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DNA · RNA Heteroduplex Containing 8-Oxo-7,8-dihydroguanosine: Base Pairing, Structures, and Thermodynamic Stability

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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 (ΔG^o) 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 ΔG^o's of 8-oxoG/dC and 8-oxoG/T were much smaller than between G/dC and G/T. CD spectra showed that DNA · RNA 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

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-oxodG) 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 dG 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

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ribonucleosides were reported in Torula yeast RNA, isolated from a RNA bacteriophage and a plasmid (Yanagawa *et al*., 1992; Schneider *et al*., 1993; Rhee *et al*., 1995). 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, RNA base damage can contribute to an elevated mutation rate in DNA, and thus interfere with correct base pairing and comprising the fidelity of replication. The potential for mutagenesis exists as a result of base misincorporation opposite an oxidatively damaged template. Recent our studies have shown that 8-oxoA and 8-oxoA-Me form base pairs with dG as well T in cDNA synthesis by reverse transcriptases, and that 8-oxoG and 8-oxoG-Me can pair not with bases (dA or T) other than dC. The base-pairing properties of 8-oxoA and 8-oxoG were reported to be dependent on reverse transcriptases (Kim *et al*., 1998; Kim *et al*., 1999; Kim *et al*., 2002).

In this paper, in order to elucidate the base pairing properties of 8-oxoG, we prepared 30 base-long oligoribonucleotides (5-CGAUACAGCUGUGGUUAAXACUUUUACCU-3, X = G, 8-oxoG or 8-oxoG-Me). We studied the incorporation properties of DNA bases opposite 8-oxoG and 8-oxoG-Me *in vitro* cDNA synthesis using HIV-RT and RAV2-reverse transcriptases. The structures of the DNA · RNA duplexes containing 8-oxoG and 8-oxoG-Me were elucidated and thermodynamic parameters of base pairing of 8-oxoG and 8-oxoG-Me with dA, dG, dC and T were determined.

Materials and Methods

Materials An oligopurification cartridge (OPC) and other chemicals for oligonucleotide synthesis were purchased from Cruachem Co. (Glasgow, Scotland). Oligonucleotides were synthesized using an Applied Biosystems synthesizer (ABI 391 PCR-MATE). RAV2 reverse transcriptase and RNase inhibitor were purchased from Takara (Otsu, Japan) and HIV-RT and [γ-^{32}P] ATP (~3000 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Seoul, Korea). An Applied Biosystems HPLC system (Foster, USA) equipped with an absorbance detector (model 783A) and gradient pump system (model 400) was employed. UV absorbance was measured using a Shimadzu UV-VIS Spectrophotometer (UV-2401 PC) and circular dichroism spectra were measured using a Jasco J710 Spectrophotometer. Sequences of 30 bases of RNA templates and 19 bases of DNA primer as used in a study of DNA base incorporation in cDNA synthesis (Kim *et al*., 1998) were employed in reverse transcription assay using HIV-RT or RAV 2-RT.

Synthesis of 8-oxo-7, 8-dihydrogunosine, and 8-oxo-7, 8-dihydro-2'-O-methylguanosine building blocks The MMT-phosphoramidites of 8-oxoG and 8-oxoG-Me, required for dodecaribonucleotide synthesis, were synthesized using reported procedures (Kim *et al*., 1998). Briefly, the treatment of 8-bromoguanosine with sodium acetate in acetic acid afforded transient hydroxyl as well as amino protected 8-oxo-guanosine in 55% yield. Treatment of N,N-dimethylcarbamoyl chloride followed by the addition of KOtBu in MeOH to the protected 8-oxo-guanosine led to 2-N-acetyl-6-O,7-N-bis(dimethylcarbamoyl)-7,8-dihydro-guanosine-8-one in good yield. Consecutive treatment of 2-N-acetyl-6-O-7-N-bis(dimethylcarbamoyl)-7,8-dihydro-guanosine-8-one by monomethoxytrityl chloride (MMT-C1) and *tert*-butyldimethylsilyl chloride converted it to the 4-methoxytrityl derivatives in 65% yield. For the synthesis of the 8-oxoG-Me building block, 2-N-acetyl-6-O,7-N-bis(dimethylcarbamoyl)-7,8-dihydroguanosine-8- one was reacted with iodomethane and sodium hydride in DMF, and then MMT-C1 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 oligonucleotides 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 deprotected 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 dodecadeoxynucleotides 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 $MgSO_4$. 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 ml). 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^o), entropies (ΔS^o) and free energies (ΔG^o) were calculated from the slope of $1/T_m$ verses ln (C_t/4) plots and by using the following equations (Aboul-ela *et al*., 1985; Koizume *et al*., 1994).

$$1/T_m = (R/\Delta H^o)\ln(C_t/4) + \Delta S^o/\Delta H^o$$

(C_t = total concentration of single strands)
$R/\Delta H^o$ = slope
$\Delta S^o/\Delta H^o$ = intercept
$\Delta G^o = \Delta H^o - T\,\Delta S^o$

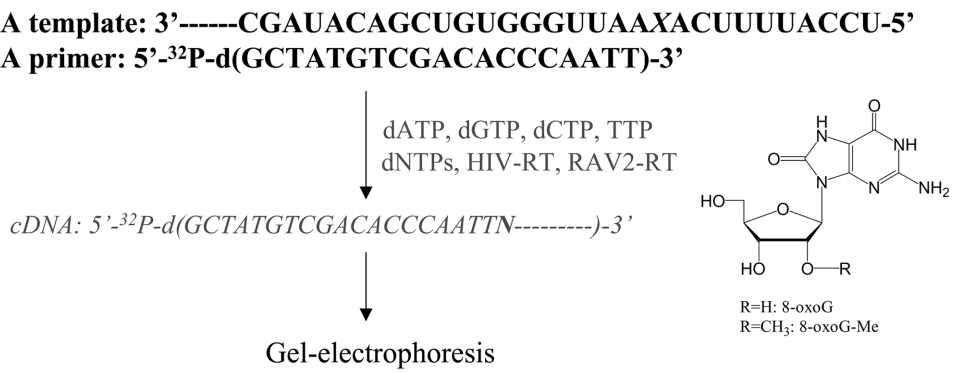
Determination of CD spectrum of the DNA · RNA heteroduplexes CD spectra were obtained using a Jasco J710 spectrophotometer, a 0.1 cm quartz cell, and the same buffer conditions used for the thermal denaturating study. Samples were prepared using the same procedure used for the thermal stability study and the concentrations of oligonucleotides corresponding to G, 8-oxoG and 8-oxoG-Me were 4 μM, 6.2 μM and 3 μM, 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 μL containing commercially presented 5 × reverse transcription buffer (1 M Tris-HCl pH 8.4, 720 mM KCl, 100 mM, $MgCl_2$), [γ-^{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 μl containing annealed template and primer hybrid solutions, DTT (100 mM), and 2.5 μM 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 μl of the aqueous aliquots with 10 μl 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-CGAUACAGCUGUGGUUAAXACUUUUACCU-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 oligonucleotides 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 denaturated 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-



Scheme 1. Synthesis of cDNA by reverse transcriptases where, X = G, 8-oxoG, or 8-oxoG-Me. The structures of 8-oxoG and 8-oxoG-Me are shown on the right.

RNA 30mer : 5'-UCCAUUUUCA*X*AAUUGG
GUGUCGACAUAGG-3'

↓

RNA 12mer : 5'--- UUUCA*X*AAUUGA-3'
DNA 12mer : 3'-d(AAAGT*Y*TTAACT)-5'

***X* = G, 8-oxoG, 8-oxoG-Me, *Y* = dA, dG, dC, T**

Scheme 2. Sequences of dodecaribonucleotides and their complementary dodecadeoxy-nucleotides.

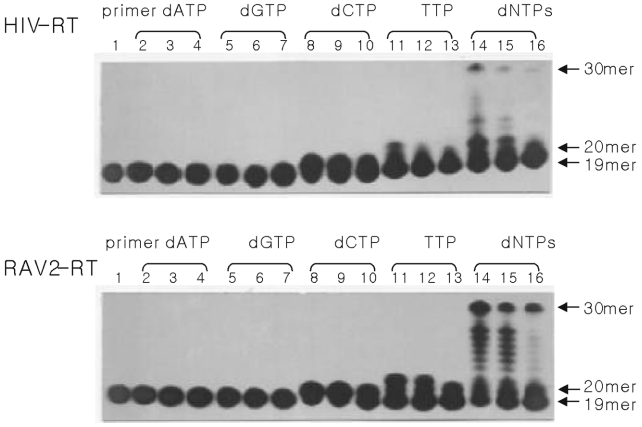


Fig. 1. Autoradiography of polyacrylamide gels shows the incorporations of dNTPs into the primer at sites opposite X. The normal template (X = G, lanes 2, 5, 8, 11, 14), the modified templates (X = 8-oxoG, lanes 3, 6, 9, 12, 15; X = 8-oxoG-Me, lanes 4, 7, 10, 13, 16) and the primer (lane 1) were incubated with HIV-RT, or RAV2-RT in the presence of dATP (lane 2-4), dGTP (lane 5-7), dCTP (lane 8-10), TTP (lane 11-13) or dNTPs (lane 14-16). 19Mers indicate unextended primer and 20 and 30mers extended primers.

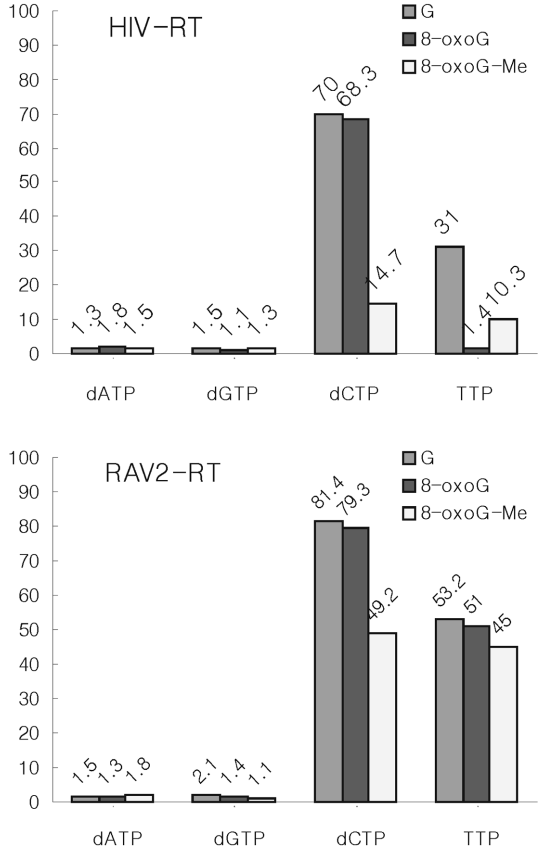


Fig. 2. Ratios of the incorporation of dNTPs into primer by HIV-RT (*left*) or RAV2-RT (*right*) from the data shown in Fig. 1. Ratios (%) were defined as = (radioactivity of newly generated spots)/(radio activity of the remaining primer + radioactivity of newly generated spots) ×100.

RT. 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 RAV2-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'-UUUCA*X*AAUUGA-3' and DNA 3'-d(AAAGT*Y*TTAACT)-5', where *X* is G, 8-oxoG, or 8-oxoG-Me, and *Y* is dA, dG, dC, or T (Table 1). T_ms were measured and thermodynamic parameters were calculated from T_m 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

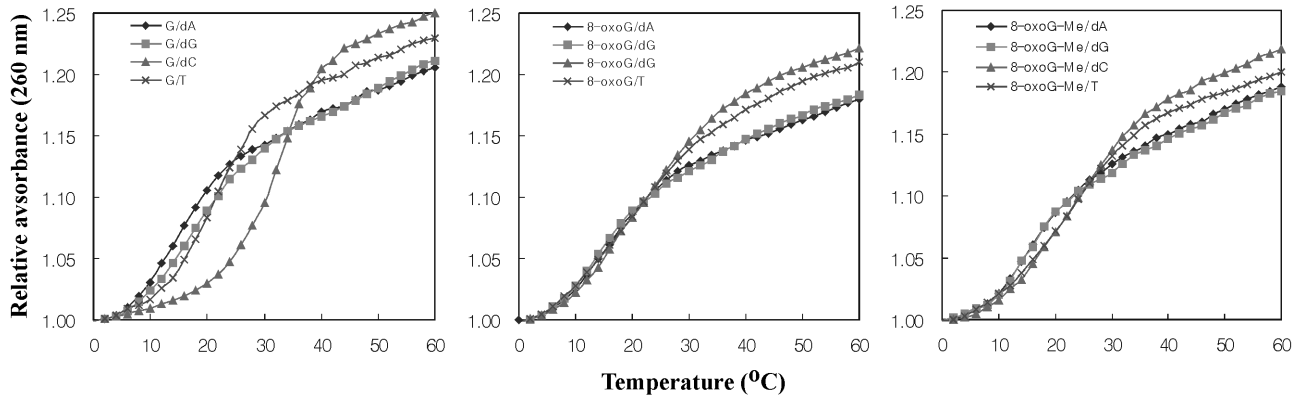


Fig. 3. Melting (T_m) curves DNA · RNA heteroduplex containing X = G (a), 8-oxoG (b), 8-oxoG-Me(c), Y = dA (◆), dG (■), dC (▲), and T (×). The DNA and RNA sequences are noted in scheme 2.

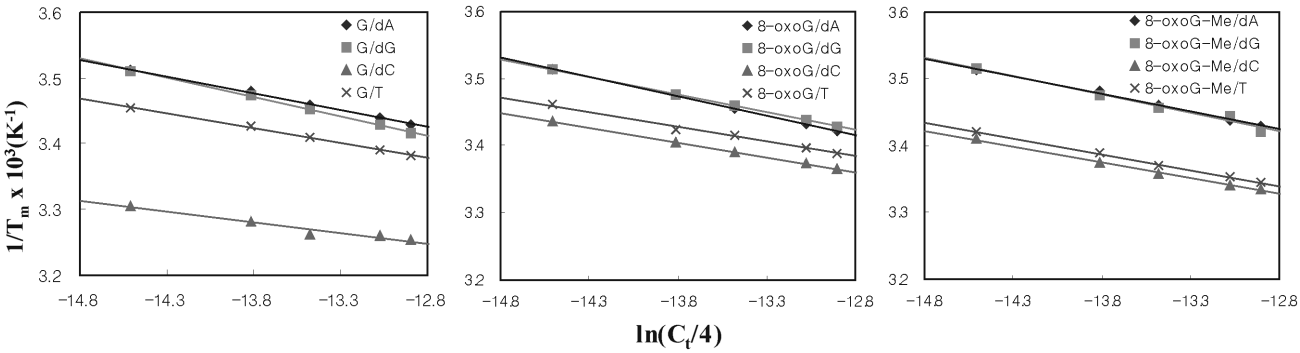


Fig. 4. Plot of the values of $1/T_m$ versus $\ln(C_t/4)$ for RNA · DNA heteroduplexes containing X = G (a), 8-oxoG (b), 8-oxoG-Me (c), Y = dA (◆), dG (■), dC (▲), and T (×). The DNA and RNA sequences are noted in scheme 2.

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 (ΔG^o) 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 (ΔG^o) 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 hetero duplexes 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 pick was observed in the spectrum of G/T

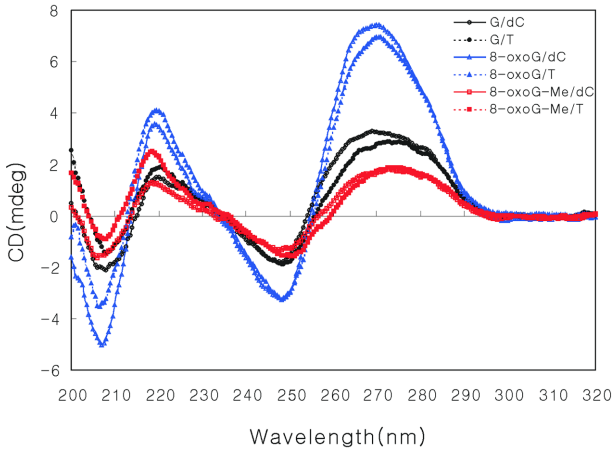


Fig. 5. CD spectra of DNA · RNA heteroduplexes, where G/dC: (○), G/T: (●), 8-oxoG/dC: (△), 8-oxoG/T: (▲), 8-oxoG-Me/dC: (□), and 8-oxoG-Me/T: (■). The DNA and RNA sequences are noted in scheme 2.

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-oxodG 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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