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Recognition of Nucleotide Analogs Containing the 7,8-Dihydro-8-oxo Structure by the Human MTH1 Protein

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The MTH1 protein catalyzes hydrolysis of oxidatively damaged purine nucleotides including 8-hydroxy-dGTP to the monophosphates. The MTH1 protein seems to act as an important defense system against mutagenesis, carcinogenesis, and cell death induced by oxidized purine nucleotides. We previously reported that the functional groups at the 2- and 6-positions of the purine ring affect the recognition by the human MTH1 protein. 8-Hydroxy-dGTP and 8-hydroxy-dATP are substrates of MTH1, and both have the "7,8-dihydro-8-oxo structure." In this study, three nucleotide analogs containing this motif were examined. A synthetic purine analog containing the 7,8-dihydro-8-oxo structure and the 2-amino function (dJTP) was hydrolyzed to the monophosphate with high efficiency by MTH1. On the other hand, two analogs that lack the two-ring system of their bases [formamidopyrimidine-dGTP (FAPY-dGTP) and 2-OH-dYTP] were poor substrates. FAPY-dGTP is a mixture of conformers and was hydrolyzed more than ten-fold less efficiently than 8-hydroxy-dGTP. These results clarify the effects of the 2-amino group and the two-ring system of the purine base on the recognition by the human MTH1 protein.

Key words: FAPY-dGTP, MTH1, nucleotide analog, oxidized nucleotide, substrate recognition.

Abbreviations: 8-OH-dGTP, 7,8-dihydro-8-oxo-2′-deoxyguanosine 5′-triphosphate; 8-OH-dATP, 7,8-dihydro-8-oxo-2′-deoxyadenosine 5′-triphosphate; 8-OH-dITP, 7,8-dihydro-8-oxo-2′-deoxyinosine 5′-triphosphate; 2-OH-dATP, 2-hydroxy-2′-deoxyadenosine 5′-triphosphate; dJTP, 2-amino-9-(2-deoxy-β-D-ribofuranosyl)-7,8-dihydro-8-oxopurine 5′-triphosphate; 2-OH-dYTP, 1-(2-deoxy-β-D-ribofuranosyl)-2,3-dihydro-2-oxo-imidazole-4-carboxamide 5′-triphosphate; FAPY-dGTP, *N*6-(2-deoxy-α,β-D-ribofuranosyl)-2,6-diamino-5-formamido-4-hydroxypyrimidine 5′-triphosphate; FAPY-Gua, 2,6-diamino-5-formamido-4-hydroxypyrimidine; hMTH1, human MTH1; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DPC, diphenylcarbamoyl; DMF, *N*,*N*-dimethylformamide.

Increasing evidence suggests that oxidative damage is associated with tumors and several neurodegenerative diseases, as well as normal aging (*1–3*). Oxidative damage is formed in the nucleotide pool and in DNA (*4*). The MTH1 protein, a mammalian homologue of *Escherichia coli* MutT, catalyzes hydrolysis of 8-OH-dGTP in the nucleotide pools to the monophosphate (*5*, *6*). A deficiency in this protein resulted in enhanced tumor formation in mice (*7*). The expression of human MTH1 suppressed H_2O_2-induced mitochondrial dysfunction and cell death in MTH1-null fibroblasts (*8*). Thus, the MTH1 protein seems to act as an important defense system against mutagenesis, carcinogenesis, and cell death induced by oxidatively damaged nucleotides.

The hMTH1 protein catalyzes hydrolysis of 2-OH-dATP and 8-OH-dATP, as well as 8-OH-dGTP (*6*). The substrate specificity of the hMTH1 protein is of great interest, not only because of its importance in the elimination of mutagenic deoxyribonucleotides, but also because these three substrates seem to lack a common hydrogen-bond accepting/donating group in their nucleobase moieties. An examination of various nucleotide analogs to address the substrate recognition mechanism of the hMTH1 protein revealed that the functional groups at the 2- and 6-positions of the purine ring affect the recognition by the human MTH1 protein (*9*).

In this study, we focused on the 7,8-dihydro-8-oxo structure within the base moieties of 8-OH-dGTP and 8-OH-dATP. Three nucleotide analogs, including FAPY-dGTP, an oxidized form of dGTP, were synthesized and incubated with the hMTH1 protein (Fig. 1). We found that an analog with a purine base containing the 7,8-dihydro-8-oxo structure and the 2-amino function (dJTP) was hydrolyzed efficiently, while the analogs lacking the two-ring system (FAPY-dGTP and 2-OH-dYTP) were poor substrates. FAPY-dGTP was weakly hydrolyzed by the MTH1 protein. These data help to clarify the substrate recognition mode of the hMTH1 protein.

MATERIALS AND METHODS

Chemicals and Instruments—Solvents were spectroscopic or HPLC grade. Reagents were purchased from

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Sigma-Aldrich (St. Louis, Missouri, USA) or Fluka (Buchs, Switzerland) and used without purification. NMR Spectra were recorded on a Bruker Avance 400 spectrometer (Bruker BioSpin, Billerica, Massachusetts) or by the Center for Instrumental Analysis, Hokkaido University. Chemical shifts are given in ppm (δ) relative to the residual solvent peak for ^{1}H and ^{13}C and to phosphoric acid as external reference for ^{31}P, coupling constants (*J*) are reported in Hertz, and the normal abbreviations are used. Mass spectra were recorded by the mass spectroscopy laboratory (CNRS-ICSN, Gif-sur-Yvette) or by the Center for Instrumental Analysis, Hokkaido University. Thin layer chromatography (TLC) was performed using silica gel plates (Kieselgel 60 F_{254}/0.2 mm thickness, Merck KGaA, Darmstadt, Germany). Spots were visualized by UV light, and then were revealed by spraying with sulfuric acid-anisaldehyde followed by heating (*10*). Preparative HPLC was carried out on a Perkin Elmer system (Series 200, Wellesley, Massachusetts, USA) with a C18 reverse phase column (Kromasil, 5μ 100 Å, 250 × 10 mm, AIT, France), using a flow rate of 5.5 ml/min and a linear gradient of CH_3CN (gradient is shown in each experimental part) in 10 mM triethylammonium acetate buffer at pH 7.5 over 20 min, or on an Agilent Technologies system (Series 1100, Palo Alto, California, USA) with an anion-exchange column (TSK-gel DEAE-2SW, 250 × 4.6 mm, Tosoh, Tokyo, Japan), using a flow rate of 1 ml/min and a linear gradient of LiCl (100 to 400 mM) in H_2O over 20 min. Eluted products were visualized using a diode array detector.

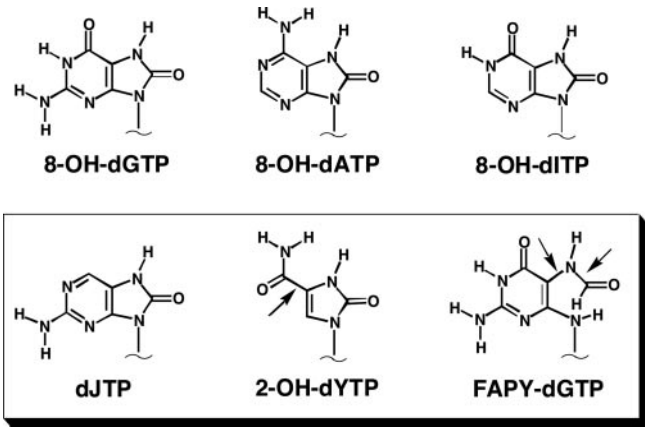
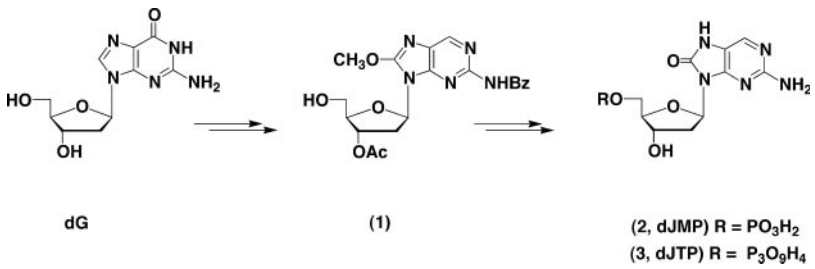


Fig. 1. **Structures of the base moieties of the nucleotide analogs.** 8-OH-dGTP and 8-OH-dATP are natural oxidized forms of dGTP and dATP, and 8-OH-dITP is a synthetic nucleotide analog that is recognized weakly by the hMTH1 protein (*9*). dJTP, 2-OH-dYTP, and FAPY-dGTP are the nucleotide analogs used in this study. Arrows indicate bonds with possible rotations (except the *N*-glycosidic bond).

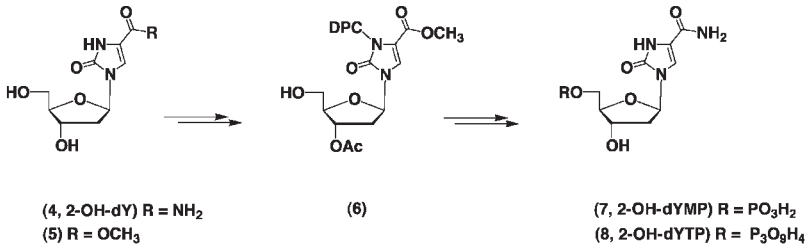
*2-Amino-9-(2-deoxy-β-D-ribofuranosyl)-7,8-dihydro-8-oxopurine 5′-phosphate (dJMP, **2**)*—To a solution of compound **1** (*11*) (Scheme 1) (0.18 g, 0.42 mmol) in anhydrous pyridine (4 ml) were added a 1 M solution of 2-cyanoethyl phosphate in pyridine (0.84 ml) and DCC (0.52 g, 2.53 mmol). After stirring for 2 days at room temperature, water (2 ml) was added to the mixture. One hour later, the insoluble material was filtered off and washed with water. The combined filtrates were evaporated. To the residue in DMF (5 ml) were added triethylamine (0.6 ml) and thiophenol (0.2 ml), and the mixture was heated at 50°C for 18 h. After evaporation to dryness, the residue was triturated in ethyl ether, and the resulting white powder was treated with saturated ammonia in methanol at 50°C for 5 days. After evaporation, the residue was purified on a C18 column (10 mM TEAA/methanol) to give the monophosphate **2** (55 mg as triethylammonium salt, 32%). ^{1}H NMR (DMSO-d_6) δ: 1.11 (t, 9H, CH_3), 2.02 (m, 1H, H2′), 2.88 (q, 6H, CH_2), 3.00 (m, 1H, H2″), 3.65 (m, 2H, H5′), 3.83 (m, 1H, H4′), 4.10 (m, 1H, H5″), 4.55 (m, 1H, H3′), 6.07 (t, J = 7.04 Hz, 1H, H1′), 6.35 (bs, 1H, OH), 7.74 (s, 1H, H6). 31P NMR (DMSO-d_6) δ: 2.81.

*2-Amino-9-(2-deoxy-β-D-ribofuranosyl)-7,8-dihydro-8-oxopurine 5′-triphosphate (dJTP, **3**)*—A solution of DCC (0.20 g, 0.97 mmol) in *t*-butanol (3.5 ml) was added dropwise over 2 h to a refluxing solution of compound **2**, as an ammonium salt (90 mg, 0.23 mmol), in a 1/1 mixture of *t*-butanol/water (4.8 ml) and morpholine (0.08 ml, 1.0 mmol). After 5 h of refluxing, water was added to the reaction mixture. The insoluble material was filtered off, and rinsed with water/*t*-butanol, and the combined filtrates were evaporated *in vacuo*. Purification on a C18 column (H_2O/CH_3CN) afforded the morpholidate derivative (110 mg as the dicyclohexylurea salt). ^{1}H NMR (D_2O) δ: 2.40 (m, 1H, H2′), 3.25 (m, 1H, H2″), 2.95 (t, 4H, CH_2-N), 3.50 (t, 4H, CH_2-O), 3.95 (m 1H, H5′), 3.77 (m, 2H, H4′ and H5″), 4.85 (m, 1H, H3′), 6.25 (t, 1H, H1′, J = 6.8 Hz), 7.90 (s, 1H, H6). ^{31}P NMR (D_2O) δ: 8.26.

The morpholidate was dried by coevaporations with pyridine, and then was added to a solution of pyrophosphoric acid (0.41 mmol) in DMF (2 ml) containing tri-*n*-butylamine (0.39 ml, 1.37 mmol). After stirring for 2 days, the mixture was loaded onto a DEAE Sephadex A-25 column (gradient from 10 to 800 mM ammonium bicarbonate, 800 ml). The appropriate fractions were lyophilized, and **3** was isolated as sodium salt after ion exchange chromatography (22 mg, 20% yield from **2**).



Scheme 1. **Key synthetic intermediates for the preparation of dJTP (3).**



Scheme 2. **Key synthetic intermediates for the preparation of 2-OH-dYTP (8).**

^{1}H NMR (D_2O) δ: 2.30 (m, 1H, H2′), 3.25 (m, 1H, H2″), 4.20 (m, 2H, H5′ and H5″), 4.30 (m, 1H, H4′), 4.70 (m, 1H, H3′), 6.25 (t, 1H, H1′, J = 6.9 Hz), 7.85 (s, 1H, H6). ^{13}C NMR (D_2O) δ: 35.42 (C2′), 66.20 (d, J = 5.3 Hz, C5′), 71.83 (C3′), 82.56 (C1′), 86.04 (d, J = 8.3 Hz, C4′), 126.54.72 (C6). ^{31}P NMR (D_2O) δ: –22.41 (t, J = 19.4 Hz), –10.53 (d, J = 19.5 Hz), –10.11 (d, J = 19.3 Hz). ES-MS, negative mode, calculated for $C_{10}H_{16}N_5O_{13}P_3$, 507; found, 528 $(M–2H+Na)^–$, 506 $(M–H)^–$.

*1-(2-Deoxy-*β*-D-ribofuranosyl)-2,3-dihydro-2-oxo-imidazole -4-carboxamide 5′-phosphate (2-OH-dYMP,* ***7****)*—To a solution of compound **6** (*12*) (0.63 g, 1.27 mmol) (Scheme 2) in anhydrous pyridine (12 ml) were added a 1 M solution of 2-cyanoethyl phosphate in pyridine (2.54 ml) and DCC (1.57 g, 7.63 mmol). After stirring for 3 days at room temperature, water (2 ml) was added to the mixture. One hour later, the insoluble material was filtered off and washed with water. The combined filtrates were evaporated. The residue was dissolved in methanol (2 ml) and was treated with 33% aqueous ammonia (13 ml). After stirring for 8 h at 37°C, the mixture was evaporated to dryness and the residue was purified by C18 reverse phase HPLC (5–25% gradient of CH_3CN in 20 mM TEAA buffer in 20 min). Compound **7** was isolated as an ammonium salt (105 mg, 26%) after cationic exchange chromatography (Dowex NH_4^+ column). Rf (isopropanol/NH_4OH/H_2O: 7:1:2): 0.17. ^{1}H NMR DMSO-d_6) δ: 1.96 (ddd, J = 2.4 Hz, J = 6.2 Hz, J = 13.1 Hz, 1H, H2′), 2.16 (ddd, J = 5.5 Hz, J = 7.6 Hz, J = 13.1 Hz, 1H, H2″), 3.56 (m, 2H, H5′), 3.70–3.75 (m, 2H, H4′ and H5″), 4.27 (m, 1H, H3′), 5.80 (t, J = 7.3 Hz, 1H, H1′), 6.70 (bs, 1H, NH_2), 7.93 (s, 1H, H5), 8.40 (bs, 1H, NH_2). ^{13}C NMR (DMSO-d_6) δ: 39.76 (C2′), 64.85 (d, J = 6.0 Hz, C5′), 72.38 (C3′), 82.71 (C1′), 86.79 (d, J = 8.2 Hz, C4′), 114.26 (C5), 117.41 (C4), 153.17 (C2), 161.65 ($CONH_2$). ^{31}P NMR (DMSO-d_6) δ: 0.07. HRMS (MALDI-TOF), positive mode, calculated for $C_9H_{15}N_3O_8P$, 324.0584; found, 324.0596 $(M+H)^+$, calculated for $C_9H_{14}N_3O_8PNa$ 346.0410; found 346.0416 $(M+Na)^+$.

*1-(2-Deoxy-*β*-D-ribofuranosyl)-2,3-dihydro-2-oxo-imidazole-4-carboxamide 5′-triphosphate (2-OH-dYTP,* ***8****)*—A solution of DCC (0.23 g, 1.12 mmol) in *t*-butanol (4.2 ml) was added dropwise over 6 h to a refluxing solution of compound **7** (90 mg, 0.28 mmol) in 5.6 ml of *t*-butanol/water (1:1) and morpholine (0.1 ml, 1.12 mmol). After 18 h of refluxing, the reaction was incomplete and the same quantities of DCC and morpholine were added. After another 18 h of refluxing, water was added to the reaction mixture. The insoluble material was filtered off and rinsed with water/*t*-butanol, and the combined filtrates were evaporated *in vacuo*. Purification on a C18 column (H_2O/methanol) followed by reverse phase HPLC (0–20% gradient of CH_3CN in 20 mM TEAA buffer in 20 min.) afforded the morpholidate derivative. ^{1}H NMR (D_2O) δ: 2.24 (m, 1H, H2′), 2.32 (m, 1H, H2″), 2.82 (t, 4H, CH_2N), 3.45 (t, 4H, CH_2O), 3.77 (m, 2H, H5′ and H5″), 3.97 (m, 1H, H4′), 4.45 (m, 1H, H3′), 5.89 (t, 1H, H1′, J = 6.8 Hz), 7.44 (s, 1H, H5). ^{13}C NMR (D_2O) δ: 39.09 (C2′), 45.03 (CH_2-N), 64.76 (d, J = 5.7 Hz, C5′), 67.31 (d, J = 7.3 Hz, CH_2-O), 71.71 (C3′), 83.14 (C1'), 85.90 (d, J = 8.8 Hz, C4′), 114.65 (C5), 117.36 (C4), 153.61 (C2), 163.11 ($CONH_2$). ^{31}P NMR (D_2O) δ: 8.65.

The morpholidate (32 mg as ammonium salt, 0.08 mmol) was dried by coevaporations with pyridine, and was added to a solution of pyrophosphoric acid (0.41 mmol) in DMF (1 ml) containing tri-*n*-butylamine (0.39 ml, 1.37 mmol). After stirring for 2 days, the mixture was diluted in water, lyophilized and purified by reverse phase HPLC (0–20% gradient of CH_3CN in 20 mM TEAA buffer in 20 min.). The appropriate fractions were lyophilized, and compound **8** was isolated as a sodium salt (12 mg, 32%). R*t* (0–10% gradient of CH_3CN in 20 mM TEAA buffer in 20 min): 11.1 min. UV (H_2O/pH 7) λ 260 nm (ε 8720). ^{1}H NMR (D_2O) δ: 2.20 (m, 1H, H2′), 2.31 (m, 1H, H2″), 3.96 (m, 1H, H5′), 3.98–4.06 (m, 2H, H4′ and H5″), 4.57 (m, 1H, H3′), 5.91 (t, 1H, H1′, J = 6.9 Hz), 7.56 (s, 1H, H5). ^{13}C NMR (D_2O) δ: 39.23 (C2′), 65.77 (d, J = 5.8 Hz, C5′), 71.57 (C3′), 83.08 (C1′), 85.82 (d, J = 9.3 Hz, C4′), 114.72 (C5), 117.39 (C4), 153.53 (C2), 163.23 ($CONH_2$). ^{31}P NMR (D_2O) δ: –21.94 (t, J = 20.1 Hz), –10.12 (d, J = 20.1 Hz), –8.92 (d, J = 20.2 Hz). HRMS (MALDI-TOF), positive mode, calculated for $C_9H_{17}N_3O_{14}P_3$, 483.9923; found, 483.9910 $(M+H)^+$, calculated for $C_9H_{16}N_3O_{14}P_3Na$, 505.9742; found, 505.9745 $(M+Na)^+$.

*N6-(2-deoxy-*α,β*-D-ribofuranosyl)-2,6-diamino-5-formamido-4-hydroxypyrimidine 5′-triphosphate (FAPY-dGTP)*—FAPY-dGTP was prepared by the oxidation of dGTP in the presence of 2-mercaptoethanol, as reported for the ribonucleotide (*13*). Purified FAPY-dGTP was quantitated by the ε_{273} value (14.3×10^3 M^{-1} cm^{-1}) reported for the ribonucleotide (*14*). ^{1}H NMR (D_2O) δ: 8.24 and 8.10 (each s, 5:1H, 1-CHO), 6.10 and 5.93 (each m, 5:1H, H1′). ^{31}P NMR (D_2O) δ: –18.40 (m), –7.62 (m), –3.41 (m). ES-MS, negative mode, calculated for $C_{10}H_{18}N_5O_{14}P_3$, 525: found, 524 $(M–H)^–$, 530 $(M–2H+Li)^–$.

MTH1 Assay—The hMTH1 protein was obtained by the *E. coli* overexpression system as described (*15*). The hMTH1 activities were assayed in a reaction mixture (100 μl) containing 20 mM Tris-HCl, pH 8.0, 4 mM $MgCl_2$, 40 mM NaCl, 80 μg/ml bovine serum albumin, 8 mM dithiothreitol, 10% glycerol, and the nucleotide substrates. Following a preincubation at 30°C for 5 min, the mixtures were incubated at 30°C with the hMTH1 protein

(final concentration, 1 nM). Reactions were terminated by the addition of 100 μl of ice-cold 5 mM EDTA. All samples were fractionated by a TSK-gel DEAE-2SW column (Tosoh, Tokyo, Japan), with isocratic elution by 75 mM sodium phosphate buffer, pH 7.0, with or without 20% acetonitrile, at a flow rate of 1 ml/min. The amounts of the nucleoside triphosphates and their hydrolyzed products were quantitated by measuring the area of UV absorbance. Lineweaver-Burk plots were drawn from the initial velocity of deoxyribonucleoside triphosphatase for the hMTH1 protein. The K_m and k_{cat} values were derived from the intercepts of regression lines.

RESULTS

Nucleotide Analogs—The hMTH1 protein catalyzes hydrolysis of 8-OH-dATP and 2-OH-dATP, as well as 8-OH-dGTP to the monophosphates (*6*). Moreover, their ribonucleotide derivatives are also its substrates (*16*). Previously, the substrate specificity of the hMTH1 protein was examined with several nucleotide analogs, and the contribution of a hydrogen-bond donating group at either the 2- or 6-position of purine bases was suggested (*9*). In the current study, three nucleotide analogs containing the 7,8-dihydro-8-oxo structure were used as possible hMTH1 substrates (Fig. 1).

The first analog, 2-amino-9-(2-deoxy-β-D-ribofuranosyl)-7,8-dihydro-8-oxo-purine 5′-triphosphate (dJTP), is a purine nucleotide analog with the 7,8-dihydro-8-oxo structure and an amino group, a hydrogen-bond donating group, at the 2-position. It corresponds to an analog of 8-OH-dGTP lacking the 6-oxo group and the *N*1-proton. The second analog, 1-(2-deoxy-β-D-ribofuranosyl)-2,3-dihydro-2-oxo-imidazole-4-carboxamide 5′-triphosphate (2-OH-dYTP), is an unnatural nucleotide with a base lacking the six-membered pyrimidine ring. The imidazole ring has a carboxamide group, and the NH_2 group may be located at the "6-position." The other analog, FAPY-dGTP, contains the formamide group. This deoxyribonucleotide has the FAPY-Gua base, which is formed by guanine oxidation. This base has the 7,8-dihydro-8-oxo structure, and the five-membered imidazole ring is opened. In addition to determining the substrate recognition mechanism, it is quite interesting to examine whether the hMTH1 protein catalyzes hydrolysis of this oxidized nucleotide, from the viewpoint of nucleotide pool sanitization.

*Syntheses of dJTP (**3**) and 2-OH-dYTP (**8**)*—The synthesis of 2-amino-9-(2-deoxy-β-D-ribofuranosyl)-7,8-dihydro-8-oxo-purine (dJ) from 2′-deoxyguanosine (dG) was previously reported (*11*, *17*). The corresponding 5′-triphosphate derivative (dJTP) was synthesized starting from the precursor **1** (Scheme 1), where the 2-amino function was protected with a benzoyl group and the 8-oxo was masked as a methoxy group (Scheme 1). 5′-Phosphorylation of **1**, followed by thiophenol/triethylamine treatment and then ammonia treatment, afforded the monophosphate **2** in 32% overall yield. The monophosphate **2** was converted into the triphosphate **3** in 20% yield. The chemical structure and the purity of **3** were confirmed by NMR and HRMS analyses.

The nucleoside 2-OH-dY (**4**, Scheme 2) was synthesized from 5-bromo-2′-deoxyuridine as previously reported (*12*). Due to the reactivity of the carboxamide function toward phosphorylating agents, the corresponding 5′-triphosphate derivative (2-OH-dYTP) was synthesized starting from the convertible methyl ester derivative **5** (Scheme 2). Prior to the phosphorylation step, for selectivity purposes we introduced a DPC group at the *N*3-position and an acetyl group at the 3′-position. 5′-Phosphorylation of the fully protected nucleoside **6**, followed by ammonia treatment of the resulting phosphodiester, allowed the complete removal of the protecting groups (DPC, Ac) and the conversion of the methyl ester into the amide. The resulting monophosphate derivative **7** was isolated in 26% overall yield after reverse phase HPLC purification. The monophosphate **7** was converted into the triphosphate **5**, which was isolated in 32% yield after reverse phase HPLC purification. The chemical structure and the purity of **8** were confirmed by NMR and HRMS analyses.

Synthesis of FAPY-dGTP—FAPY-dGTP was prepared by direct oxidation of dGTP in the presence of a reducing agent, as reported for the corresponding ribonucleotide (*13*). FAPY-dGTP was unstable, and ~10% of impurities were detected when the isolated FAPY-dGTP fraction was analyzed by anion exchange HPLC (data not shown). FAPY-dGTP would be a mixture of the α- and β-anomers (*18*, *19*). The FAPY-Gua base might be a mixture of four conformers, due to rotation around the C5-N7 and N7-C8 bonds (Fig. 1) (*19*). Due to the impurities and conformers, complete identification of the NMR signals was difficult. However, the existence of FAPY-dGTP in the isolated fraction was proved by its UV spectrum (λ_{max} 273 nm), ES-MS, and ^{1}H-NMR signals that indicate the 1-CHO (8.24 and 8.10 ppm). FAPY-dGTP was quantitated by the ε_{273} value (14.3×10^3 M^{-1} cm^{-1}) reported for the ribonucleotide (*14*).

Hydrolysis of Nucleotide Analogs by hMTH1—First, dJTP (10 μM) was incubated with the hMTH1 protein (1 nM) at 30°C for 5 min at pH 8.0. As shown in Fig. 2, this nucleotide was hydrolyzed to the monophosphate by the hMTH1 protein with higher efficiency than 8-OH-dGTP. Nearly 40% of dJTP was hydrolyzed when ~20% of 8-OH-dGTP was consumed. Under our experimental conditions, hydrolysis of dJTP was catalyzed by hMTH1 linearly with time up to 5 min (Fig. 3). The dJTP nucleotide is an analog of 8-OH-dGTP lacking the 6-oxo group and the *N*1-proton (Fig. 1). This result suggests that the 6-oxo group and/or the *N*1-proton of 8-OH-dGTP may cause a slightly unfavorable interaction with the protein.

On the other hand, hydrolysis of the nucleotide analog 2-OH-dYTP by the hMTH1 protein was below estimated detection limit of the analysis (~0.2%, data not shown). Moreover, addition of 2-OH-dYTP did not inhibit the hydrolysis of 8-OH-dGTP by the hMTH1 protein, suggesting weak, if any, binding to the protein. The loss of the six-membered ring reduces the affinity to the hMTH1 protein.

Next, FAPY-dGTP, one of the oxidized forms of dGTP, was incubated with the hMTH1 protein. As shown in Fig. 4, very slight (~1%) hydrolysis of FAPY-dGTP was observed when 10 μM FAPY-dGTP and 1 nM hMTH1 were used. The product was identified as the monophosphate derivative based on its chromatographic behavior and that of the sample prepared by partial dephosphorylation of FAPY-dGTP with bacterial alkaline phosphatase, and its amount increased depending on incubation time with hMTH1 (data not shown). Thus, the hMTH1 protein

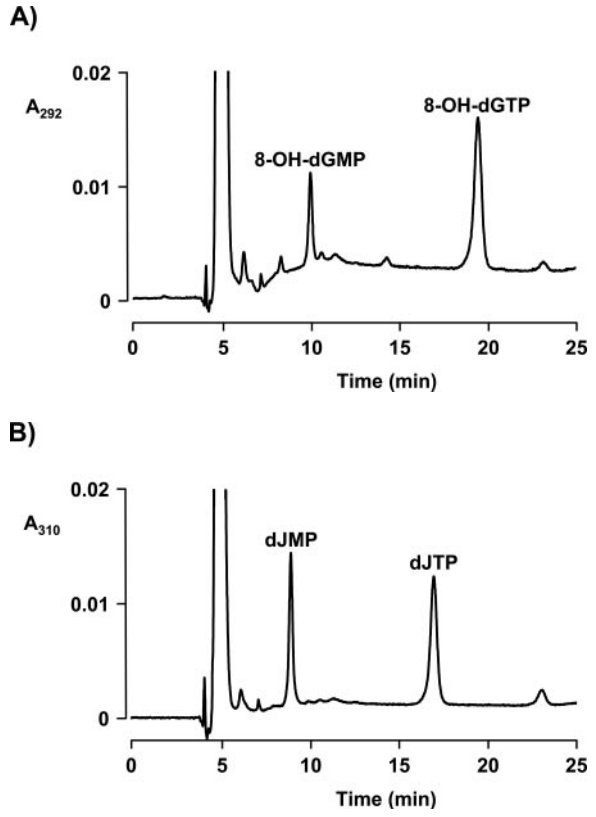


Fig. 2. **Hydrolysis of nucleotide analogs by the hMTH1 protein, monitored by anion exchange HPLC.** Each nucleotide (10 μM) was incubated with 1 nM of the hMTH1 protein at 30°C for 5 min. (A) 8-OH-dGTP, (B) dJTP. The elution solution was 20% acetonitrile, 75 mM sodium phosphate buffer, pH 7.0 (isocratic).

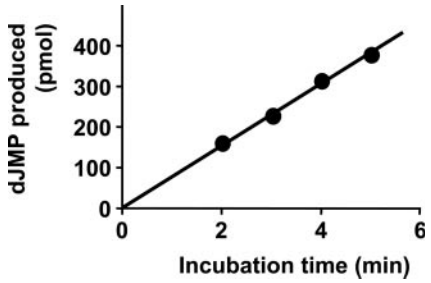


Fig. 3. **Time course of hydrolysis of dJTP by the hMTH1 protein.** dJTP (10 μM) was incubated with 1 nM of the hMTH1 protein at 30°C. Reactions were terminated at the time points indicated and analyzed by anion exchange HPLC.

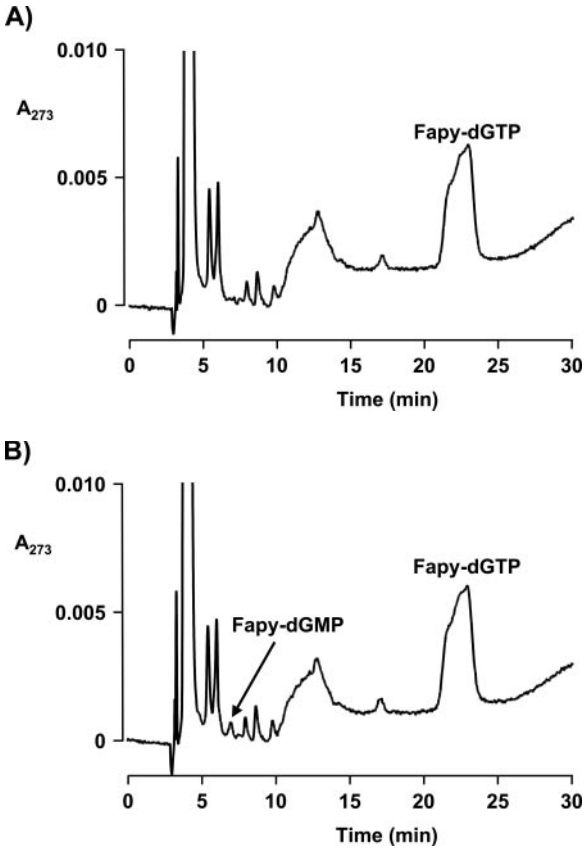


Fig. 4. **Hydrolysis of FAPY-dGTP by the hMTH1 protein, monitored by anion exchange HPLC.** FAPY-dGTP (10 μM) was incubated in the presence (panel B) or absence (panel A) of 1 nM of the hMTH1 protein at 30°C for 5 min. The elution solution was 75 mM sodium phosphate buffer, pH 7.0 (isocratic). Note that FAPY-dGTP was eluted as a broad peak due to the presence of isomers (see text). Its elution pattern occasionally altered even when the same lot of the compound was analyzed. The absorption spectrum was very similar independent of the positions in the peak.

Table 1. **Substrate Specificity of hMTH1.**

Nucleotide	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot\mu M^{-1}$)
dJTP	9.3 (9.2, 9.4)[a]	24.5 (24.4, 24.6)[a]	2.63
FAPY-dGTP[b]	70.8 (68.1, 73.4)[a]	2.2 (2.1, 2.2)[a]	0.03
8-OH-dGTP[c]	17.3	27.1	1.57
8-OH-dITP[c]	123.6	15.0	0.12

[a]Experiments were done in duplicate and the mean values are represented. The data obtained in a single experiment are shown in parentheses. [b]The parameters are average values for all isomers (see text). [c]Data from Ref. *9*.

may eliminate FAPY-dGTP from the nucleotide pool in human cells. This result suggests that the presence of the two aromatic rings is strongly, but not absolutely, required for hydrolysis by the hMTH1 protein.

The kinetic parameters indicated that the K_m value for dJTP, the synthetic nucleotide, was 9.3 μM, ~1.9-fold lower than that for 8-OH-dGTP (Table 1). The k_{cat} values for the two nucleotides were similar, resulting in a ~1.7-fold higher k_{cat}/K_m value for dJTP. The kinetic parameters of FAPY-dGTP were also determined assuming that its all conformations are equally active. The obtained parameters are average values for all isomers. The K_m value for FAPY-dGTP was 70.8 μM, ~4.1-fold higher than that for 8-OH-dGTP (Table 1). Importantly, the k_{cat} value for FAPY-dGTP was ~12.3-fold lower than that for 8-OH-dGTP. Consequently, the k_{cat}/K_m value for FAPY-dGTP was ~50-fold lower than that for 8-OH-dGTP.

DISCUSSION

In this study, three nucleotide analogs were tested to clarify the recognition mechanism of the hMTH1 protein.

The functional groups at the 2- and 6-positions of the purine base are important factors in the substrate recognition mechanism of the hMTH1 protein (*9*). This study was designed to examine the analogs containing the 7,8-dihydro-8-oxo structure.

It has been shown that 8-OH-dITP (Fig. 1) is hydrolyzed by the hMTH1 protein with ten-fold less efficiency than 8-OH-dGTP (Table 1) (*9*). This result suggests that the 2-amino group of 8-OH-dGTP is very important for the recognition of 8-OH-dGTP. dJTP also has the 2-amino group, and was expected to be recognized well by the hMTH1 protein. Indeed, this unnatural nucleotide analog was hydrolyzed efficiently by hMTH1 (Fig. 2). Thus, the kinetic parameters for 8-OH-dGTP, 8-OH-dITP, and dJTP indicate the significant contribution of the 2-amino group for competent recognition. In addition, dJTP was hydrolyzed more efficiently than 8-OH-dGTP. The K_m value of hMTH1 for dJTP was ~1.9-fold lower than that for 8-OH-dGTP (Fig. 2 and Table 1). 8-OH-dGTP contains the 6-oxo and 1-imino functions. This result suggests that a slightly unfavorable interaction may exist between the 6-oxo group and/or the *N*1-proton of 8-OH-dGTP and the hMTH1 protein.

These results agree with our previous observations that 2-OH-dATP and d*iso*ITP are hydrolyzed more efficiently, and with lower K_m values, than 8-OH-dGTP or 8-OH-dATP (*9*). The enol tautomers of 2-OH-dATP and d*iso*ITP present the "(donor)-acceptor-donor" hydrogen-bonding pattern that is shared with dJTP (the *N*1 acceptor and the 2-amino donor).

The importance of the amino group (also hydroxy group) at the 2-position may be explained by an interaction between the phosphate group and the 2-amino group. This explanation is supported by a computational study of dGMP based on the extended Hückel theory (*20*). The authors concluded that the *syn* conformation is favored due to the interaction. Since hydrolysis of 2-amino-dATP by hMTH1 was undetectable (*9*), the presence of the 2-amino group does not absolutely determine the hydrolysis. However, the putative interaction may assist the "fixation" of the *syn* conformation of dJTP, and this may be one of the reasons of preferential hydrolysis of the nucleotide.

FAPY-Gua is one of the major damaged purine bases produced by ROS (*21–24*). FAPY-Gua in DNA facilitates the misincorporation of dATP *in vitro* (*25*), and FAPY-dGTP may be a mutagenic deoxyribonucleotide. FAPY-dGTP might be present in the nucleotide pool as well as 8-OH-dGTP. In addition, DNA glycosylases that recognize 8-OH-Gua also excise FAPY-Gua (*26–29*). Thus, it is important to examine whether the hMTH1 protein catalyzes hydrolysis of FAPY-dGTP. As shown in Fig. 4 and Table 1, FAPY-dGTP was hydrolyzed, albeit to a lesser degree, by the hMTH1 protein. This result suggests a potential role of the hMTH1 protein in eliminating this oxidized dGTP from the nucleotide pool.

FAPY-dGTP and 8-OH-dGTP contain the same functional groups at the same positions of the pyrimidine ring. The only difference is in the opened and closed imidazole rings. However, the hMTH1 protein catalyzed hydrolysis of the two oxidized deoxyribonucleotides with quite different efficiencies. FAPY-dGTP seems to be a mixture of the α- and β-anomers (*18*, *19*). Moreover, the FAPY-Gua base can exist as four conformers, due to rotation around the C5-N7 and N7-C8 bonds (Fig. 1) (*19*). The presence of four of the eight isomers was observed for a FAPY-Gua nucleoside derivative and for the ribonucleoside triphosphate (*14*, *19*). The existence of a variety of structures would cause a weak interaction between FAPY-dGTP and the hMTH1 protein. Interestingly, the k_{cat} value of hMTH1 for FAPY-dGTP was 2.2 s^{-1}, a ~12.3-fold smaller value than that for 8-OH-dGTP (Table 1). On the other hand, the K_m value for FAPY-dGTP (70.8 μM) was ~4.1-fold higher than that for 8-OH-dGTP (17.3 μM). Thus, the opening of the imidazole ring results in the reduction of the k_{cat} value, rather than the increase in the K_m value. This is in contrast to the deoxyribonucleotide analogs examined in the previous study (*9*). The k_{cat} values of hMTH1 for the analogs were within 3-fold of that for 8-OH-dGTP. In the case of oxidized ribonucleotides, the k_{cat} values are lower than those for the corresponding deoxyribonucleotides (*16*). The k_{cat} value for 8-hydroxy-ATP is ~10-fold lower than that for 8-OH-dATP and 8-OH-dGTP.

Note that the FAPY-dGTP used in this study contained ~10% impurities. Thus, the concentration of FAPY-dGTP calculated by the absorbance at 273 nm was likely to be overestimated. Therefore, the actual K_m value for FAPY-dGTP could be less than the determined value, 70.8 μM.

FAPY-dGTP was weakly recognized, and 2-OH-dYTP was not hydrolyzed. Previously, the importance of the Trp-117 residue was shown in the binding of 8-OH-dGTP to the hMTH1 protein (*30*). Thus, a π–π interaction occurred between the Trp residue and the 8-hydroxyguanine base. The loss of the two-ring system would impair the stacking interaction, and this might be a reason for the weak hydrolysis of FAPY-dGTP and 2-OH-dYTP.

We calculated the kinetic parameters of FAPY-dGTP assuming that all isomers of FAPY-dGTP are active or neutral. However, some of the isomers of this nucleotide could be inhibitors, which alter the kinetic parameters of the active species. In addition, the ~10% impurities contained in the FAPY-dGTP used might inhibit the reaction. The discussion of the kinetic parameters of FAPY-dGTP would be invalid if some isomers and/or the impurities act as inhibitors.

In this study, we showed that the 2-amino group and the two-ring system of the 7,8-dihydro-8-oxo-purine bases are important factors in the substrate recognition mechanism of the hMTH1 protein. The solution structure of the hMTH1 protein has been determined (*31*). To reveal the actual binding mode, the structural analysis of the complex of a substrate analog and the protein will be very useful. Experiments toward this goal are in progress.

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