Electronic Supplementary Information (ESI) for

Detection and Structural Analysis of Pyrimidine-Derived Radicals Generated on DNA using a Profluorescent Nitroxide Probe

Kosho Yamauchi,[a] Yuta Matsuoka,[a] Masatomo Takahashi,[b] Yoshihiro Izumi,[b] Hideto Naka,[a] Yosuke Taniguchi,[c] Kazuaki Kawai,[d] Takeshi Bamba[b] and Ken-ichi Yamada*[a]

[a.] *Physical Chemistry for Life Science Laboratory, Faculty of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi Higashi-ku, Fukuoka 812-8582, Japan*

[b.] *Metabolomics Laboratory, Research Center for Transomics Medicine, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi Higashi-ku, Fukuoka 812-8582, Japan*

[c.] *Frontier in Biofunction of Nucleic Acid and Organic Chemistry, Faculty of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi Higashi-ku, Fukuoka 812-8582, Japan*

[d.] *Department of Environmental Oncology, Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Japan, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu, 807-8555, Japan*

Correspondence should be addressed to: Ken-ichi Yamada

Physical Chemistry for Life Science Laboratory, Faculty of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi Higashi-ku, Fukuoka 812-8582, Japan

Tel: +81-92-642-6624

Fax: +81-92-642-6626

E-mail: kenyamada@phar.kyushu-u.ac.jp

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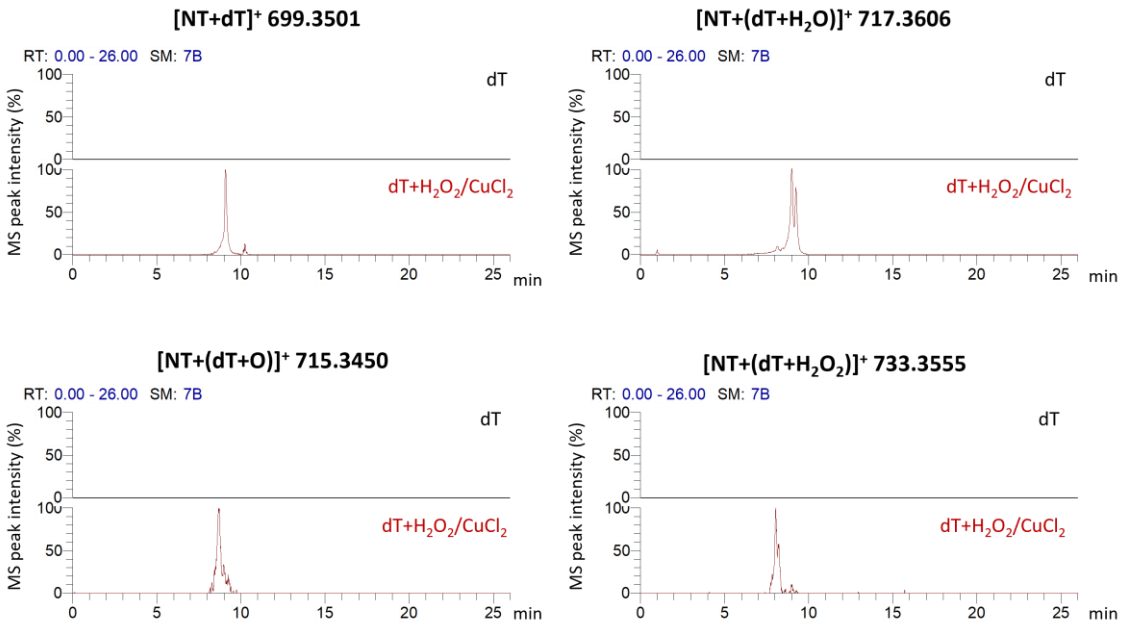
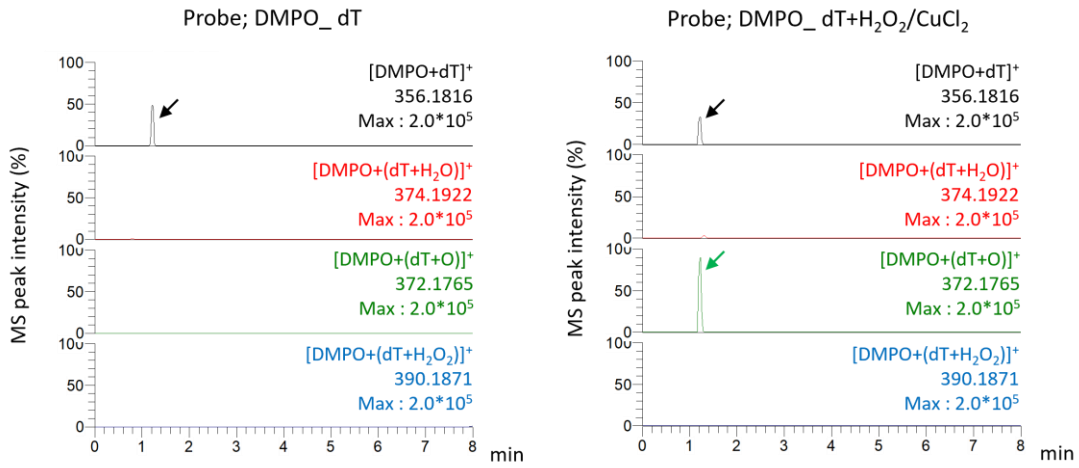
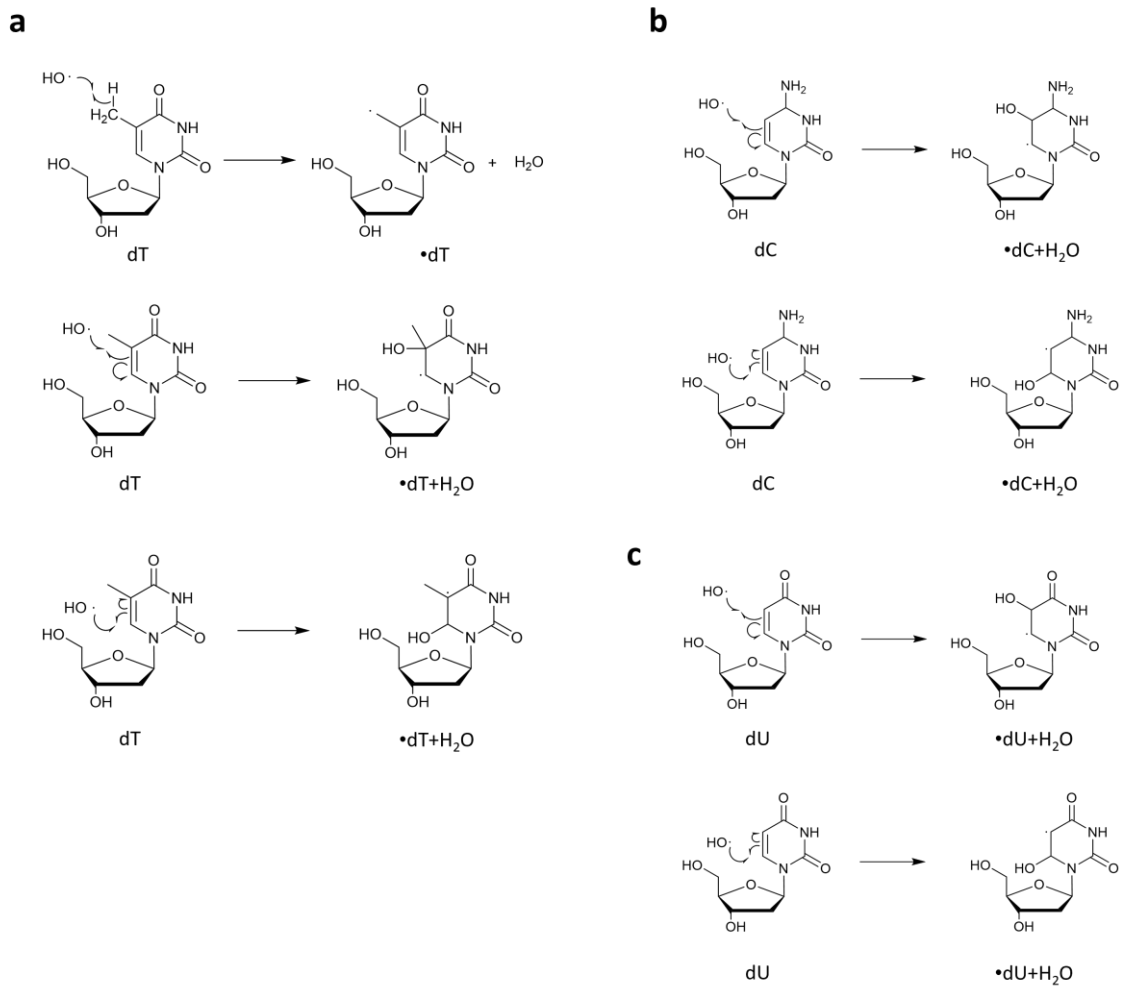


Supplementary Figure 1. Extracted ion chromatograms of the reaction adducts between the NT and dT-derived radicals ($[NT+dT]^+$, 699.3501; $[NT+(dT+O)]^+$, 715.3450; $[NT+(dT+H_2O)]^+$, 717.3606; and $[NT+(dT+H_2O_2)]^+$, 733.3555) generated by $H_2O_2 + CuCl_2$ system.



Supplementary Figure 2. Extracted ion chromatograms of the reaction adducts between the dT-derived radicals and DMPO. $[DMPO+dT]^+$ 356.1816, $[DMPO+(dT+H_2O)]^+$ 374.1922, $[DMPO+(dT+O)]^+$ 372.1765, $[DMPO+(dT+H_2O_2)]^+$ 390.1871. dT-derived radicals were generated by the addition of 200 µM H_2O_2 and 300 µM $CuCl_2$ to the reaction solution containing 600 µM dT and 50 µM DMPO.



Supplementary Scheme 1. Plausible generation mechanisms of dT- (a), dC- (b), and dU- (c) derived radicals by the hydroxyl radical.

Supplementary Table 1. LC/HRMS information on the reaction adducts between the NT and pyrimidine nucleoside-derived radicals generated by the H_2O_2 + $CuCl_2$ system.

Yellow; unreported nucleoside-derived radicals before our experiments

ID	reaction adducts	retention time, min	Monoisotopic mass	calculated $[M]^+$, *m/z*	Observed ion, *m/z*
1	NT+dT	9.1	698.3423	699.3501	699.3506
2	NT+(dT+O)	8.7	714.3372	715.3450	715.3458
3	NT+(dT+H_2O)	8.0-10.0	716.3528	717.3606	717.3611
4	NT+(dT+H_2O_2)	8.1	732.3477	733.3555	733.3560
5	NT+dC	7.1	683.3426	684.3504	684.3510
6	NT+(dC+H_2O)	5.9-7.0	701.3532	702.3610	702.3613
7	NT+dU	7.5-10	684.3266	685.3344	685.3349
8	NT+(dU+H_2O)	7.5-8.5	702.3372	703.3450	703.3453
9	NT+(dU+H_2O_2)	8.1	718.3321	719.3399	719.3404

Materials and Methods

Reagents

9-(Ethylimino)-N-(2,2,6,6-tetramethylpiperidin-1-oxyl)-10-methyl-9H-benzo[a]phenoxazin-5-amine hydrochloride (NT) and (9-(ethylimino)-N-(1-methoxy-2,2,6,6-tetramethylpiperidin-1-oxyl)-10-methyl-9H-benzo[a]phe-noxazin-5-amine hydrochloride (NT-NOMe) were synthesized according to a previously reported method.[1] 2-Deoxythymidine (dT), 2-deoxyadenosine (dA), 2-deoxyguanosine (dG), 2-deoxycytidine (dC), 2-deoxyuridine (dU), H_2O_2, $CuCl_2$, and nuclease P1 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); calf thymus DNA was purchased from Sigma-Aldrich (St. Louis, MO); and alkaline phosphatase was purchased from Takara Bio Inc. (Shiga, Japan). Acetonitrile (LC/MS grade, ≥99.9%), ultrapure water (LC/MS grade), and ammonium acetate were purchased from Wako Pure Chemical Industries, Ltd.

Oxidation of nucleosides and calf thymus DNA using the H_2O_2 + $CuCl_2$ system

For nucleoside oxidation, a solution containing 600 μM nucleosides, 50 μM NT, 300 μM $CuCl_2$, and various concentrations of H_2O_2 was incubated to phosphate buffered saline (PBS) (pH 7.4) at 37 °C for 1 h. Thereafter, the individual reaction mixtures were extracted using equal amounts of $CHCl_3$ and MeOH containing 1 mM ethylenediaminetetraacetic acid (EDTA) as an antioxidant. The extracted reaction mixtures were dried under a stream of nitrogen gas; the residue was dissolved in methanol (200 μL) and stored at −80 °C before injecting it to the high-performance liquid chromatography (HPLC) column.

For the oxidation of calf thymus DNA, PBS solution (pH 7.4) containing 150 ng/mL calf thymus DNA and 50 μM NT was mixed with 300 μM $CuCl_2$ and various concentrations of H_2O_2, and incubated at 37 °C for 1 h. Thereafter, 1 mM EDTA was added to the mixture to quench the reaction. The DNA in

the reaction solution was digested with nuclease P1 (2U) and alkaline phosphatase (2U) for 30 min at 37 °C. Subsequently, the individual reaction mixtures were extracted using equal amounts of $CHCl_3$ and MeOH. The extracted solutions were dried under a stream of nitrogen gas; the residue was dissolved in methanol (200 μL) and stored at −80 °C before injecting it to the HPLC column.

Structural analysis of nucleoside-derived radicals by liquid chromatography-fluorometry and high-resolution tandem mass spectrometry (LC/FL/HRMS/MS)

LC/FL/HRMS/MS was performed using a Nexera LC system (Shimadzu Co., Kyo-to, Japan) coupled with a fluorescence detector (RF-20Axs, Shimadzu Co.) and a high-performance benchtop quadrupole Orbitrap mass spectrometer (Q Exactive; Thermo Fisher Scientific, Waltham, MA). The mass spectrometer was equipped with an electron spray ionization source. The LC conditions were as follows: injection volume, 10 μL; autosampler temperature, 4 °C; column, InertSustain C18 column (2.1 mm × 150 mm with a particle size of 3 μm; GL sciences, Tokyo, Japan); column temperature, 40 °C; mobile phase, A: 5 mM ammonium acetate in H_2O and B: 5 mM ammonium acetate in ACN:H_2O = 95:5; time program, B% for 0–15 min: 30%–65%, for 15.01–20 min: 100%, for 20.5–25 min: 30%); and flow rate, 0.4 mL/min). The fluorescence detector parameter settings were as follows: excitation/emission, 627/651 nm; cell temperature, 30 °C; and response, 500 ms. The ionization conditions were as follows: ionization mode, negative or positive; sheath gas flow rate, 40 arbitrary units; auxiliary gas flow rate, 10 arbitrary units; spray voltage, 2000 V; capillary temperature, 265 °C; S-lens level, 50; and heater temperature, 425 °C. The experimental conditions for full-scan MS were as follows: resolving power, 70000; automatic gain control target, 1×10^6; trap fill time, 100 ms; and scan range, *m/z* 120–1500. The experimental conditions for MS/MS were as follows: resolving power, 17500; automatic gain control target, 1×10^6; trap fill time, 80 ms; isolation width, ±0.6 Da; fixed first mass, *m/z* 80; normalized collision energy, 20 eV; intensity threshold of precursor ions for MS/MS

analysis, 3100; apex trigger, 2–4 s; and dynamic exclusion, 2 s. The intensity thresholds of the precursor ions for the MS/MS analysis and dynamic exclusion were set to 1×10^4 and 1 s, respectively. The LC/HRMS/MS analysis was controlled using Xcalibur 4.2.47 software (Thermo Fisher Scientific).

Semiquantification of the reaction adducts between the NT and pyrimidine nucleoside-derived radicals by liquid chromatography–tandem mass spectrometry (LC/MS/MS)

LC/MS/MS was conducted using LCMS-8060 (Shimadzu Co., Kyoto, Japan). The LC/MS/MS analyses were performed using LabSolutions version 5.80 (Shimadzu Co.). The LC parameters and equipment applied were as follows: injection volume, 10 μL; autosampler temperature, 4 °C; column, InertSustain (2.1 mm × 150 mm (C18) with a particle size of 3 μm; GL Sciences); column temperature, 40 °C; mobile phase, A: 5 mM ammonium acetate in H_2O and B: 5 mM ammonium acetate in ACN:H_2O = 95:5; time program, B% for 0–15 min: 30–65%, for 15.01–20 min: 100%, for 20.5–25 min: 30%; and flow rate, 0.4 mL/min. The mass spectrometer was equipped with an electron spray ionization source. The extracted solution was measured by LC/MS/MS with multiple reaction monitoring (MRM).

Agarose gel electrophoresis

We modified a previously described method for electrophoresis and the evaluation of mobility of oxidized DNA[3]; A brief description of the method is presented herein. The electrophoretic mobility of DNA was determined by electrophoresis on 4% agarose gel. The reaction solutions (10 μL) was applied to the gel well and electrophoresed for 30 min at 50 V. The electrophoresis was performed using Mupid®-2x (Mupid Co., Ltd., Tokyo. Japan). Tris-acetate-EDTA (TAE) buffer was used as the electrophoretic buffer. The fluorescent intensity of the gel was detected using the ChemiDoc™ XRS+ System (EtBr staining, λex: 300 nm/λem: 605 nm; DNA radical adducts, λex: 652 nm/λem: 672 nm

(Dylight650)) (Bio-Rad Laboratories, Inc.) and analyzed using Image Lab™ Software (Bio-Rad Laboratories, Inc.). After the electrophoresis, the gel was stained with ethidium bromide and colorimetrically detected using the ChemiDoc™ XRS+ System (Bio-Rad Laboratories, Inc.).

Supplementary References

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