Oligoribonucleotides containing 8-oxo-7,8-dihydroguanosine (8-oxoG) and 8-oxo-7,8-dihydro-2'-O-methylguanosine (8-oxoG-Me) were synthesized. The base pairing properties of 8-oxoG and 8-oxoG-Me in oligoribonucleotide in cDNA synthesis by reverse transcriptases were studied. dCMP was preferentially incorporated into the site opposite 8-oxoG or 8-oxoG-Me than into other dNMPs. TMP and dCMP were inserted preferentially into sites opposite 8-oxoG or 8-oxoG by reverse transcriptases. HIV-RT did not incorporate TMP, but RAV2-RT incorporated 50% more TMP than dCMP into the site opposite 8-oxoG. In the site opposite 8-oxoG-Me TMP was substantially incorporated by HIV-RT or RAV2-RT. Thermodynamic analysis of the DNA · RNA heteroduplex containing 8-oxoG revealed that 8-oxoG and 8-oxoG-Me formed base pairs with cytidine and thymidine with similar stability. The thermodynamic parameter ($\Delta G^\circ$) demonstrated that the formation of duplexes between 8-oxoG or 8-oxoG-Me and cytidine or thymidine is more thermodynamically favorable than with adenosine and guanosine. However, differences in the melting temperature and $\Delta G^\circ$'s of 8-oxoG/dC and 8-oxoG/T were much smaller than between G/dC and G/T. CD spectra showed that RNA · DNA containing 8-oxoG or 8-oxoG-Me duplexes showed similarities between the A-type RNA and B-type DNA conformations.

**Keywords:** Base pairing, CD spectra, Melting temperature, 8-Oxo-7,8-dihydroguanosine, 8-Oxo-7,8-dihydro-2'-O-methylguanosine, Thermodynamic parameter

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**Introduction**

Reactive oxygen species generated by ionizing radiation or endogenous oxidative processes react with nucleic acid and other cellular components. Oxidative damage of DNA or RNA bases play an important role in cellular processes, and causes miscoding lesions that are potentially mutagenic, carcinogenic, or generate interstrand cross linking (Ames 1983; Kasai and Nishimura, 1984; Fraga et al., 1990; Lee, 2001). Unrepaired DNA damage leads to the accumulation of mutation and may contribute to the development of cancer and other degenerative deceases associated with cellular aging (Lin et al. 1985; Kuchino et al., 1987; Shibutani et al., 1991; Tudek, 2003). C8 residues of purine nucleosides are easily hydroxylated by oxidizing species and this more frequently occur in guanine (8-oxoG) than adenine (8-oxoA). 8-OxodG and 8-oxodA differ significantly with respect to their respective mutagenic potentials. The mutagenic properties of 8-oxodG show lesion stability when paired in the syn conformation with dA (anti), and this is combined with the relative resistance of this complex to proof leading exonucleases and subsequent repair by 8-oxodG-DNA glycosylase (Durate et al., 1998). In contrast, 8-oxodA promotes a nonmutagenic event-insertion of dTMP (anti) opposite the lesion, forming a Watson-Crick pair. Recently, base pairing and conformational changes of C8 oxidized deoxy purine nucleosides in polymerization reactions or in duplex formation were well characterized (Oda et al., 1991; Shibutani et al., 1993; Koizumi et al., 1994). Misreading of 8-oxodG with dA was shown in insertion reaction with polymerases and the degree of dATP insertion was depended on enzymes. The pairing properties of 8-oxodA with T or G were also dependent on enzymes (Kamiya et al., 1995).

In contrast to DNA, little is known about base lesions in RNA strands. 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 damage to RNA can contribute to an elevated mutation rate in DNA, and interfere with correct base pairing, which comprises the accuracy of the cellular process with respect to the replication of genomic material. A potential for mutagenesis exists as a result of base misincorporation due to oxidatively damaged templates. Oxidative base damaged
by the addition of KOTBu in MeOH to the protected 8-oxo-
guanosine led to 2-N-acetyl-6-O-bis(dimethylcarbamoyl)7,8-
dihydro-8-one in good yield. Consecutive treatment of 2-
 N-acetyl-6-O-bis(dimethylcarbamoyl)-7,8-dihydro-
guanosine-8-one by monomethoxytrityl chloride (MMACT) and tert-
butyldimethylsilyl chloride converted it to the 4-methoxytrityl
derivatives in 65% yield. For the synthesis of the 8-oxo-G-Me
building block, 2-N-acetyl-6-O-bis(dimethylcarbamoyl)-7,8-
dihydroguanosine-8-one was reacted with iodomethane and
sodium hydride in DMF, and then MMACT-1 was added.

The required phosphoramidites were obtained in quantitative
yield as a diastereomeric mixture, using a standard procedure
described elsewhere (Atkinson and Smith, 1984).

**Synthesis and identification of oligonucleotides**

Dodecaribonucleotides were synthesized using standard solid phase
phosphoramidite chemistry on an automated oligonucleotide
synthesizer using a slightly modified protocols and 4,5-dicyanoimidazole
as a coupling agent. The phosphoramidites were cleaved from the
solid support and deprotected using 3 : 1 (v/v) ammonia/ethanol at
55°C for 20 h. The mixture was then concentrated and dried in a
lyophilizer. The 2’ hydroxyl group was depolymerized by overnight
incubation with 1 M tetrabutylammonium fluoride for 48 h,
followed by n-butanol precipitation. The crude oligonucleotides
were purified by an OPC and 20% polyacrylamide/gel-
electrophoresis containing 7 M urea. Extracted oligonucleotides
from gel slices were desalted by reverse phase HPLC. Complementary
sequences of dodeca deoxyxynucleotides to the dodecaribonucleotides
were also prepared using standard solid phase phosphoramidite
chemistry. The purities of dodecaribonucleotides were identified by
20% denaturating polyacrylamide-7 M urea gel-electrophoresis and
by reverse phase HPLC. The nucleoside compositions of the
dodecaribonucleotides were analyzed by reverse phase HPLC after
combined treatment with snake venom phosphodiesterase and
alkaline phosphatase.

**Determination of the thermodynamic stabilities** ($T_m$) of the
DNA · RNA heteroduplex Oligonucleotides were dissolved in a
buffer containing 10 mM sodium cacodylate (pH 7.0), 100 mM
NaCl and 10 mM MgSO₄. The concentrations of oligonucleotides
were 1, 2, 2.8, 3.8 and 5 µM. Each sample was heated at 70°C for
10 min and then cooled gradually to 0°C. UV absorbance verses
temperature (melting curves) at 260 nm were measured using a
spectrophotometer equipped with a 6 cuvettes, a thermoelectric
controller (Shimazu, Tokyo, Japan), and 1 cm path-length quartz
cells (600 µL). To prevent water condensation at low temperatures,
dry nitrogen gas was purged into the sample compartment, and to
prevent solvent evaporation at high temperature, a layer of silicon
oil was placed on the surface the sample solutions. Temperature
was increased from 0°C to 60°C with a heating rate of 1°C /min.

**Determination of thermodynamic parameters** Van’t Hoff
transition enthalpies ($ΔH_T$), entropies ($ΔS_T$) and free energies
($ΔG_T$) were calculated from the slope of 1/$T_m$ verses ln(C/4) plots and by
using the following equations (Aboul-el et al., 1985; Koizume et
al., 1994).

$$\frac{1}{T_m} = \frac{(R/\Delta H)}{\ln(C/4)} + ΔS/ΔH$$
(Ct = total concentration of single strands)

\[ R/\Delta H = \text{slope} \]

\[ \Delta S/\Delta H = \text{intercept} \]

\[ \Delta G = \Delta H - T \Delta S \]

**Determination of CD spectrum of the DNA·RNA heteroduplexes**

CD spectra were obtained using a Jasco J710 spectrophotometer, a 0.1 cm quartz cell, and the same buffer conditions used for the thermal denaturating study. Samples were prepared using the same procedure used for the thermal stability study and the concentrations of oligonucleotides corresponding to G, 8-oxoG and 8-oxoG-Me were 4 \( \mu \)M, 6.2 \( \mu \)M and 3 \( \mu \)M, respectively. Each sample was heated at 70°C for 10 min and then cooled gradually to 5°C. Spectra were recorded at 200-320 nm at least 10 times using a buffer blank. All spectra were noise-reduced using the software supplied by Jasco, Inc., and molar ellipticities were calculated using the same software.

**Synthesis of cDNA by reverse transcriptases**

For the synthesis of cDNA, a solution of 20 \( \mu \)L containing commercially presented 5 \( \times \) reverse transcription buffer (1 M Tris-HCl pH 8.4, 720 mM KCl, 100 mM, MgCl\(_2\)), \( [\gamma-^{32}P]ATP \) (20000 cpm) 5 end-labeled, unlabelled 19 mer DNA primer (40 pmol) and 30 mer RNA template (40 pmol) was preheated at 90°C for 10 min, followed by slow cooling to room temperature. Primer extension was carried out in a volume of 40 \( \mu \)L containing annealed template and primer hybrid solutions, DTT (100 mM), and 2.5 \( \mu \)M of dATP, dGTP, dCTP, or TTP, or mixed dNTPs, Rnase inhibitor (20 units), and reverse transcriptases (20 units). After incubation at 39°C for 6 h, the reactions were extracted with phenol/chloroform, and then 10 \( \mu \)L of the aqueous aliquots with 10 \( \mu \)L loading buffer containing 9 M-urea, 5 mM Tris-borate (pH 8.3), 1 mM EDTA, 0.02% XC and 0.02% BPB were loaded onto 20% denaturating polyacrylamide gels. The gels were electrophoresed and exposed to X-ray film at –70°C for autoradiography.

**Results and Discussion**

**Synthesis of oligoribonucleotides containing 8-oxoG or 8-oxoG-Me**

In order to elucidate the base pairing properties of 8-oxoG, we prepared 30 base-long oligoribonucleotides (5'-CGAUACAGCUGUGGGUAAAXACUUUUACCU-3', X = G, 8-oxoG, or 8-oxoG-Me). Oligoribonucleotides containing 8-oxoG or 8-oxoG-Me were synthesized using the 8-oxo-7,8-dihydroguanosine or 8-oxo-7,8-dihydro-2’-O-methyl-guanosine phosphoramidite building blocks, respectively. PAGE and HPLC showed that the purities of oligoribonucleotides produced were over 98%. The presence of 8-oxoG or 8-oxoG-Me in oligonucleotides was analyzed by digesting oligoribonucleotides with a combination of phosphodiesterase I and alkaline phosphatase, and separating the component nucleosides by reverse phase HPLC (data not shown), as shown in a previous report. In order to determine the thermodynamic stabilities of base pairing of 8-oxoG or 8-oxoG-Me with dA, dG, dC or T, DNA·RNA heteroduplexes containing 8-oxoG and 8-oxoG-Me were determined. Hetero duplexes were designed as shown in Scheme 2. The sequences of the oligoribonucleotides produced were a part of 30-mer oligoribonucleotides used as templates for the cDNA synthesis using reverse transcriptases (Scheme 1). Each oligoribonucleotide contained G, 8-oxoG or 8-oxoG-Me at the center of its sequence.

**Incorporation of dNTPs opposite 8-oxoG or 8-oxoG-Me**

The investigation of the incorporation of dNTPs opposite 8-oxoG or 8-oxoG-Me during cDNA synthesis was carried out using reverse transcriptases (Scheme 1). 5'-End labeled 19 base DNA-primers were extended along the modified or unmodified RNA templates using reverse transcriptases, and the 20th bands of the inserted dNMPs to the 3-end primers were analyzed by denatured PAGE (Fig. 1). The amount of dNMP inserted was also calculated from the ratios of the radioactivities of PAGE bands (Fig. 2). In the control reaction, dCMP was preferentially inserted into the site opposite the unmodified guanosine by reverse transcriptases as was expected (Fig. 2). Surprisingly, TMP was also substantially inserted into the site opposite the unmodified guanosine by reverse transcriptases. When oligoribonucleotides containing 8-oxoG or 8-oxoG-Me were used as templates, dCMP was also preferentially incorporated into the site opposite 8-oxoG or 8-oxoG-Me as compared to other dNMPs. TMP was also substantially inserted into the site opposite 8-oxoG by RAV2-
However, when HIV-RT was used, the 8-oxoG residue did not direct the insertion of TMP. In the site opposite 8-oxoG-Me, TMP was substantially incorporated by both HIV-RT and RA V2-RT. Our previous report revealed that the 8-oxoG residue itself directed the insertion of TMP instead of dCMP by MMRV-RT, and when AMV-RT was used the 8-oxoG residue directed the insertion of the correct dCMP (Kim et al., 1999). Therefore, the amount of TMP insertion into the site opposite 8-oxoG or 8-oxoG-Me in cDNA synthesis was dependent on the reverse transcriptase involved. These base pairing properties of 8-oxoG with TMP by reverse transcriptase in cDNA synthesis closely parallel those of 8-oxodG by *Escherichia coli* DNA polymerase I during in vitro DNA synthesis (Kuchino et al., 1987).

### Thermodynamic analysis of the DNA - RNA heteroduplex

In order to determine the incorporation properties of 8-oxoG or 8-oxoG-Me with TMP or dCMP, we carried out a melting temperature experiment and analyzed the thermodynamic stabilities of DNA/RNA hetero-duplexes containing 8-oxoG or 8-oxoG-Me. 12 DNA·RNA hetero duplexes were constructed with RNA 5'-UUUCAXAAUUGA-3' and DNA 3'-d(AAAGTYTTAACT)-5', where X is G, 8-oxoG, or 8-oxoG-Me, and Y is dA, dG, dC, or T (Table 1). Tₘₛ were measured and thermodynamic parameters were calculated from Tₘ values using the methods described by Aboul-ela and Koizumi (Aboul-ela et al., 1985; Koizumi et al., 1994). Guanosine most stably formed base pairs with cytidine as we expected. Indeed, 8-oxoG formed base pairs with cytidine and thymidine with similar stabilities (Fig. 3, Fig. 4), and 8-OxoG-Me also formed base pairs with cytidine and thymidine with...
similar stabilities. When 8-oxoG or 8-oxoG-Me or guanosine formed base pairs with adenosine or guanosine, the base pairs remarkably destabilized the duplexes compared with duplexes containing cytidine or thymidine. The thermodynamic parameter ($\Delta G$) demonstrated that the formation of duplexes between 8-oxoG and 8-oxoG-Me and cytidine and thymidine is more thermodynamically favorable compared with other two bases. These results are coincident with the incorporation of bases opposite 8-oxoG and 8-oxoG-Me in cDNA synthesis by reverse transcriptases. We noted that differences between the melting temperatures and thermodynamic parameters ($\Delta G$) of 8-oxoG/dC and 8-oxoG/T were much smaller than between G/dC and G/T. Therefore, the amount of TMP insertion into the site opposite 8-oxoG or 8-oxoG-Me in cDNA synthesis was dependent on the reverse transcriptase used.

**CD spectra** CD spectra of DNA · RNA heteroduplexes with various base pairs of 8-oxoG and 8-oxoG-Me were measured. All the duplexes showed that the conformation of the DNA · RNA heteroduplex lies between the conformations of A-type RNA and B-type DNA (Wang and Keiderling, 1992; Cummins et al., 1995) (Fig. 5). The CD spectral patterns of duplexes containing G/dC, 8-oxoG/dC, and 8-oxoG-Me/dC pairing were similar. Between 250-280 nm, a small broad red shifted peak was observed in duplexes containing 8-oxoG/T or 8-oxoG-Me/T pairing, and a much broader and shift peak was observed in the spectrum of G/T.
pairing. On the other hand, the CD spectra of duplexes with corresponding dA or dG were similar to the spectra of 8-oxoG/dC or 8-oxoG-Me/dC (data not shown). These results suggest that both 8-oxoG and 8-oxoG-Me contribute to the hybrid or base stacking of duplexes when they pair with dC or T without disturbing the DNA · RNA duplex structure.

Several groups have proposed a conformation of 8-oxodG in DNA duplexes in which 8-oxoG exists in the keto form with an anti of syn conformation. Even though structures for 8-oxoG with T have been proposed (Gannett and Sura, 1993), the structures of base pairing of 8-oxoG and 8-oxoG-Me with cytidine and thymidine are not fully understood. Based on the conformation of the 8-oxoA (syn)/G/C triad, which was reported recently (Kim et al., 2002), we presume that the base pairing of 8-oxoG in the RNA/DNA duplex may adopt the same 8-oxoA (syn) /G/C triad conformation.

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