. Leuratti, C.; Singh, R.; Lagneau, C.; Farmer, P. B.; Plastaras, J. P.; Marnett, L. J.; Shuker, D. E. *Carcinogenesis* **1998**, *19*, 1919.
26. Floyd, R. A. *Biochem. Biophys. Res. Commun.* **1981**, *99*, 1209.
27. Arkin, M. R.; Stemp, E. D.; Pulver, S. C.; Barton, J. K. *Chem. Biol.* **1997**, *4*, 389.
28. Maeda, M.; Nushi, K.; Kawazoe, Y. *Tetrahedron* **1974**, *30*, 2677.
29. Bentel, J. M.; Rhodes, G. C.; Markus, I.; Smith, G. J. *Int. J. Cancer* **1990**, *46*, 251.
30. Bilsborough, J.; Van Pel, A.; Uyttenhove, C.; Boon, T.; Van den Eynde, B. J. *J. Immunol.* **1999**, *162*, 3534.
31. Lei, K. F.; Wang, Y. F.; Zhu, X. Q.; Lu, P. C.; Sun, B. S.; Jia, H. L.; Ren, N.; Ye, Q. H.; Sun, H. C.; Wang, L.; Tang, Z. Y.; Qin, L. X. *BMC Cancer* **2007**, *7*, 172.
32. De Luca, A.; Sacchetta, P.; Nieddu, M.; Di Ilio, C.; Favaloro, B. *BMC Mol. Biol.* **2007**, *8*, 39.
33. Allain, F. H.; Yen, Y. M.; Masse, J. E.; Schultze, P.; Dieckmann, T.; Johnson, R. C.; Feigon, J. *EMBO J.* **1999**, *18*, 2563.
34. Holliday, R.; Ho, T. *Somat. Cell Mol. Genet.* **1991**, *17*, 537.