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Reaction of 2′-deoxycytidine with peroxynitrite in the presence of ammonium bromide

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Abstract—Peroxynitrite, a reactive nitrogen species generated from nitric oxide and superoxide anion radical, is an endogenous potential risk factor for human cancer. When 2′-deoxycytidine was incubated with peroxynitrite at neutral pH and 37 °C, the reaction was greatly enhanced by the addition of ammonium bromide. Both ammonium ion and bromide ion were required to exert the enhancing effect. In addition to ammonium ion, methylamine and dimethylamine exerted the enhancing effect in the presence of bromide ion. Two major products were identified as 5-hydroxy-2′-deoxycytidine and 5-bromo-2′-deoxycytidine. Hypochlorite solution and bromine water reacted with 2′-deoxycytidine generating 5-hydroxy-2′-deoxycytidine and 5-bromo-2′-deoxycytidine in the presence of ammonium bromide with the yields similar to those of the reaction of peroxynitrite with ammonium bromide. Fenton reaction of 2′-deoxycytidine was suppressed by the addition of ammonium bromide. Nitrogen dioxide gas did not react with 2′-deoxycytidine in the presence or the absence of ammonium bromide. These results suggest that in the presence of ammonium ion or amines, bromide ion interacts with peroxynitrous acid, which is a protonated form of peroxynitrite, but not with hydroxyl radical or nitrogen dioxide generated by homolysis of peroxynitrous acid, to form hypobromous acid. In the presence of ammonium ion or amines, bromide ion may play a role in enhancing the genotoxic effects of peroxynitrite in humans.
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1. Introduction

Nitric oxide (·NO) is synthesized in various types of cells by the enzyme nitric oxide synthase and is involved in numerous biological functions, including vasodilation, neurotransmission, and inflammation.[1,2] Although ·NO is a radical, the reactivity of ·NO per se is relatively low.[3] Superoxide anion radical $\cdot O_2^-$ is synthesized by NADPH oxidase, a key enzyme involved in the generation of reactive oxygen species during the respiratory burst in phagocytic cells, such as monocytes and macrophages.[4] $\cdot O_2^-$ is also generated by xanthine oxidoreductase as an antimicrobial agent.[5] $\cdot O_2^-$ per se has a limited reactivity with most biological molecules.[6] When ·NO and $\cdot O_2^-$ encounter, they react at an almost diffusion-controlled rate resulting in peroxynitrite ($ONOO^-$).[7] In a neutral solution, peroxynitrite exists as a mixture of $ONOO^-$ and peroxynitrous acid (ONOOH), a protonated form of peroxynitrite, since the pK_a of ONOOH is 6.5.[8] ONOOH is highly reactive and readily causes oxidation and nitration of various biological molecules such as fatty acids and amino acids.[9,10] For DNA components, guanine base reacts effectively with peroxynitrite.[11,12] Many studies have examined the effects of additive compounds on the reaction of ONOOH. Urate, cystein, and ascorbate were effective scavengers of ONOOH.[13–15] CO_2 or $NaHCO_3$ enhanced the reactions of amino acids with ONOOH resulting in an increase of nitration products.[16,17] We examined the effects of various compounds on the reaction of nucleosides with peroxynitrite at neutral pH, and found that ammonium bromide could act as an enhancer of peroxynitrite reaction.

In the present study, we show that ammonium bromide efficiently accelerates the reaction of 2′-deoxycytidine (dCyd) with ONOOH. We discuss the reactive species formed in the reaction system based on the product analysis on the reactions of dCyd with several reactive species such as hypobromous acid, hydroxyl radical, and nitrogen dioxide in the presence of ammonium bromide.

Keywords: Peroxynitrite; Ammonium; Bromide; Deoxycytidine.
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2. Results

2.1. Reaction of dCyd with ONOOH in the presence of NH_4Br

A dCyd (100 μM) solution in 100 mM potassium phosphate buffer (pH 7.4) was incubated with 1 mM $ONOO^-$ at 37 °C for 5 h. The reaction was monitored by the reversed phase high performance liquid chromatography (RP-HPLC) with detection at 260 nm. The dCyd consumption was low (ca. 2%). However, when the reaction was carried out in the presence of 100 mM NH_4Cl, the consumption of dCyd increased greatly and several product peaks were observed in the RP-HPLC chromatogram (Fig. 1). A product (termed **1**) eluted at an HPLC retention time of 9.5 min showed λ_{max} = 291 nm in the on-line detected UV spectrum (Fig. 1, inset). Another product (termed **2**) with a retention time of 16.1 min showed λ_{max} = 287 nm. Compounds **1** and **2** were isolated and subjected to MS measurements. Figure 2A and B show the electrospray ionization time of flight mass spectrometry (ESI-TOF/MS) spectra of compounds **1** and **2**, respectively. Authentic samples of 5-OH-dCyd and 5-Br-dCyd were synthesized. On the basis of coincidence of their retention times, UV spectra, and MS spectra, compounds **1** and **2** were identified as 5-OH-dCyd and 5-Br-dCyd, respectively. The reaction scheme and the structures of 5-OH-dCyd and 5-Br-dCyd are shown in Figure 3.

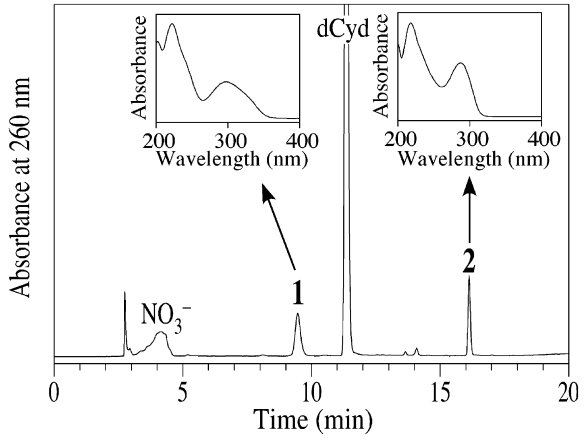

Figure 1. RP-HPLC chromatogram of a reaction solution of dCyd with ONOOH in the presence of NH_4Br. The insets are the on-line UV spectra of **1** and **2**. A solution of 100 μM dCyd containing 100 mM NH_4Br was incubated with 1 mM $ONOO^-$ in 100 mM potassium phosphate buffer (1 mL, pH 7.4) at 37 °C for 5 h in a capped microtube. The RP-HPLC chromatogram was detected at 260 nm.

2.2. NH_4Br and $ONOO^-$ dose dependence

Figure 4A shows the concentrations of unreacted dCyd and produced 5-OH-dCyd and 5-Br-dCyd in the reaction of dCyd with $ONOO^-$ with various concentrations of NH_4Br. The consumption of dCyd and the yields of the products increased with increasing HN_4Br dose. When the NH_4Br dose was 100 mM, the yields of 5-OH-dCyd and 5-Br-dCyd were 12.6 ± 0.1 μM and 6.8 ± 0.1 μM, respectively, while 45.0 ± 0.7 μM dCyd was consumed. Figure 4B shows the $ONOO^-$ dose dependence of concentrations of dCyd and the products in the reaction of dCyd with $ONOO^-$ in the presence of 100 mM NH_4Br. The consumption of dCyd and the yields of the products increased with increasing $ONOO^-$ dose.

2.3. Time course of the reaction of dCyd with ONOOH in the presence of NH_4Br

Figure 5 shows the time courses of concentrations of unreacted dCyd and produced 5-OH-dCyd and 5-Br-dCyd when dCyd was incubated at 37 °C with $ONOO^-$ in the presence of 100 mM NH_4Br. The concentrations of dCyd and 5-Br-dCyd were constant for incubation times from 1 to 12 h. The concentration of 5-OH-dCyd increased up to 5 h and then decreased slightly.

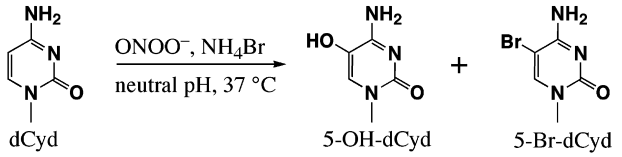

Figure 3. Reaction scheme and structures of the reaction products, 5-OH-dCyd and 5-Br-dCyd.

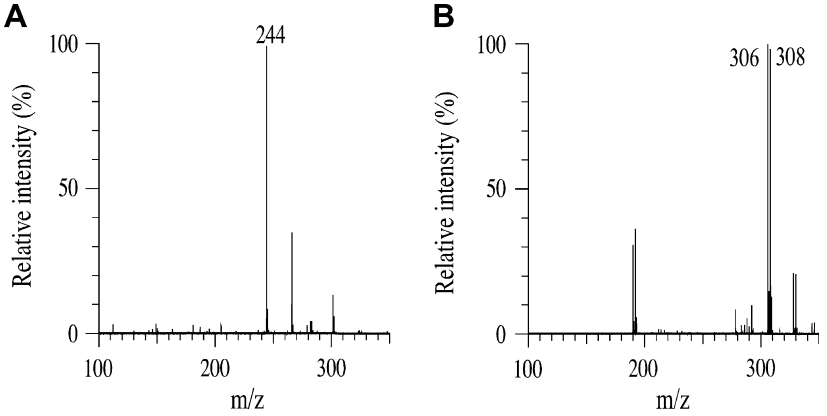

Figure 2. Positive-ion electrospray ionization TOF (time of flight) mass spectrometry spectra of **1** (A) and **2** (B). The samples isolated by RP-HPLC were directly infused into the MS system using a syringe pump.

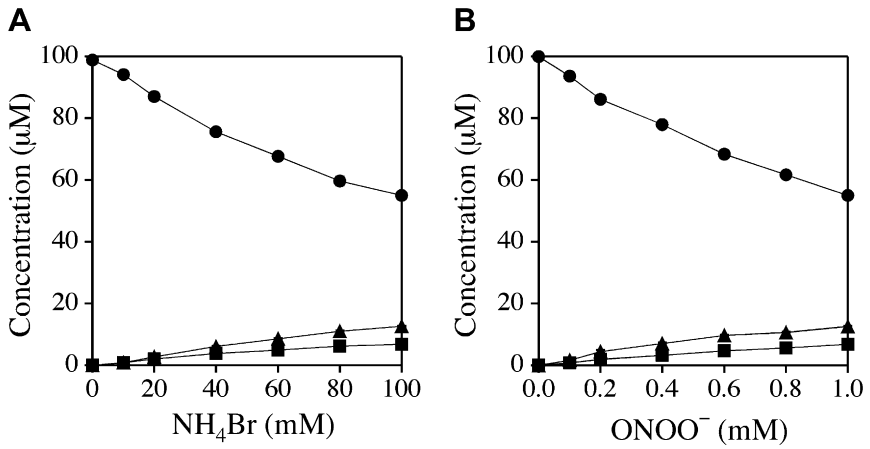

Figure 4. (A) NH_4Br dose dependence of the yields of 5-OH-dCyd (triangle) and 5-Br-dCyd (square) with unreacted dCyd (circle). A solution of 100 μM dCyd containing 0–100 mM NH_4Br was incubated with 1 mM $ONOO^-$ in 100 mM potassium phosphate buffer (pH 7.4) at 37 °C for 5 h in a capped microtube. (B) $ONOO^-$ dose dependence of the yields of 5-OH-dCyd (triangle) and 5-Br-dCyd (square) with unreacted dCyd (circle). A solution of 100 μM dCyd containing 100 mM NH_4Br was incubated with 0–1 mM $ONOO^-$ in 100 mM potassium phosphate buffer (pH 7.4) at 37 °C for 5 h in a capped microtube. The concentrations were determined by RP-HPLC analysis. Means ± SD (n = 3) are presented.

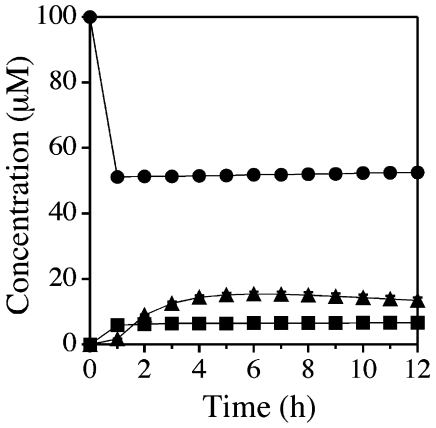

Figure 5. Time course of the yields of 5-OH-dCyd (triangle) and 5-Br-dCyd (square) with unreacted dCyd (circle). A solution of 100 μM dCyd containing 100 mM NH_4Br was incubated with 1 mM $ONOO^-$ in 100 mM potassium phosphate buffer (pH 7.4) at 37 °C for 0–12 h in a capped microtube. The concentrations were determined by RP-HPLC analysis. Means ± SD (n = 3) are presented.

2.4. Effects of NH_4^+ and Br^-

To clarify whether both ammonium ion (NH_4^+) and bromide ion (Br^-) are required for the acceleration ability, dCyd and ONOOH were reacted in the presence of various concentrations of NH_4Cl and NaBr. Figure 6A shows the concentrations of dCyd and the products when dCyd and $ONOO^-$ were incubated with 100 mM NH_4Cl and various concentrations of NaBr. At 0 mM NaBr, the consumption of dCyd was ca. 2%. As the NaBr concentration increased, the reaction was enhanced. At 100 mM NaBr, the concentrations of dCyd and the products were comparable to those of the reaction in the presence of 100 mM NH_4Br. Figure 6B shows the concentrations of dCyd and the products with 100 mM NaBr and various concentrations of NH_4Cl. At 0 mM NH_4Br, the consumption of dCyd was ca. 5%. When the concentration NH_4Cl increased, the consumption of dCyd and the yields of the products were increased rapidly up to the 20 mM dose. From 20 to 100 mM of NH_4Cl, the reaction was not affected by the NH_4Cl concentration.

2.5. Amines in lieu of ammonium ion

To know whether amines exert an effect in lieu of ammonium ion, reaction of dCyd and ONOOH was performed in the presence of methylamine, dimethylamine, trimethylamine, or tetramethyl ammonium with 100 mM NaBr. Table 1 shows the concentrations of unreacted dCyd and produced 5-OH-dCyd and 5-Br-dCyd in the reaction with 20 mM of the amines. Methylamine and dimethylamine accelerated the reaction, although their efficiencies were less than that of NH_4^+.

2.6. Reaction of dCyd with HOBr

To investigate the possibility of production of the hypobromous acid (HOBr) as a reactive species in the ONOOH/NH_4Br system, two HOBr production systems, $HOCl/Br^-$ and Br_2 at neutral pH were applied. When dCyd was incubated with sodium hypochlorite solution (100 μM OCl^-) at neutral pH in the presence of 100 mM NH_4Br, dCyd was consumed, and 5-OH-dCyd and 5-Br-dCyd were produced in a similar manner to the ONOOH/NH_4Br system (Table 2). When dCyd was incubated with bromine water (100 μM Br_2) at neutral pH in the presence of 100 mM NH_4Br, a similar result was obtained. Figure 7A shows the Br_2 dose dependence of concentrations of dCyd and the products in the reaction of dCyd with bromine water in the presence of 100 mM NH_4Br. At 75 μM Br_2, the concentrations of dCyd and the products were very similar to those of 1 mM $ONOO^-$ reaction with 100 mM NH_4Br. Figure 7B shows the time course of concentrations of dCyd and the products when dCyd was incubated at 37 °C with 100 μM Br_2 in the presence of 100 mM NH_4Br. The concentrations of dCyd and the products

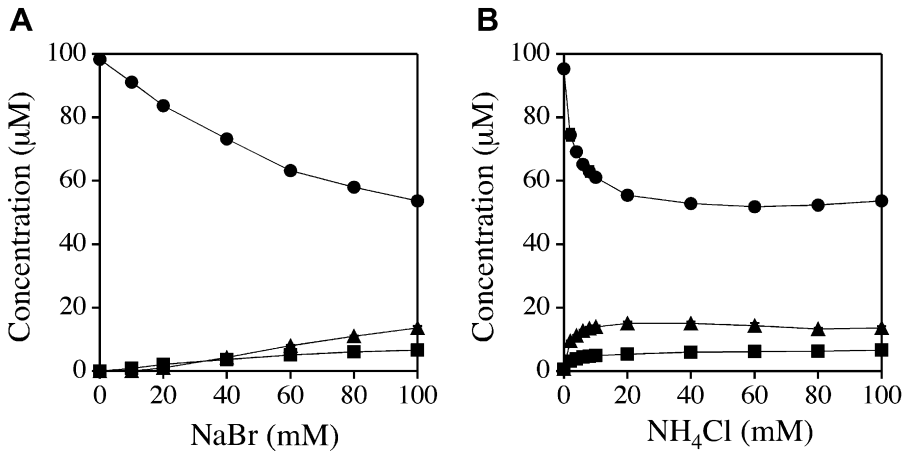

Figure 6. (A) NaBr dose dependence of the yields of 5-OH-dCyd (triangle) and 5-Br-dCyd (square) with unreacted dCyd (circle) in the presence of NH_4Cl. A solution of 100 μM dCyd containing 0–100 mM NaBr and 100 mM NH_4Cl was incubated with 1 mM $ONOO^-$ in 100 mM potassium phosphate buffer (pH 7.4) at 37 °C for 5 h in a capped microtube. (B) NH_4Cl dose dependence of the yields of 5-OH-dCyd (triangle) and 5-Br-dCyd (square) with unreacted dCyd (circle) in the presence of NaBr. A solution of 100 μM dCyd containing 100 mM NaBr and 0–100 mM NH_4Cl was incubated with 1 mM $ONOO^-$ in 100 mM potassium phosphate buffer (pH 7.4) at 37 °C for 5 h in a capped microtube. The concentrations were determined by RP-HPLC analysis. Means ± SD (n = 3) are presented.

Table 1. Effects of amines on the reaction of dCyd with peroxynitrite in the presence of NaBr[a]

Amines (20 mM)	dCyd (μM)	5-OH-dCyd (μM)	5-Br-dCyd (μM)
None	95.4 ± 1.2	0.63 ± 0.20	0.45 ± 0.04
NH_4Cl	55.4 ± 0.8	16.3 ± 0.2	5.92 ± 0.08
CH_3NH_2·HCl	71.2 ± 0.5	3.90 ± 0.07	3.77 ± 0.03
$(CH_3)_2NH$·HCl	83.5 ± 0.8	0.36 ± 0.09	2.17 ± 0.12
$(CH_3)_3N$·HCl	94.8 ± 0.2	<0.05	0.28 ± 0.01
$(CH_3)_4NCl$	94.4 ± 0.3	0.62 ± 0.13	0.40 ± 0.05

[a] A solution (1 mL) of 100 μM dCyd containing 100 mM NaBr and 20 mM amines in 100 mM potassium phosphate buffer (pH 7.4) was incubated with 1 mM $ONOO^-$ at 37 °C for 5 h. The concentration was determined by RP-HPLC. Means ± SD (n = 3) are presented.

Table 2. Reaction of dCyd with Hypochlorite Solution or Bromine Water in the Presence of NH_4Br[a]

Reaction	dCyd (μM)	5-OH-dCyd (μM)	5-Br-dCyd (μM)
HOCl/NH_4Br	45.5 ± 0.2	16.8 ± 0.0	7.57 ± 0.01
Br_2/NH_4Br	43.8 ± 0.8	20.5 ± 0.2	6.94 ± 0.07

[a] A solution of 100 μM dCyd containing 100 mM NH_4Br was incubated with sodium hypochlorite solution (100 μM OCl^-) or bromine water (100 μM Br_2) in 100 mM potassium phosphate buffer (pH 7.4) at 37 °C for 5 h. The concentration was determined by RP-HPLC. Means ± SD (n = 3) are presented.

showed changes similar to those in the ONOOH/NH_4Br reaction (Fig. 5).

2.7. Effect of NH_4Br on the reaction with OH or NO_2

To obtain information about the mechanism for the generation of the reactive species in the ONOOH/NH_4Br system, the reactions of dCyd with Fenton system and $^{\cdot}NO_2$ gas in a solution at neutral pH were investigated with or without NH_4Br. For the Fenton system, the concentrations of unreacted dCyd and the products are listed in Table 3. NH_4Br and NaBr (100 mM each) suppressed the Fenton reaction greatly. NH_4Cl also suppressed the reaction but with a lower efficiency. For the reaction with $^{\cdot}NO_2$ gas, the dCyd consumption and the production of 5-OH-dCyd and 5-Br-dCyd were not detected with or without 100 mM NH_4Br (data not shown).

3. Discussion

In the present study, we found that NH_4Br greatly enhanced the reaction of dCyd with $ONOO^-$ at neutral pH. Two of the major products were identified as 5-OH-dCyd and 5-Br-dCyd, although the total yield of the products was ca. 50% of the consumed dCyd. Both NH_4^+ and Br^- were required to exert the effect. While Br^- enhanced the reaction dose-dependently, a small amount of NH_4^+ relative to Br^- was enough to exert the effect (Fig. 6A and B) suggesting that NH_4^+ acts as a catalyst for the reaction, while Br^- is a substrate. Two HOBr production systems, HOCl/Br^- and Br_2 at neutral pH[18,19] reacted with dCyd resulting in the consumption of dCyd and the formation of 5-OH-dCyd and 5-Br-dCyd with a similar ratio to the ONOOH/NH_4Br system (Table 2). The time course of the change of product yields in the Br_2 reaction was also similar to that in the ONOOH/NH_4Br system (Figs. 5 and 7). For both time courses, the concentrations of 5-OH-dCyd increased up to 5 h, although dCyd and 5-Br-dCyd were constant for incubation times from 1 to 12 h. The delay in the 5-OH-dCyd formation can be explained because 5-OH-dCyd is formed via a relatively stable intermediate, dCyd glycol. The reported half-life of dCyd glycol is 50 min at 37 °C in a neutral buffer solution.[20] These results strongly suggest that the reactive species formed in the ONOOH/NH_4Br system is HOBr. In general, the strong reactivity of peroxynitrite is explained by the formation of two reactive species, $^{\cdot}OH$ and $^{\cdot}NO_2$, generating from homolysis of ONOOH with 30% yield.[21–23] Since it

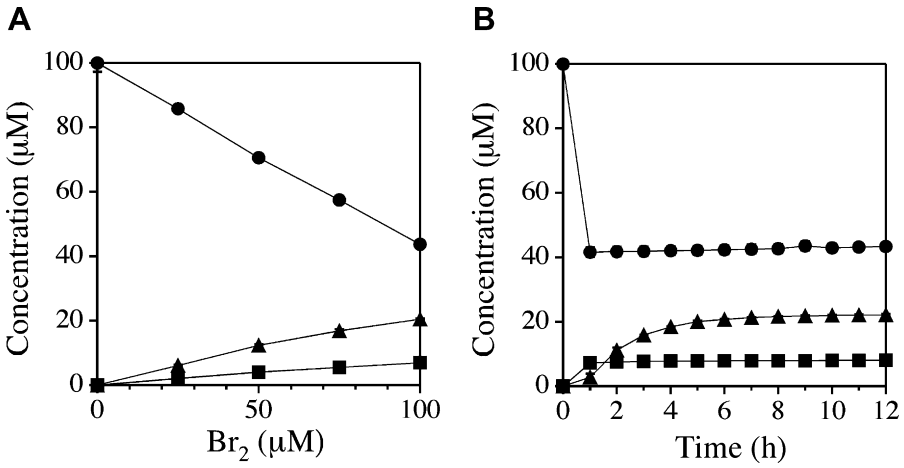

Figure 7. (A) Br_2 dose dependence of the yields of 5-OH-dCyd (triangle) and 5-Br-dCyd (square) with unreacted dCyd (circle). A solution of 100 μM dCyd containing 100 mM NH_4Br was incubated with 0–100 μM Br_2 in 100 mM potassium phosphate buffer (pH 7.4) at 37 °C for 5 h in a capped microtube. (B) Time course of the yields of 5-OH-dCyd (triangle) and 5-Br-dCyd (square) with unreacted dCyd (circle). A solution of 100 μM dCyd containing 100 mM NH_4Br was incubated with 100 μM Br_2 in 100 mM potassium phosphate buffer (pH 7.4) at 37 °C for 0–12 h in a capped microtube. The concentrations were determined by RP-HPLC analysis. Means ± SD (n = 3) are presented.

Table 3. Effects of Additives on the Reaction of dCyd with Fenton System[a]

Additives (100 mM)	dCyd (μM)	5-OH-dCyd (μM)	5-Br-dCyd (μM)
None	47.5 ± 1.9	<0.05	<0.05
NH_4Br	98.7 ± 0.6	<0.05	<0.05
NaBr	97.2 ± 0.3	<0.05	<0.05
NH_4Cl	63.7 ± 1.4	0.27 ± 0.01	<0.05

[a] A solution (1 mL) of 100 μM dCyd, 1 mM $FeSO_4$, and 2 mM H_2O_2 with 100 mM additives in 100 mM potassium phosphate buffer (pH 7.4) was incubated at 37 °C for 5 h. The concentration was determined by RP-HPLC. Means ± SD (n = 3) are presented.

has been reported that ·OH reacts with Br^- to form a bromine radical ·Br,[24] we studied the Fenton reaction of dCyd in the presence of Br^-. However, NH_4Br and NaBr greatly suppressed the Fenton reaction of dCyd (Table 3). Another reactive species $\cdot NO_2$ did not react with dCyd in the presence or the absence of Br^-. The results show that the reactive species, generated in the ONOOH/NH_4Br system, is not generated by the reaction of Br^- with ·OH or $\cdot NO_2$. It implies that, in the presence of NH_4^+, HOBr is generated by the reaction of Br^- with ONOOH, a protonated form of $ONOO^-$, but not with ·OH or $\cdot NO_2$ generated from ONOOH.

$$Br^- \xrightarrow[NH_4^+]{ONOOH} HOBr$$

Ammonia (NH_3/NH_4^+) is toxic and converted to urea by the urea cycle in the liver. Thus, the ammonia concentration in humans is low. For instance, the ammonia concentration of human plasma is 10–60 μM.[25,26] The plasma concentration of ammonia would be too low to exert an enhancing effect on the $ONOO^-$ reaction of dCyd even in the presence of sufficient amount of Br^-. However, the present study showed that, in lieu of ammonium ion, a primary amine methylamine and a secondary amine dimethylamine also accelerated the dCyd/ONOOH reaction in the presence of Br^- in humans, many biomolecules containing amino groups exist at high concentrations. For instance, the intracellular total concentration of the 20 naturally occurring free amino acids is 34 mM in healthy human muscle.[27] This seems to be a sufficient amount of amines to activate the ONOOH reaction in the presence of Br^-. in human intra- and intercellular fluids. Bromine is one of the most abundant and ubiquitous trace elements. Seawater contains 65–67 mg/kg Br^-, about three hundredth the concentration of Cl^-.[27,28] In the nineteenth century, bromides, in the forms of KBr, NaBr, and NH_4Br, were widely used as an antiepileptic drug with good success.[29,30] Bromides are no longer a mainstay of epilepsy therapy because of their significant toxicity[31] and the availability of safer agents. However, bromides are still used in the treatment of patients with refractory seizures, particularly in pediatrics. Although there is evidence that bromides act to stabilize excitable membranes through hyperpolarization of neurons,[32,33] the precise mechanism of the action remains unknown. Essential roles of Br^- in cells have been difficult to demonstrate, since Br^- can replace Cl^- as a substrate in many biological processes including enzyme activation and inhibition.[28] The first identified use of Br^- by enzymes in humans appears to be a role in defense mechanisms against parasites mediated by the preferential oxidation of Br^- by eosinophil peroxidase.[34] An eosinophil peroxidase–H_2O_2–Br^- system in the presence of a plasma concentration of Cl^- can react with nucleosides to form brominated nucleosides.[35–37] More recently, it has been reported that a myeloperoxidase–H_2O_2–Cl^- system in the presence of a plasma concentration of Br^- generates 5-Br-dCyd from dCyd.[38] The present study indicated that ONOOH reacts efficiently with dCyd in the presence of bromide ion and ammonium ion or amines, forming 5-OH-dCyd and 5-Br-dCyd. The concentration of Br^- in human plasma is low ranging over levels of 39–84 μM.[39] At the plasma concentration of Br^-, the efficiency enhancing the ONOOH reaction of dCyd by Br^- would be low. On the other hand, the therapeutic serum concentration of Br^- is high

and ranges over levels of 10–35 mM.[35] At the therapeutic dose level, ONOOH can be converted to HOBr and can harm biological molecules such as dCyd more efficiently.

4. Conclusion

We investigated the effects of NH_4Br on the reaction of dCyd with $ONOO^-$ at neutral pH, and showed that NH_4Br accelerated the reaction resulting in oxidation and bromination on dCyd. In addition to NH_4^+, amines exert an enhancing effect in the presence of Br^-. The proposed reactive species in the system was HOBr. The present results draw attention to the contribution of Br^- in human cancers.

5. Experimental

5.1. Materials

2′-Deoxycytidine (dCyd) was obtained from Sigma (MO, USA). Ammonium bromide and ammonium chloride were purchased from Nacalai Tesque (Osaka, Japan), and sodium bromide from Aldrich (WI, USA). All other chemicals of reagent grade were purchased from Sigma, Aldrich, Nacalai Tesque, Wako (Osaka, Japan), Cica (Tokyo), and used without further purification. Nitrogen dioxide (99.9%) was purchased from Sumitomo Seika (Tokyo). Water was distilled and then purified with a Millipore Milli-Q deionizer.

5.2. Synthesis of $ONOO^-$

$ONOO^-$ was synthesized by the reaction between acidified H_2O_2 and $NaNO_2$ followed by rapid mixing with excess NaOH in a quenched-flow reactor as previously described.[40] Unreacted H_2O_2 was removed by MnO_2. The alkaline solution of $ONOO^-$ was preserved in liquid nitrogen. The concentration of $ONOO^-$ was determined spectrophotometrically from its absorbance at 302 nm in 0.1 M NaOH using a molar extinction coefficient of 1670 M^{-1} cm^{-1}.[40]

5.3. HPLC and MS conditions

The HPLC system consisted of Shimadzu LC-10ADvp pumps and an SCL-10Avp system controller. On-line UV spectra were obtained with a Shimadzu SPD-M10Avp UV–vis photodiode-array detector. For the RP-HPLC, an Inertsil ODS-3 octadecylsilane column of 4.6×250 mm and particle size 5 μm (GL Science, Tokyo) was used. For analyses, 20 mM triethylammonium acetate buffer (pH 7.0) containing methanol was used as the eluent. The methanol concentration was increased from 0% to 50% for 15 min in linear gradient mode and kept at 50% until 20 min. The column temperature was 40 °C and the flow rate was 1.0 mL/min. The ESI-TOF/MS measurements were performed on a Micro-TOF spectrometer (Bruker, Bremen, Germany) in the positive mode. The sample isolated by RP-HPLC using 20 mM ammonium acetate buffer (pH 7.0) containing methanol as the eluent was directly infused into the MS systems by a syringe pump without a column at a flow rate of 2 μL/min.

5.4. Reaction conditions

For the reaction of dCyd with ONOOH (Fig. 1), a solution of 100 μM dCyd containing 100 mM NH_4Br was incubated with 1 mM $ONOO^-$ in 100 mM potassium phosphate buffer (1 mL, pH 7.4) at 37 °C for 5 h in a capped microtube. The reaction was started by mixing the solution excluding $ONOO^-$ with the alkaline stock solution of $ONOO^-$. The same conditions were applied except for the NH_4Br concentration (0–100 mM) as depicted in Figure 4A, the $ONOO^-$ concentration (0–1 mM) as in Figure 4B, and the incubation time (0–12 h) for Figure 5. As shown in Figure 6A, 100 mM NH_4Cl and 0–100 mM NaBr was used in lieu of 100 mM NH_4Br. For Figure 6B, 100 mM NaBr and 0–100 mM NH_4Cl were used in lieu of 100 mM NH_4Br. The final pHs were 7.2–7.5. For the effect of amines on the reaction of dCyd with ONOOH (Table 2), a solution of 100 μM dCyd containing 20 mM amines and 100 mM NaBr was incubated with 1 mM $ONOO^-$ in 100 mM potassium phosphate buffer (1 mL, pH 7.4) at 37 °C for 5 h in a capped microtube. The final pHs were 7.2–7.4. For the reaction of HOCl and Br_2 (Table 1), a solution of 100 μM dCyd containing 100 mM NH_4Br was incubated with 100 μM OCl^- (sodium hypochlorite solution) or 100 μM Br_2 (bromine water) in 100 mM potassium phosphate buffer (1 mL, pH 7.4) at 37 °C for 5 h in a capped microtube. The OCl^- concentration was determined from the UV absorbance at 290 nm, assuming $\varepsilon_{290} = 350$ M^{-1} cm^{-1}.[41] The Br_2 concentration was determined at 390 nm, assuming $\varepsilon_{390} = 172$ M^{-1} cm^{-1}.[42] The same conditions were applied except for the Br_2 concentration (0–100 μM) as exhibited in Figure 7A and the incubation time (0–12 h) as in Figure 7B. The final pHs were 7.2–7.4. For Fenton reaction (Table 3), a solution of 100 μM dCyd containing 1 mM $FeSO_4$ and 2 mM H_2O_2 in 100 mM potassium phosphate buffer (pH 7.4) with or without 100 mM NH_4Br, NaBr, or NH_4Cl was incubated at 37 °C for 5 h in a capped microtube. The H_2O_2 concentration was determined from the UV absorbance at 240 nm, assuming $\varepsilon_{240} = 39.4$ M^{-1} cm^{-1}.[43] The final pHs were 7.2–7.4. For $^{\cdot}NO_2$ reaction, 100 μM dCyd was dissolved in 10 mL of 100 mM potassium phosphate buffer (pH 7.4) with or without 100 mM NH_4Br in an open vessel, and $^{\cdot}NO_2$ was bubbled at a flow rate of 1 mL/min into a stirred solution under aerobic conditions. The pH was held in the range 7.2–7.5 by titration of 1 M NaOH. The reaction was terminated when 100 μL of 1 M NaOH (100 μmol) was added. The solution (1 mL) was incubated in a capped microtube at 37 °C for 5 h before RP-HPLC analysis. All experiments were carried out in triplicate.

5.5. Preparation of the authentic 5-OH-dCyd and 5-Br-dCyd

Authentic 5-hydroxy-2′-deoxycytidine (5-OH-dCyd) and 5-bromo-2′-deoxycytidine (5-Br-dCyd) were prepared according to the method reported previously.[44,45] For 5-OH-dCyd, bromination of dCyd was carried out by the

drop-by-drop addition of Br_2 water to dCyd (500 mg) at 0 °C until the solution changed to a persistently light yellow color. To convert corresponding bromohydrin to 5-OH-dCyd, 10% (v/v) 2,4,6-collidine was added and the solution was stirred at room temperature for 2 days. The product was purified by HPLC using the ODS column with 5% methanol in water as the mobile phase, and dried on a vacuum pump. For 5-Br-dCyd, bromination of dCyd was carried out by the drop-by-drop addition of Br_2 water to dCyd (200 mg) at 0 °C until the solution changed to a persistently light yellow color. The solution was stirred at room temperature for 1 day. The product was purified by HPLC using the ODS column with 10% methanol in water as the mobile phase, and dried on a vacuum pump. Spectrometric data for 5-OH-dCyd: ^{1}H NMR (500 MHz, in DMSO-d_6): δ (ppm/TMS) 7.50 (s, H6, 1H), 6.15 (dd, H1′, 1H), 4.23 (m, H3′, 1H), 3.81 (m, H4′, 1H), 3.57 (m, H5′,5″, 2H), 2.12 (m, H2′ or 2″, 1H), 2.01 (m, H2′ or 2″, 1H); UV: λ_{max} = 291 (pH 7.0); ESI-TOF/MS (positive) *m*/*z* 244 $[M+H]^+$. Spectrometric data for 5-Br-dCyd: ^{1}H NMR (500 MHz, in DMSO-d_6): δ (ppm/TMS) 8.28 (s, H6, 1H), 6.06 (dd, H1′, 1H), 4.20 (m, H3′, 1H), 3.77 (m, H4′, 1H), 3.58 (m, H5′,5″, 2H), 2.13 (m, H2′ or 2″, 1H), 2.00 (m, H2′ or 2″, 1H); UV: λ_{max} = 287 (pH 7.0); ESI-TOF/MS (positive) *m*/*z* 308 $[M(^{81}Br)+H]^+$, 306 $[M(^{79}Br)+H]^+$.

5.6. Quantitative procedures

The concentrations of the products were evaluated from integrated peak areas on HPLC chromatograms detected at 260 nm and the molecular extinction coefficients at 260 nm. The ε_{260nm} of 7400 M^{-1} cm^{-1} was used for dCyd. The ε_{260nm} values for 5-OH-dCyd and 5-Br-dCyd were estimated to be 2970 and 3960 M^{-1} cm^{-1}, respectively, from the integration of H1′ proton signal and the HPLC peak area detected at 260 nm relative to those of dCyd.

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