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Base Pairing Properties of 8-Oxo-7,8-dihydroadenosine in cDNA Synthesis by Reverse Transcriptases

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Abstract—Incorporation of nucleotides opposite 8-oxo-7,8-dihydroadenosine (8-oxoA) in oligonucleotides with dNTPs by three reverse transcriptases (AMV-, MMRV-, RAV2-RT) in cDNA synthesis was studied. Guanine as well as thymine was incorporated preferentially by all reverse transcriptases. In the melting temperature experiment, 8-oxoA and 8-oxoA-Me formed base pairs with thymine and guanine with similar stabilities.

Oxidative base damages of nucleic acids play an important role in cellular processes and the damage creates miscoding lesions that are potentially mutagenic, carcinogenic, or generating interstrands crosslinking.[1–3] C8 residues of purine deoxynucleosides are easily hydroxylated by the oxidative damages and more frequently occurred in deoxyguanosine (8-oxodG) than deoxyadenosine (8-oxodA). Unrepaired DNA damage leads to accumulation of mutation and may attribute to the development of cancers and other degenerated diseases with cellular aging.[4–7] The base pairing properties and conformational change of C8 oxidized deoxypurine nucleosides in polymerization reactions or in duplex formation were well characterized.[8–10] 8-OxodG and 8-oxodA differ significantly with respect to their respective mutagenic potential. The mutagenic properties of 8-oxodG reflect the stability of the lesion when paired in the *syn* conformation with dA (*anti*), coupled with the relative resistance of the complex to proof reading exonucleases and subsequent repair by 8-oxodG-DNA glycosylase.[11] In contrast, 8-oxodA promotes a nonmutagenic event-insertion of TMP (*anti*) opposite the lesion forming a Watson–Crick pair. 8-OxodG with other than dATP was proved in insertion reaction with polymerase and the degree of dATP insertion was depended on enzymes. Base-pairing properties of the 8-oxodA was similar aspects to those of the 8-oxodG. Several research groups revealed the inserted deoxynucleotides opposite 8-oxodA using various DNA polymerases.[12,13]

In contrast to DNA, the base lesion in RNA strands is not well understood. Recently, oxidative base damaged ribonucleosides were reported by Torula yeast RNA, isolated from a RNA bacteriophages and a plasmid.[14–16] RNA may be considered to have enhanced potential for oxidative attack due to its widespread cytosolic distribution within various organelles. In the case of retroviral replication, base damages of RNA can contribute to an elevated mutation rate in DNA, interfering with correct base pairing which comprises the accuracy of cellular process such as the genomic material. The potential for mutagenesis exists as a result of base misincorporation opposite oxidatively damaged templates. In our previous reports, we reported that 8-oxoG and 8-oxoG-Me in a predetermined position in 30 base-long oligoribonucleotides could pair not only with dC but also with other bases (dA or T) and the base-pairing properties were dependent on the reverse transcriptases.[17,20]

In this paper, in order to extend understanding of the base pairing properties of oxidatively damaged purine ribonucleotides, we prepared 24 base-long oligoribonucleotides [5′-AUUUUCXGAAUUGGGUGUCGAC-AU-3′, X = A(ORNs-1), X = 8-oxo-A(ORNs-2) or X = 8-oxoA-Me(ORNs-3)] containing 8-oxoA and 8-oxoA-Me. We analyzed the incorporation properties of DNA bases opposite the modified adenosine in vitro cDNA synthesis with AMV-, MMLV-, and RAV2-reverse transcriptases.

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The 8-oxo-7,8-dihydroadenosine and 8-oxo-7,8-dihydro-2′-*O*-methyladenosine phosphoramidite building blocks were prepared according to the previous report.[17] Oligoribonucleotides were synthesized using solid phase phosphoramidite approach with an automated synthesizer (ABI, DNA Synthesizer, and model 391). 4,5-Dicyanoimidazole was used for an activator and double coupling was performed for both the 8-oxoA and 8-oxo-A-Me amidites coupling steps. Oligoribonucleotides were deprotected by subsequent treatment of ethanol ammonia and triethylamine trihydrofluoride. Oligonucleotides were purified using an OPC, denatured polyacrylamide gel-electrophoresis (PAGE) and reverse phase HPLC. The purities of oligoribonucleotides were identified over 98% by PAGE and HPLC (data not shown). The presence of 8-oxoA and 8-oxoA-Me in oligonucleotides were analyzed by digestion of oligonucleotides with a combination of phosphodiesterase I and alkaline phosphatase, followed by separation of the component nucleosides by reverse phase HPLC (data not shown). Alkali degradation of oligonucleotides also proved the presence of the modified adenosine derivatives. The 8-oxoA and 8-oxoA-Me in oligonucleotides have shown a distinctive mobility in PAGE (Fig. 1).

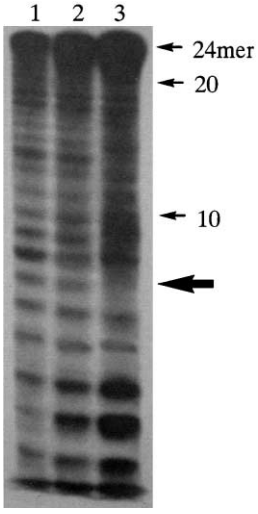


Figure 1. Autoradiography of polyacrylamide-gel of the modified and unmodified oligoribonucleotides. Lane 1: ORNs-1, lane 2: ORNs-2, lane 3: ORNs-3. 20000 cpm/μL of 5′-end ^{32}P labeled ORNs-1,-2 and -3 were incubated in a solutions of 50 mM of $NaHCO_3$ (pH 9.0) and 1 mM DETA at 90 °C for 11 min. The bold arrow of lane 3 indicated the 8-oxoA-Me position.

Investigation of the incorporation of dNTPs opposite the modified adenosine during cDNA synthesis was carried out with reverse transcriptases (Scheme 1). 5′-End labeled 17 base-long of DNA primer was extended along the modified or unmodified RNA templates by reverse transcriptases, and the 18th bands of the inserted dNMPs to 3′-end primer were analyzed by PAGE (Fig. 2). The amount of inserted dNMPs was also calculated from the ratio of radioactivity of PAGE bands (Fig. 3).

In control reaction, TMP was preferentially inserted into the site opposite the unmodified adenosine by all reverse transcriptases as we expected. TMP was also incorporated preferentially compared to other dNMPs opposite 8-oxoA or 8-oxoA-Me by the reverse transcriptases. However, in the site opposite 8-oxoA and 8-oxoA-Me, dGMP was also incorporated in substantial amount by the reverse transcriptases. The amount of dGMPs insertion was dependent on the reverse transcriptases. AMV-reverse transcriptase incorporated

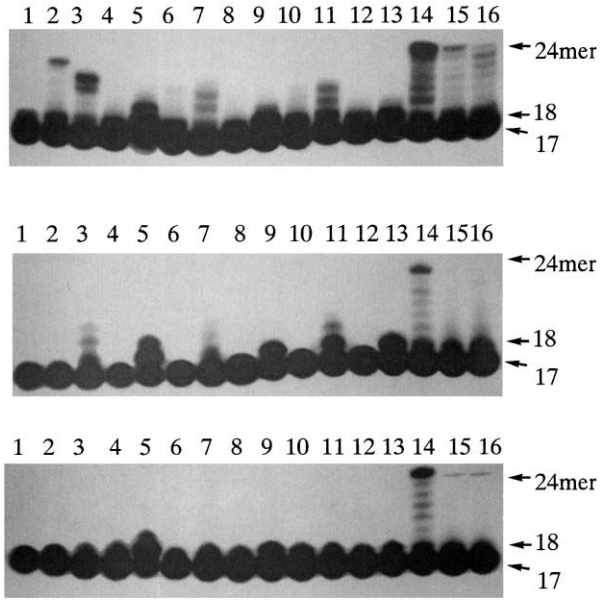
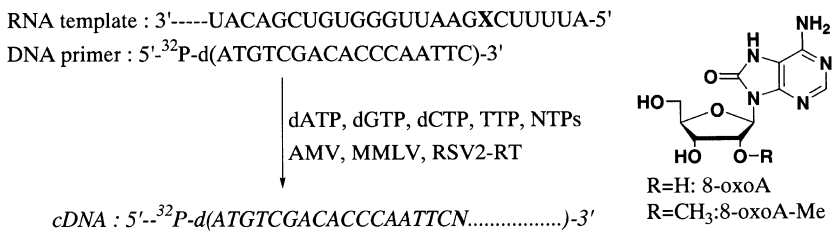


Figure 2. Autoradiograms of polyacrylamide gels showing incorporations of dNTPs into the primer at the site opposite **X** the normal template ORNs-1 (**X** = A, lanes 2, 5, 8, 11, 14), modified template ORNs-2 (**X** = 8-oxoA, lanes 3, 6, 9, 12, 15) or ORNs-3 (**X** = 8-oxoA-Me 4, 7, 10, 13, 16) and the primer (lane 1) were incubated with AMV-RT (*upper*), MMLV-RT (*middle*) or RAV2-RT (*lower*) in the presence of dATP (lanes 2–4), dGTP (lanes 5–7), dCTP (lanes 8–10), TTP (lanes 11–13) and dNTPs (lanes 14–16). 17mers indicate not extended primer and 18mers and 24mers indicate extended primers.[18]



Scheme 1. Synthesis of cDNA by reverse transcriptases where, **X** = A, 8-oxoA or 8-oxoA-Me and structures of 8-oxoA and 8-oxoA-Me are shown on right.

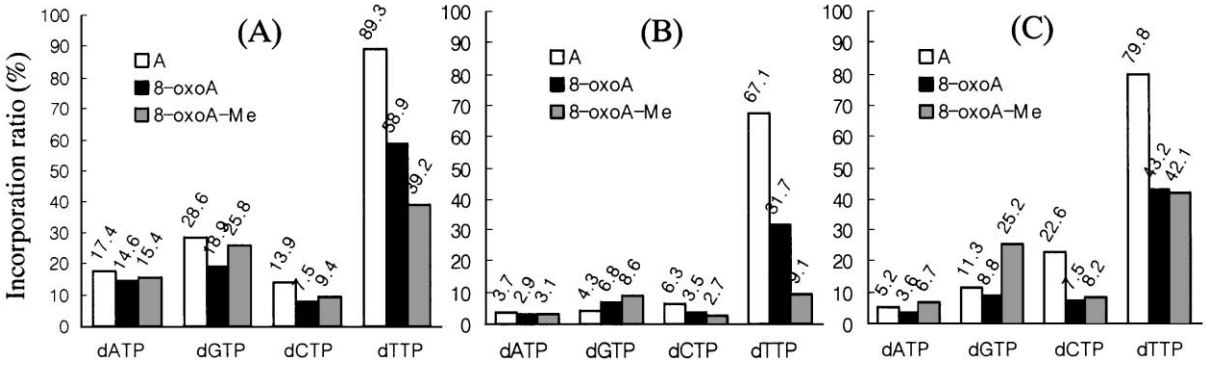


Figure 3. Ratio of incorporation of dNTPs to the primers by AMV-RT (A), MMLV-RT (B) and RAV2-RT (C). The ratio (%) was calculated as Ratio = (radioactivity of newly generated spots)/(radio activity of the remaining primer + radioactivity of newly generated spots) × 100.[19]

Table 1. Melting temperatures (T_m) and thermodynamic parameters[a] of duplex[b] oligonucleotides

Base pair of *X*·*Y* in duplex	T_m[c] (°C)	$\Delta H°$ (*K*cal/mol)	$\Delta S°$ (cal/mol)[c]	$\Delta G°$ (25 °C) (*K*cal/mol)
A·dA	15.5	−35.6	−96.6	−6.8
A·dG	21.3	−47.7	−135.0	−7.4
A·dC	15.9	−37.0	−101.2	−6.8
A·T	32.7	−67.2	−192.8	−9.7
8-oxoA·dA	15.4	−36.1	−98.4	−6.8
8-oxoA·dG	20.2	−37.5	−124.3	−7.2
8-oxoA·dC	15.7	−44.3	−103.1	−6.8
8-oxoA·T	22.3	−46.9	−131.9	−7.6
8-oxoA-Me·dA	14.5	−36.9	−101.5	−6.6
8-oxoA-Me·dG	21.1	−39.0	−117.0	−7.4
8-oxoA-Me·dC	14.7	−42.3	−108.7	−6.6
8-oxoA-Me·T	23.7	−47.0	−131.6	−7.8

[a]Thermodynamic parameters were calculated from the slop of a $1/T_m$ versus $\ln(C_t/4)$ plot and the following equations.[8,19] $\Delta S° = \Delta H°/T_m - R\ln(C_t/4)$ (C_t = total concentration of single strands). $\Delta G° = \Delta H° - T\Delta S°$.

[b]Duplexes were constructed with RNA (5′-UUUUC*X*GAAUUA-3′) and DNA [3′-d(A-AAAG*Y*CTTAAT)-5′]. *X* = A, 8-oxoA, 8-oxoA-Me, *Y* = dA, dG, dC, T.

[c]Thermal denaturation experiment was carried out in 10 mM Na-carcodylate (pH 7.0), 100 mM NaCl and 20 mM $MgCl_2$. The concentrations of duplexes were 2.8 μM.

dGMP up to one third of TMP. In the case of 8-oxoA-Me, dGMP was more incorporated than 8-oxoA by the all reverse transcriptases. The order of incorporation of dNMP opposite 8-oxoA by AMV-RT was TMP > dGMP > dAMP > dCMP and by MMLV-RT was TMP > dGMP > dCMP ≥ dAMP, respectively. But in the case of RAV2-RT, the other of dNMPs insertion was variable. These base-pairing properties of 8-oxoA with dGMP by the reverse transcriptase in cDNA synthesis closely parallel those of 8-oxodA by DNA polymerase α and β in vitro DNA synthesis.[21] Our results were also agreed with other report that 8-oxoA recognized guanosine which paired with cytidine to form a triplex.[22]

In order to find out the incorporation properties of 8-oxoA and 8-oxoA-Me with dGMP as well as TMP, we carried out the melting temperature experiment. We prepared dodecaribonucleotides containing 8-oxoA and 8-oxoA-Me and constructed 12 RNA·DNA heteroduplexes (Table 1). We measured T_m of the heteroduplexes and calculated thermodynamic parameters from T_m values. Adenosine formed base pairs with thymidine with the most stability as we expect. Indeed, 8-oxoA formed base pairs with thymidine and guanosine with nearly similar stabilities. 8-OxoA-Me formed also base pairs with thymidine and guanosine with similar stabilities. When 8-oxoA and 8-oxoA-Me form base pairs with adenosine or cytidine, the base pairs remarkably destabilized the duplexes compared to the duplexes containing thymidine and guanosine. Thermodynamic parameter ($\Delta G°$) demonstrates that formation of duplexes between 8-oxoA and 8-oxA-Me and thymidine or guanosine is more thermodynamically favorable compared with other bases. These results were coincident with the incorporation of bases opposite 8-oxoA and 8-oxoA-Me in cDNA synthesis by the reverse transcriptase.

Several groups proposed the conformations of 8-oxodA in DNA duplex in which 8-oxodA exist in keto form with *anti* or *syn* conformation.[12,22,23] The conformations of base pairing of 8-oxoA with thymidine and guanosine have not understood yet. Based on the conformation of 8-oxoA (*syn*)·G·C triad which was reported recently,[22] we assumed that base-pairing of 8-oxoA in RNA/DNA duplex may exists the same conformation as 8-oxoA (*syn*)·G·C triad.

Acknowledgements

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18. 5 × RT buffers in this reaction were 250 mM Tris–HCl, pH 8.3, 40 mM $MgCl_2$, 250 mM NaCl, 5 mM DTT for AMV-RT, 250 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT for MMLV-RT and 250 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT for RAV2-RT in respective. cDNA synthesis was performed as a mixture of primer (40 pmol) 2 μL, template (40 pmol) 2 μL in a RT buffer 4 μL and sterile water 4 μL was denatured at 90 °C for 5 min and then slowly cooled. After adding dNTPs (2.5 nmol) 2 μL and RNase In (10 U, Amersham Pharmacia biotech) 2 μL, the volume of the mixture was adjusted to 20 μL with sterile water. RT enzyme (10 U, Amersham Pharmacia biotech) was then added and the mixture was incubated for 6 h at 39 °C. The mixture was extracted with phenol-chloroform and aqueous layer was loaded on a 20% PAGE containing 7 M urea. The gel was auto-radiographed onto an X-ray film.
19. The incorporation ratio was calculated by measuring the radio activities of the gel band using an image analyzer (Bio Image 50S Series, B. I. System Corp) equipped with a computer system of SPARC station 5 and a scanner of XRS.
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