PREPARATION OF NEW 7-HYDROXYGUANINE DERIVATIVES AND THEIR BIOLOGICAL ACTIVITIES

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We prepared new 7-hydroxyguanine derivatives, 7-hydroxyguanosine 5'-monophosphate and N^2-tetrahydropyranyl-7-hydroxyguanine, and compared biological activities of 7-hydroxyguanine derivatives including nucleosides acquired previously. 7-Hydroxyguanine and its nucleotide inhibited the focus formation of Rous sarcoma virus. Antitumor activities of these derivatives against mouse leukemia L1210 were not so different from one another. Anti-proliferative activities of the derivatives on various human cell lines were significantly different from one another.

An antitumor antimetabolite, 7-hydroxyguanine has been discovered by several laboratories[1~4]. To investigate more desirable antitumor compounds, we have modified 7-hydroxyguanine using purine nucleoside phosphorylase[5,6].

In this paper we describe the preparation of new derivatives, 7-hydroxyguanosine 5'-monophosphate and N^2-tetrahydropyranyl-7-hydroxyguanine, and biological activities of 7-hydroxyguanine derivatives.

Materials and Methods

Chemicals

7-Hydroxyguanine[2], 7-hydroxyguanosine and 7-hydroxy-2'-deoxyguanosine[6] were prepared as described previously. 5-Phospho-α-D-ribosyl diphosphate sodium salt and hypoxanthine phosphoribosyltransferase (EC 2.4.2.8) were purchased from Sigma Chemical Company.

HPLC Analysis

The HPLC instrumention consisting of the Nihonbunko V system and YMC pack A-612 column 6 × 150 mm (Yamamura Chemical Co., Ltd.) was used. The mobile phase was 95 mM $NH_4H_2PO_4$ buffer (pH 4.0) containing 5% methanol at a flow rate of 1.0 ml/minute. 7-Hydroxyguanosine 5'-monophosphate, N^2-tetrahydropyranyl-7-hydroxyguanine and 7-hydroxyguanine were detected by UV absorption at 254 nm and their retention times were 8.9, 7.1 and 4.4 minutes, respectively.

Preparation of 7-Hydroxyguanosine 5'-Monophosphate Disodium Salt (1)

7-Hydroxyguanine (180 mg) and 5-phospho-α-D-ribosyldiphosphate sodium salt (1,300 mg) were dissolved in 350 ml of 50 mM Tris buffer (pH 7.8) containing 12.5 mM dithiothreitol and 2.5 mM $MgCl_2$. The reaction was started by the addition of 50,000 U of hypoxanthine phosphoribosyltransferase. After incubation for 14 hours at 30°C, 70% of 7-hydroxyguanine was converted to 7-hydroxyguanosine 5'-monophosphate.

The reaction mixture was applied to a column of DEAE-Sephadex A-25 (HCO_3^-, 200 ml) and eluted with 0.2 M NH_4HCO_3. The eluate was evaporated *in vacuo* to 3 ml. The concentrate was applied to the column of Sephadex LH-20 (600 ml) and developed with 20 mM NH_4OH. The eluate was evaporated and dried, yielding white powder of crude 7-hydroxyguanosine 5'-monophosphate (280 mg). The white powder

(160 mg) was dissolved in 1.2 ml of water. To this solution 80 μl of 1 M NaOH and 3.2 ml of ethanol were added. After 16 hours in the refrigerator **1** precipitated as colorless crystals (127.5 mg).

Preparation of N^2-Tetrahydropyranyl-7-hydroxyguanine (**2**)

7-Hydroxyguanine (1,000 mg) was dissolved in 25 ml of DMSO with 2 ml of hydrogen chloride in dry dioxane (6.0 M). 2,3-Dihydropyran (1.12 ml) was added to the stirred solution. The mixture was stirred at 15°C under argon atmosphere for 4 hours. After neutralization with anhydrous $NaHCO_3$ (1,200 mg), ethyl acetate (250 ml) and hexane (250 ml) were added to the solution. The resulting precipitate was filtered off, dried *in vacuo* and purified by silica gel column chromatography ($CHCl_3$ - methanol, 10 : 1). The eluate was evaporated and dried, yielding a white powder of N^2-tetrahydropyranyl-7-hydroxyguanine (270 mg).

Anti-rous Sarcoma Virus Assay

The effects of 7-hydroxyguanine and its derivatives on growth of chick embryonic fibroblast were determined by trypan blue staining method. Focus-forming assay for Rous sarcoma virus (RSV) was carried out according to the procedure descrived by SAMBROOK and POLLACK[7].

Cell Lines and Media

The following cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD): A549, human lung carcinoma (ATCC CCL 185); MCF-7, human breast adenocarcinoma (ATCC HTB 22); PANC-1, human pancreas epitheloid carcinoma (ATCC CRL 1469); HeLa S3, human cervix epitheloid carcinoma (ATCC CCL 2.2); G361, human malignant melanoma (ATCC CRL 1424); and CCRF-CEM, human acute lymphoblastic leukemia (ATCC CCL 119). MRC-5, human embryonic lung and Colo320DM, human colon adenocarcinoma were obtained from Japan Cancer Research Resources Bank (JCRB).

A549, PANC-1 and G361 were maintained in DULBECCO's modified EAGLE's medium (DMEM, Nissui Pharmaceutical Co., Ltd., Tokyo), MCF-7, HeLa S3 and MRC-5 in EAGLE's minimal essential medium (MEM, Nissui) and Colo320DM and CCRF-CEM in RPMI1640 (Nissui). Cells were cultured in medium supplemented with 2 mM L-glutamine, 50 μg/ml kanamycin and 10% fetal calf serum (FCS, Flow Laboratories, Rockville, MD).

In Vitro Cell Proliferation Assay

0.1 ml aliquots of tumor cells ($1 \sim 2 \times 10^4$/ml) were dispensed into 96-well microtiter plates in their cell-specific media and serial 2-fold dilutions of 7-hydroxyguanine derivatives were added to a final volume of 0.2 ml, respectively. Cells were incubated at 37°C for 5 ~ 7 days in a 5% CO_2 - humidified air incubator.

The methylene blue dye assay was performed on adherent tumor cell lines. Adherent tumor cells were fixed with 2.5% glutaraldehyde and stained with 0.05% methylene blue for 10 minutes. After the cells were thoroughly washed with tap water, the methylene blue dye was eluted with 0.1 ml of 0.33 M HCl. The absorbance at 655 nm was measured on a Titertek Multiscan photometer (Flow Laboratories).

Anti-proliferative activity to non adherent tumor cells Colo320DM and CCRF-CEM was measured by colorimetric assay, which is based on the ability of mitochondrial enzymes to transform MTT tetrazolium salt into a formazan[8]. Tetrazolium salt (MTT) (Sigma Chemical Company) was prepared fresh at 5 mg/ml in phosphate-buffered saline in a darkened tube. The MTT was added to all wells of the assay plates in a 20-μl volume. Then, plates were incubated at 37°C for 4 hours. At the end of the MTT incubation, 0.1 ml aliquots of the medium were removed by aspiration and 0.1 ml of acidified isopropyl alcohol (0.4 M HCl in 2-propanol) was added to solubilize the MTT formazan. After the complete solubilization of the MTT formazan using a multichannel pipet, the absorbance was measured at 540 nm on a Titertek Multiscan photometer.

Antitumor Assay

Lymphoid leukemia L1210, 10^5 cells per mouse were inoculated intraperitoneally into female mice (BDF_1 strain 18 ~ 22 g weight). 7-Hydroxyguanine derivatives were administered intraperitoneally 24 hours after the tumor inoculation. The treatment was done once daily for 5 days.

Results and Discussion

Physico-chemical Properties

7-Hydroxyguanosine 5'-monophosphate disodium salt (**1**) (Fig. 1) is freely soluble in water. **1** gives positive reactions with Folin-Ciocalteu reagent and sodium molybdate-sulfuric acid. Physico-chemical properties of **1** are summarized in Table 1.

Positive ion FAB-MS of **1** gave a base ion at m/z 424 $(M+H)^+$. Elemental analysis showed **1** to form a pentahydrate. Proton magnetic resonance study indicated that 7-hydroxyguanosine does not have an 8-H signal in aqueous solution[6], but **1** has an 8-H signal at 8.47 ppm in aqueous solution because the 5'-phosphoryl group has a specific deshielding effect on the 8-H proton of guanine nucleotides[9]. The ^{13}C NMR study indicates that the resonance of C-4' is split as a result of ^{13}C-^{31}P scalar coupling ($J_{cp}=6.8$ Hz).

To prepare new derivatives which we will not be able to prepare by enzymatic methods, we have tried chemical modifications of 7-hydroxyguanine. Most attempts failed to yield bio-active derivatives. In many cases, 7-hydroxyguanine was substituted by the agents at the 8-position. The derivatives substituted at the 8-position lost antibacterial activity on synthetic medium and antitumor activity against mouse leukemia L1210.

2,3-Dihydropyran gave an active derivative, N^2-tetrahydropyranyl-7-hydroxyguanine (**2**) (Fig. 2). The physico-chemical properties of **2** were as follows: MP 184~186°C (dec); IR (KBr) cm^{-1} 3340, 2985, 1695, 1600, 1440; ^1H NMR (90 MHz, DMSO-d_6) δ 7.95 (1H, s), 6.74 (1H, d), 5.02 (1H, t), 3.2~3.8 (2H, m), 1.2~1.8 (6H, m); MS (m/z) M^+ 251; *Anal* calcd for $C_{10}H_{13}N_5O_3$: C 47.80, H 5.24, N 27.81; found: C 47.80, H 5.22, N 27.88. The solubility of **2** in organic solvents was increased compared to 7-hydro-

Table 1. Physico-chemical properties of 7-hydroxyguanosine 5'-monophosphate disodium salt.

MP (dec)	135~140°C
$[\alpha]_D^{20}$ (c 0.5, H_2O)	−44.6°
FAB-MS (m/z)	424 $(M+H)^+$
Molecular formula	$C_{10}H_{12}N_5O_9PNa_2\cdot 5H_2O$
Anal Calcd:	C 23.40, H 4.32, N 13.64
Found:	C 23.51, H 4.40, N 13.48
UV $\lambda_{max}^{H_2O}$ nm (ε)	230 (17,600), 276 (8,800)
$\lambda_{max}^{0.1\,M\,HCl}$ nm (ε)	258 (11,600), 278 (sh)
$\lambda_{max}^{0.1\,M\,NaOH}$ nm (ε)	232 (16,600), 276 (8,600)
IR (KBr) cm^{-1}	3350, 3150, 1710, 1650, 1610, 1540, 1405, 1190, 1090, 780
^1H NMR (D_2O) δ	3.96 (5'-2H), 4.22 (4'-1H), 4.35 (3'-1H), 4.54 (2'-1H), 5.91 (1'-1H), 8.47 (8-1H)
^{13}C NMR (D_2O) δ	66.7 (5'), 73.1 (3'), 77.6 (2'), 87.4 (4'), 90.6 (1'), 110.5 (5), 128.7 (8), 149.9 (4), 157.6 (2), 158.1 (6)

Fig. 1. Structure of 7-hydroxyguanosine 5'-monophosphate disodium salt.

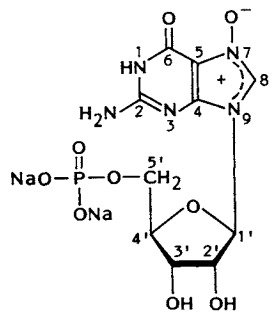

Fig. 2. Structure of N^2-tetrahydropyranyl-7-hydroxyguanine.

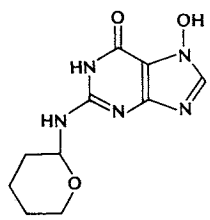

Table 2. Anti-viral activity of 7-hydroxyguanine derivatives against RSV.

	IC_{50}[a] (μg/ml)		
	7-Hydroxy-guanine	7-Hydroxy-guanosine	**1**
RSV	2.0	8.5	6.0
CEF	26.0	11.5	62.0

[a] IC_{50} of focus formation (RSV) or growth (CEF).

Table 3. Anti-proliferative activity of 7-hydroxyguanine derivatives on various human cell lines.

Cell line	Origin	IC_{50} (μg/ml)			
		7HG	7HGR	7HGD	**2**
A549	Lung carcinoma	6.52	3.29	15.71	19.61
Colo320DM	Colon adenocarcinoma	0.95	0.53	3.35	2.33
MCF-7	Breast adenocarcinoma	1.64	2.24	8.91	4.64
PANC-1	Pancreatic carcinoma	4.71	8.41	19.81	15.11
HeLa S3	Cervix carcinoma	0.27	0.47	2.10	0.86
G361	Melanoma	0.04	0.30	0.24	0.09
CCRF-CEM	Lymphoblastic leukemia	>32	>64	>64	>64
MRC-5	Lung diploid	1.33	24.04	20.8	4.24

7HG: 7-Hydroxyguanine, 7HGR: 7-hydroxyguanosine, 7HGD: 7-hydroxy-2'-deoxyguanosine.

xyguanine.

Biological Activities

As shown in Table 2, 7-hydroxyguanine and **1** inhibited the focus formation of RSV. In this system, 7-hydroxyguanosine did not show anti-viral activity because of its high toxicity to host cells. The difference in toxicity to chicken embryonic fibroblasts (CEF) among these derivatives suggest a possibility of differential metabolism of 7-hydroxyguanine derivatives in the cell. HASOBE *et al.* reported the anti-viral activity of guanine 7-*N*-oxide, a tautomer of 7-hydroxyguanine, against herpes virus and rhabdo virus which were infectious to salmonids[10]. We showed that 7-hydroxyguanine and its nucleotide inhibited the focus formation of oncogenic retro virus. Further study will be necessary to clarify the differential effects of 7-hydroxyguanine derivatives on the metabolism of host cells and viruses.

Table 4. Antitumor activity of 7-hydroxyguanine derivatives against mouse leukemia L1210.

Dose[a]	T/C (%)[b]			
	7-Hydroxy-guanine	**1**	**2**	Fluoro-uracil
0.5	124	NT	NT	NT
1	135	NT	118	NT
2	145	NT	145	125
4	151	141	139	140
8	119	145	147	157
16	Toxic	148	147	172
32	NT	105	99	113
64	NT	Toxic	Toxic	NT

[a] mg/kg/day, day 1~5 (ip). 6 mice in each group.
[b] T/C (%) means survival period of treated/mean survival period of controls.
NT: Not tested.

As shown in Table 3, 7-hydroxyguanine derivatives have anti-proliferative activity on various human cell lines. In the tested cell lines, G361 (malignant melanoma) was most sensitive to these compounds. 7-Hydroxyguanosine was more effective to A549 (lung carcinoma) than 7-hydroxyguanine. 7-Hydroxyguanosine was less toxic to normal type cell line, MRC-5 (embryonic-lung-diploid cell). JACKSON *et al.* reported that 7-hydroxyguanine did not inhibit the rate of synthesis of DNA and RNA in mouse leukemia L1210 cells[11]. They showed that the major effect of 7-hydroxyguanine on L1210 cells was inhibition of protein biosynthesis. HASOBE *et al.* reported that 7-hydroxyguanine might be regarded as an analog of 7-methylguanosine in the cap structure of eucaryotic and viral m-RNA[12]. It is interesting to clarify the mechanism of action of 7-hydroxyguanine derivatives on eucaryotic cells and viruses.

As shown in Table 4, **1** and **2** have antitumor activity against mouse leukemia, but the effects are not superior to that of 7-hydroxyguanine.

The acute toxicity of **1** and **2** were determined in male mice (ICR strain) by single intraperitoneal administration, the LD_{50} values of **1** and **2** are in the range 150~300 and 160~320 mg/kg, respectively.

In the *in vivo* L1210-system, 7-hydroxy-2'-deoxyguanosine was slightly superior to other derivatives[6], but anti-proliferative activities of 7-hydroxyguanine derivatives on human cancer cell lines were significantly different from one another. *In vivo* study with human cancer cells will be important to further evaluation of 7-hydroxyguanine derivatives.

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