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Action Mechanism of 2-(2-Hydroxy-5-*n*-hexylphenyl)-8-quinolinol-4-carboxylic Acid—with Special Reference to Selective Inhibition of DNA Synthesis in Ascites Hepatoma AH 13 Cells in Culture[1]

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The mode of action of 2-(2-hydroxy-5-*n*-hexylphenyl)-8-quinolinol-4-carboxylic acid (HQ II) was studied using mainly ascites hepatoma AH 13 (AH 13) cells in culture.

1) *In vitro*, HQ II causes hemolysis of rabbit erythrocytes and injures cancer cells. However, these effects are not appeared in the medium containing some amounts of serum. 2) Even in the culture containing serum, HQ II inhibits the proliferation of AH 13 cells and causes selective inhibition of desoxyribonucleic acid (DNA) synthesis. These actions are mostly restored by washing or addition of Fe salts. 3) Uptake of ^{3}H-thymidine, ^{3}H-deoxycytidine (^{3}H-CdR), and ^{3}H-cytidine (^{3}H-CR) to the cellular acid-solubles are not decreased by HQ II treatment. However, the incorporations to DNA in turn are prominently inhibited. 4) The conversion of ^{3}H-CR to ^{3}H-CdR nucleotides is inhibited in the cells treated with HQ II. 5) The exogenous addition of a certain composition of deoxyribonucleosides to the medium can suppress the inhibitory effect of HQ II on DNA synthesis. 6) The reduction of ^{14}C-CR diphosphate to ^{14}C-CdR nucleotides in cell-free extracts of AH 13 cells is prominently inhibited by the agent.

HQ II causes the cell membrane injury and/or inhibits selectively DNA synthesis in AH 13 cells. The results suggest that the inhibition of DNA synthesis caused by HQ II having metal- chelate-forming ability may be due to the interaction of the agent with the extracellular and/or intracellular Fe (in which ribonucleotide reductase may be involved.).

2-(2-Hydroxy-5-*n*-hexylphenyl)-8-quinolinol-4-carboxylic acid (HQ II), an analogue of 2-(2-hydroxyphenyliminomethyl)-4-*n*-hexylphenol (HP),[3] is an anticancer, antimicrobial and metal complex-forming compound.[4] It was suggested in the previous paper[4] that cell membrane injury and inhibition of nucleic acid synthesis with HQ II might participate in mode of action of HQ II on cancer cells.

The further studies to elucidate the mechanism of action of HQ II were mainly concentrated to macromolecular synthesis in cancer cells.

Materials and Methods

Materials——HQ II and related compounds were prepared by one of the authors (T.U.).[4] Hydroxyurea (HU) (Sigma Chemical Co.), Trypan blue (G. Gruebler and Co.), Nigrosine (Nakarai Chemical Co.), Crystal violet (Dr. G. Gruebler and Co.). The unlabeled nucleosides, nucleotides and other biochemicals were purchased from Sigma or Carbiochem Inc. Labeled compounds, thymidine-6-^{3}H (sp. act., 16.0 Ci/mmole), uridine-5-^{3}H (sp. act., 5.0 Ci/mmole), L-leucine-^{14}C(U) (sp. act., 198 mCi/mmole) were purchased from Daiichi Pure Chemicals Co. Deoxycytidine-5-^{3}H (sp. act., 26.5 Ci/mmole), cytidine-5-^{3}H (sp. act., 25.8 Ci/mmole) and cytidine-^{14}C(U)-5′-diphosphate trisodium salt (sp. act., 367.3 mCi/mmole) were all products of New England Nuclear.

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2) Location: *Takara-machi, 13-1, Kanazawa.*

3) T. Ujiie, *Chem. Pharm. Bull.* (Tokyo), **16**, 165 (1968).

4) T. Ujiie, *Chem. Pharm. Bull.* (Tokyo), **23**, 62 (1975).

Animals——20—22 g mice (ddYs, male) and 100—120 g rats (Donryu, male) were used.

Tumor Cells——Ehrlich ascites carcinoma (EA) (from Kitazato Institute) was maintained by weekly transplanting to mice and ascites hepatoma AH 13 (AH 13) (from Sasaki Institute) was passaged by intraperitoneally transplanting to rats every 5 days.

Hemolysis of Rabbit Erythrocytes——The method was described in the previous paper.[4]

Tumor Cell Suspension——Peritoneal fluid withdrawn from 7-day-old EA bearing mice or from 5-day-old AH 13 bearing rats was centrifuged and the sedimented cells, after desrupting blood ceels by hypotonic shock, were washed 2 times with PBS and once with an appropriate medium (PBS, Hanks, Eagle's minimum essential medium (EM) and EM containing fetal calf serum), subsequently in which the cells were suspended to give an appropriate cell density. The cell suspension in a test tube covered with a rubber stopper was incubated with gentle shaking. In the experiments on the primary culture of AH 13 cells, 1 ml of the cell suspension in EM supplemented with 20% fetal calf serum (EF) was incubated still in a test tube (13×90 mm) with a rubber stopper at 37°.

Cell Counting (Estimation of Cell Injury)——1) Dye Exclusion Method: Dead cells were estimated by staining with Nigrosine solution (0.2% in PBS) or with Trypan blue (0.5% in PBS).

2) Release of Radioactivity from ^{51}Cr-loaded Cells: According to the method of Wigzell,[5] to 20 ml of EA cells in Hanks solution (pH 7.4) (2.3×10^6 cells/ml) was added 400 μCi of Na_2CrO_4-^{51}Cr (Daiichi Pure Chemicals). The cell suspension was incubated at 37° for 1 hr with gentle shaking. The suspension was then allowed to stand for 5 min in cold. The cells collected by centrifugation were washed 2 times with 10 ml of Hanks solution and resuspended in 20 ml of the solution. After the cell suspension was incubated with agents at 37°, an aliquot of that was taken up and centrifuged. The supernatant was subjected to assay its radioactivity by means of γ-ray radiation counter (Aloka universal scaler model TDC-5). Cells in 1 ml of the suspension gave 13.6×10^4 cpm of radioactivity.

3) Naked Nuclei Counting: AH 13 cells cultured in a test tube were centrifuged at 2000 rpm for 30 sec and treated woth 0.05% Crystal violet–0.1 M citric acid at 37° for 1 hr with moderate shaking. Cell nuclei were counted using Buerker hemocytometer.

Agents Tested——Agent dissolved in dimethyl sulfoxide (DMSO) was added to the cell suspension or the cell culture in the final concentration of 1% or 0.5% DMSO, the amount of which did not cause any obvious effects.

Cellular Uptake of Radioactive Precursors——The cells were incubated with ^{3}H-thymidine (^{3}H-TdR), ^{3}H-uridine (^{3}H-UR) and ^{14}C-leucine (^{14}C-Leu) for determination of cellular activities on desoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein, respectively. The cells were incubated with a radioactive precursor at 37° for an appropriate time and incorporation was terminated by adding 100 μg of unlabeled precursor to 1 ml of the medium and/or cooling. The cells were washed 3 times with cold PBS and treated with cold 5% trichloroacetic acid (TCA). After 30 min-standing in cold, the sediment was washed twice with cold 5% TCA, once with EtOH and twice with EtOH: ether (3: 1 v/v). The residue was dissolved in 2 N NH_4OH or Hyamine hydroxide. The radioactivity of the residue was determined in a modified Bray's solution using Beckman liquid scintillation system LS-200B.

In some cases for the experiments on DNA synthesis, the cold TCA-insoluble fraction was incubated in 1 N KOH at 37° for 17 hr. After the alkaline solution was neutralized with 6 N HCl, treated with cold TCA (final, 5%) and centrifuged, the resulting sediment and supernatant were used as DNA and RNA fraction, respectively. They were counted for their radioactivities. In the cases of low cell concentrations (10^4—10^5 cells/ml), filter disc method was employed for isotopic experiments. 1 ml portion of the cell suspension was pipetted onto Whatman glass fiber paper (GF/C, 2.4 cm) or Gelman glass fibre filter (type A) on a suction filter apparatus. 3 washes (3 ml each) with cold 5% TCA followed by 3 washes (3 ml each) with EtOH: ether (3: 1 v/v). After drying, the filter was placed in a scintillation vial. 10 ml of a 2,5-diphenyloxazole (PPO)-toluene scintillation mixture was added and radioactivity was measured.

Uptake of ^{3}H-Cytidine into the Acid Soluble Fraction of AH 13 Cells and Its Conversion to ^{3}H-Deoxycytidine Nucleotides——AH 13 cells were suspended in EF in a cell density of 1.6×10^6 cells/ml. The cells, after preincubation with agents for 30 or 60 min at 37°, were incubated with ^{3}H-cytidine (^{3}H-CR) (2 μCi/ml, 0.45 μM) at 37° for 60 min. The cells were sedimented by centrifugation, washed 3 times with cold PBS and then treated with cold 5% TCA or 5% perchloric acid (PCA). In order to determine the amount of ^{3}H-deoxycytidine (^{3}H-CdR) nucleotides in the acid soluble fraction, PCA solution which was separated by centrifugation was neutralized with 2.5 N KOH. After removal of deposited $KClO_4$ by centrifugation, the supernatant was evaporated to dryness at less than 30° by Taiyo concentrator model TC-8. Following Cory, *et al.*,[6] the residue was incubated with 3.75 mg of *Crotalus adamanteus* venom extract (Sigma) in 0.5 ml of a solution containing 50 mM Tris buffer (pH 9.0) and 50 mM $MgSO_4$ at 37° for 2 hr. The reaction was stopped by heating in a boiling water bath for 3 min. The reaction mixture was allowed to cool and 50 μg of CdR was added to the mixture, which was centrifuged. The supernatant was applied on a Dowex 1-

5) H. Wigzell, *Transplantation*, 3, 423 (1965).
6) J.G. Cory and T.W. Whitford, Jr., *Cancer Res.*, 32, 1301 (1972).

borate (×8, 200—400 mesh) column (0.6×7.5 cm), followed by eluting with 3 ml of water. The radioactivity of the effluent was counted in 17 ml of a modified Bray's solution.

Protein Determination——The protein concentrations were estimated by the method of Lowry, *et al.*[7] using the albumin standard solution (Daiichi Pure Chemicals) as standard.

Preparation of Cell-free Extracts——Tumor cells which were collected by centrifugation of peritoneal fluids of rats bearing 5-day-old AH 13 tumor were washed with a cold 0.015 M NaCl solution to remove blood cells. After being washed twice with PBS, the tumor cells were then packed by centrifugation at 2000 rpm for 5 min. The packed cells were suspended in 2 volumes of 1 mM dithioerythritol (DTE) and homogenized by passage through a motor driven Potter Elvehjem homogenizer with a teflon pestle at 2900 rpm for 1.5 min under cooling with ice water. The homogenate was centrifuged in the Beckman preparative ultracentrifuge at 100000×*g* for 2 hr at 4°. The supernatant fluid was used as the source for ribonucleotide reductase.

Assay for Ribonucleotide Reductase Activity——The reaction mixture described by Moore[8] was used with some modifications. The reaction mixture contained in a final volume of 125 μl; 8.3 mM K phosphate (pH 7.0), 4.4 mM ATP, 4.2 mM Mg acetate, 8.3 mM NaF, 0.06 mM $FeCl_3$, 6.2 mM DTE, 0.4 mM cytidine diphosphate (CDP)-^{14}C (0.1 μCi/tube) and 50 μl of the cell-free extracts (350 μg of protein). The mixture was incubated at 37° for 1 hr and the reaction was terminated by heating in a boiling water bath for 3 min. After cooling, 50 μg of CdR dissolved in 50 μl of a mixture of 24 mM Tris buffer (pH 8.7) and 80 mM $MgSO_4$ and 25 μl of the snake venom extract suspension (50 mg/ml) were added to the reaction mixture. Subsequently, this mixture was incubated at 37° for 2 hr. The reaction was again stopped by heating. The reaction mixture obtained was diluted with 0.8 ml of water and subjected to chromatography on a Dowex 1-borate column (0.6×7.5 cm) using water as a solvent. The absence of $FeCl_3$ in assay mixture did not give obvious effect on the enzyme activity.

Results

As reported previously,[4] HQ II causes hemolysis of rabbit erythrocytes *in vitro*. Table I and Fig. 1 show that the hemolytic activity of HQ II depends on incubation time and temperature. The dye staining and the release of radioactivity from ^{51}Cr-loaded cells were adopted as criteria of cancer cells injury. These two methods gave the similar results as indicated in Fig. 2. EA cells and AH 13 cells were injured by incubation with HQ II and these actions were affected by incubation temperature, time and cell density, the result being similar to that with the erythrocytes (Table II).

TABLE I. Comparison of Hemolytic Activity of HQ II, SDS, SDC and $HgCl_2$

Agent	Concentration (μg/ml)	Hemolytic activity (% hemolysis)[a] incubated for 30 min at	
		37°	0°
HQ II	50	15	14
	100	100	34
SDS[b]	10	3	2
	20	19	15
	50	69	77
	100	100	100
SDC[c]	100	38	29
$HgCl_2$	10	100	2
	100	100	5

a) rabbit erythrocytes: 1.4×10^8 cells/ml of PBS *b*) sodium dodecyl sulfate
c) sodium desoxycholate

Now, it is natural to suppose that the activity of HQ II may be affected by several biological factors. Then, the effect of serum as one of the factors on the cell injury by HQ II

7) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, 193, 265 (1951).
8) E.C. Moore, "Methods in Enzymology," Vol. XII, part A, ed. by L. Grossman and K. Moldave, Academic Press, Inc., New York and London, 1967, pp. 155—174.

TABLE II. Correlation between Cell Damage by HQ II and Concentration of Ehrlich Ascites Carcinoma Cell Suspension

HQ II (μg/ml)	Incubation		Per cent of dye-stained cell concentration of cell suspension (per ml of PBS)		
	Temperature (°C)	Time (min)	1.3×10^7	4.5×10^6	1.3×10^6
100	37	90	22.5	100.0	100.0
100	0	90	8.9	3.6	100.0
None	0	90	4.8	0.7	6.0

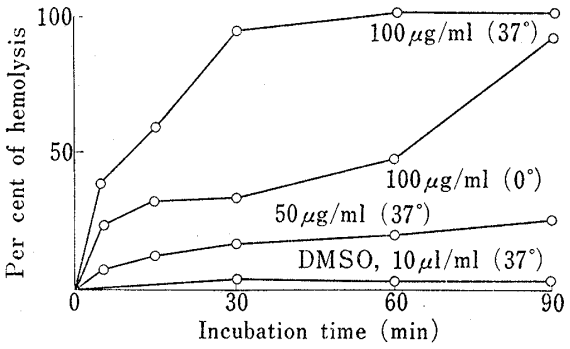

Fig. 1. Kinetics of Hemolysis by HQ II

rabbit erythrocytes, 1.4×10^8 cells/ml of PBS (total hemolysis; $A_{550nm}=2.475$ unit)

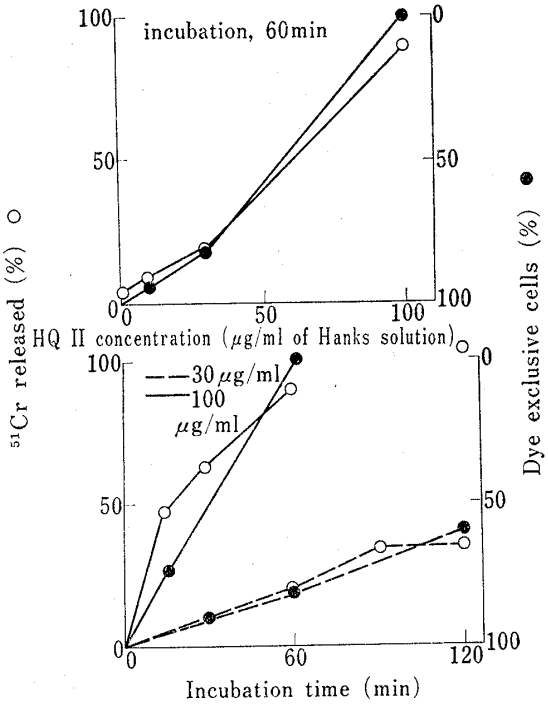

Fig. 2. Release of Radioactivity from ^{51}Cr-loaded Ehrlich Ascites Carcinoma Cells by HQ II Treatment

was investigated. As shown in Fig. 3, the presence of serum was seemed to protect the cells against HQ II. However, even in the presence of serum DNA synthesis in EA or AH 13 cells was prominently inhibited by HQ II with no significant cell injury (Fig. 3, Table III).

Further studies were performed with AH 13 cells in their primary culture. AH 13 cells in EF did exponentially grow at least for 3 days after planting without refeeding. As shown in Fig. 4, HQ II gave growth inhibition of AH 13 cells at a concentration of more than 0.14mM. More than 48 hr's incubation of the cells with HQ II yielded cytolysis, karyorrhexis and pyknosis. Subsequently, macromolecular synthesis in AH 13 cells in culture was examined by the radioactive precursor incorporation method. DNA synthesis examined by using ^{3}H-TdR as a precursor was selectively inhibited by 0.27mM HQ II, while RNA and protein syntheses were slightly affected (Table IV). Fig. 5 represents kinetics of DNA and protein syntheses and effects of HQ II on them. These results may indicate that the inhibition of DNA synthesis by HQ II occurs instantly after addition of the agent but the cessation of DNA synthesis requires at least 2 hr's intervals. In the comparison with the incorporation of ^{3}H-TdR into DNA, as another DNA precursor, ^{3}H-CdR was also examined for its incorporation into DNA. As shown in Table V, the incorporation of ^{3}H-CdR to TCA-insoluble fraction was prevented by HQ II as well as that of ^{3}H-TdR. On the contrary, these radioactivites were accumulated in the acid soluble fractions with 2 or 3 fold amounts of control. The results suggest that the inhibition of DNA synthesis by HQ II may not be resulted from preventing uptake of DNA precursors into the cells but from obstruction of the following process(es).

Reversibility of the inhibitory effect of HQ II on the DNA synthesis and the cellular growth was investigated as follows: After preincubation of the cells with HQ II for several

Table III. Effect of HQ II on DNA Synthesis and Cell Viability of Ascites Hepatoma AH 13 Cells *in Vitro*

Agent	Concentration (μg/ml)	Incubation time (hr) at 37°	^{3}H-TdR[a] incorporated (cpm/cells in 1 ml of medium[b])	Inhibition (%)	Dead cells (%)
None	—	1	6530	—	1.3
		2	12222	—	0.5
DMSO[c]	5 (μl)	1	4745	—	0.8
		2	10477	—	1.9
HQ II	1	1	5584	—	—
		2	8159	—	—
	10	1	4985	—	—
		2	7965	24.0	1.4
	100	1	2103	55.7	3.0
		2	2668	74.5	3.3

a) ^{3}H-TdR, 1 μCi/ml (0.2 μM) *b*) 2.2×10^6 cells/ml of Eagle's medium containing 10% fetal calf serum *c*) dimethyl sulfoxide

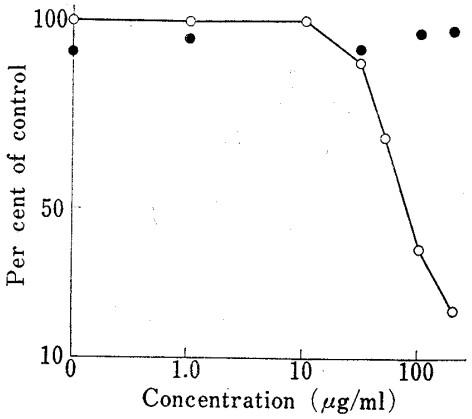

Fig. 3. Effect of HQ II on DNA Synthesis and Cell Viability of Ehrlich Ascites Carcinoma Cells *in Vitro*

2.0×10^6 cells/ml of Eagle's MEM supplemented with 10% fetal calf serum
incubation: 37°, 60 min
○: ^{3}H-TdR incorporation;
●: Trypan blue-excluding cell

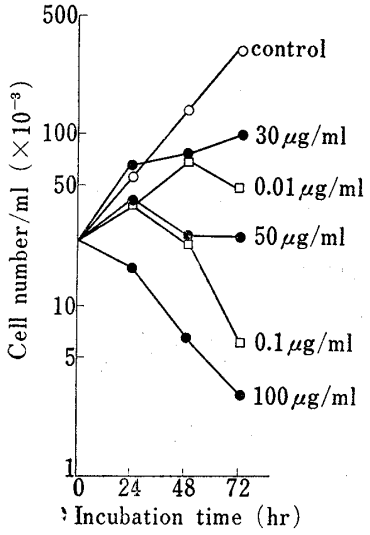

Fig. 4. Effect of HQ II on Growth Profiles of Ascites Hepatome AH 13 Cells in Culture

●: HQ II □: mitomycin C

Table IV. Effect of HQ II on Growth and DNA, RNA and Protein Syntheses of Ascites Hepatoma AH 13 Cells in Culture

Agent	Concentration (μg/ml)	Incubation time (hr)	Cell No. /ml (×10⁻⁴)	Labeled precursor incorporated to TCA-insolubles (% of control)		
				^{3}H-TdR	^{3}H-UR	^{14}C-Leu
None	—	4	15.34	100	100	100
		24	33.75	—	—	—
DMSO	5 (μl)	4	—	98.4	94.3	94.2
		24	34.75	—	—	—
HQ II	50	4	—	27.6	106.9	90.2
		24	26.13	—	—	—
	100	4	—	4.3	133.6	80.2
		24	18.25	—	—	—

AH 13 cells were exposed to HQ II and incubated with a radioactive precursor for 4 hr. ^{3}H-TdR, ^{3}H-UR and ^{14}C-Leu were added to give 1 μCi/ml (0.2 μM), 1 μCi/ml (0.2 μM) and 1 μCi/ml, (0.5 mM), respectively. The radioactivities of control samples of DNA, RNA and protein were 2.3×10^4, 2.3×10^3, and 4.5×10^4 cpm/ml of culture, respectively. All samples were in duplicate.

hr, the cells were washed with EF containing or not containing HQ II and incubated to assay for ^{3}H-TdR incorporation and cell proliferation. As presented in Table VI, washing with the medium without HQ II gave significant restoration of the cell growth and the DNA synthesis. Moreover, the inhibitory effect of HQ II was cancelled by the addition of $FeCl_3$ or $FeSO_4$ to the culture medium. The addition of Fe salts to the medium even at 6 hr after the exposure of the cells to HQ II caused restoration of the cell growth (Table VII). Fig. 6 indicates the profiles of the cellular DNA synthesis and the influences of HQ II, $FeCl_3$ and HQ II plus $FeCl_3$ on them. It is thought from these data that the inhibitory effect of HQ II on the DNA synthesis and the cellular growth is partly due to depriving Fe ions from the medium or the cells (see Discussion).

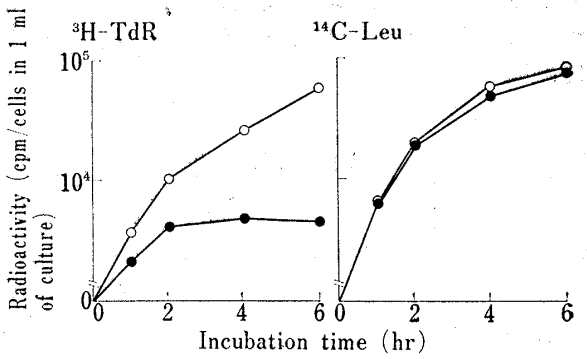

Fig. 5. Effect of HQ II on Macromolecular Synthesis by Ascites Hepatoma AH 13 Cells in Culture

condition, see Table IV ●: HQ II (0.27 mM)
○: DMSO, (5 μl/ml)

Table V. Effect of HQ II on DNA Synthesis and the Acid Soluble Fraction by Ascites Hepatoma AH 13 Cells in Culture

Concentration of HQ II (μg/ml)	Incubation time (hr)	Incorporation to DNA ($\times 10^{-3}$ cpm/ml of culture)		Acid-soluble pool ($\times 10^{-3}$ cpm/ml of culture)	
		^{3}H-TdR[a)]	^{3}H-CdR[b)]	^{3}H-TdR	^{3}H-CdR
0	1	16.7	9.1	7.5	8.3
0	2	31.1	17.6	6.5	13.2
0	4	54.0	29.0	5.1	19.8
100	1	5.2(31.1)[c)]	4.3(47.2)	10.8(144)	16.6(200)
100	2	5.8(18.6)	4.4(25.0)	15.1(232)	34.2(259)
100	4	5.1(9.4)	4.3(14.8)	18.9(371)	62.0(313)

a) ^{3}H-TdR, 1 μCi/ml (0.2 μM) b) ^{3}H-CdR, 1 μCi/ml (0.04 μM) c) Numbers in parentheses are the percentage of control.

Further studies concerning with uptake of ^{3}H-CR to the acid-solubles of AH 13 cells revealed that HQ II inhibited the conversion of ^{3}H-CR into ^{3}H-CdR nucleotides with moderate extent. An anticancer agent, hydroxyurea, which has been reported as a ribonucleoside diphosphate reductase (reductase) inhibitor,[9] inhibited prominently the conversion with no effect on RNA synthesis (Table VIII). The uptake of ^{3}H-CR into the acid soluble fraction was scarcely affected by HQ II in good accordance with the data shown in Table V indicating no inhibition by HQ II of uptake of DNA precursors into the acid-solubles. In turn, in the presence of deoxyribonucleosides with appropriate amounts (see Table IX) in medium DNA synthesis in the cultured cells was not inhibited by HQ II. However, a low concentration of deoxyadenosine (AdR) (2 mM) could not reverse the inhibitory action of HQ II even in the presence of 1 mM CdR and 2 mM deoxyguanosine (GdR). As these results suggested that one inhibitory site of HQ II on the DNA synthesis might be the enzymatic reduction of ribonuleotides to deoxyribonucleotides, the investigation of the conversion of ^{14}C-CDP into ^{14}C-deoxycytidine diphosphate (^{14}C-dCDP) in cell-free extracts of AH 13 cells was carried out.

9) E.C. Moore, *Cancer Res.*, **29**, 291 (1969).

^{14}C-CDP reduction in 100000×*g* supernatant of the cell homogenate was inhibited by HQ II (ID_{50}, *ca.* 0.035 mM) at a less concentration than that of HU (0.38 mM) (Fig. 7).

The results described above may indicate that HQ II inhibits the reductase activity. It is thought that as a result of inhibition of the enzyme activity the production of deoxyribonucleotides, precorsors of DNA synthesis, is stopped, resulting in the cessation of DNA synthesis.

Table VI. Effect of HQ II on DNA Synthesis by Ascites Hepatoma AH 13 Cells in Culture. Reversal of Inhibitory Action of HQ II After Removal of the Agent by Washing

Concentration of HQ II (μg/ml)	Preincubation time (hr)	Washing with medium containing (+) or not containing (−) HQ II	Incubation time (hr)	^{3}H-TdR incorporated to TCA-insolubles (% of control)	Cell No. $\times 10^{-4}$/ml
0	—	—	0	—	20.38
0	—	—	4	100	—
0	—	—	24	—	43.75
100	—	—	4	19.7	—
100	—	—	24	—	26.25
0	0.5	—	4	100	—
100	0.5	+	4	10.7	—
100	0.5	−	4	73.0	—
100	0.5	−	24	—	41.00
0	2	—	4	100	—
0	2	—	24	—	43.25
100	2	+	4	1.6	—
100	2	+	24	—	20.00
100	2	−	4	83.4	—
100	2	−	24	—	45.51

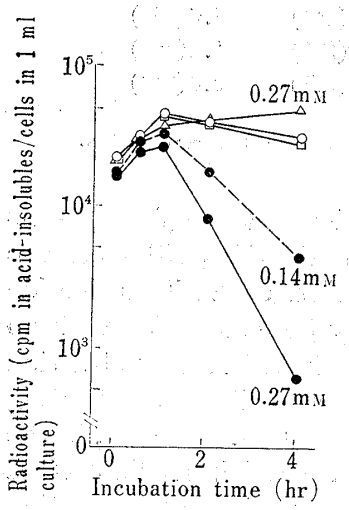

Fig. 6. Influence of $FeCl_3$ on the Inhibition of ^{3}H-Thymidine Incorporation by HQ II in AH 13 Cells

AH 13 cells (3.5×10^5 cells/ml of EF) exposed with HQ II (●): 0.47 mM $FeCl_3$ (□) and both (△) were labeled with ^{3}H-TdR (2 μCi/ml, 0.125 μM) for 30 min. ○: control

Table VII. Effect of $FeCl_3$ on Growth Inhibition of AH 13 Cells by HQ II

Concentration (mM)		Cell number/ml of culture ($\times 10^{-4}$)[a]
HQ II	$FeCl_3$	
—	—	13.45
—	0.14	13.15
—	0.43	12.25
0.14	—	2.10
0.14	0.14	13.50
0.14	0.14[b]	13.70
0.14	0.43	13.70
0.14	0.43[b]	13.40
0.27	—	0.65
0.27	0.01	0.70
0.27	0.14	13.00

a) Incubation time is 72 hr. *b*) added 6 hr after HQ II exposure

Table VIII. Effect of HQ II on the Incorporation of ^{3}H-Cytidine into the Acid-solubles of Ascites Hepatoma AH 13 Cells in Culture and Its Conversion to ^{3}H-Deoxycytidine Nucleotides

Agent	Concentration (mM)	Preincubation time (min)	dpm/ml of culture ($\times 10^{-3}$)			
			Acid-solubles	Deoxycytidine	RNA	DNA
—	—	30	92.1(100)	1.73(100)	84.6(100)	1.38(100)
DMSO	(5 μl/ml)	30	93.6(102)	1.57(91)	67.6(80)	—
HQ II	0.08	30	89.0(97)	1.75(101)	87.0(103)	—
	0.27	30	89.5(97)	1.48(86)	86.7(102)	0.65(47)
HU[a]	2.0	30	90.0(98)	0.34(20)	73.1(86)	0.20(14)
—	—	60	103.0(100)	2.55(100)	102.2(100)	1.44(100)
DMSO	(5 μl/ml)	60	103.0(100)	2.67(104)	101.7(100)	1.34(93)
HQ II	0.08	60	105.0(102)	2.25(88)	101.2(99)	1.26(88)
	0.27	60	110.0(107)	1.65(65)	92.6(91)	0.62(43)
HU	2.0	60	121.0(117)	0.76(30)	104.5(102)	0.25(17)

a) hydroxyurea
AH 13 cells were suspended in Eagle's MEM containing 20% fetal calf serum in a cell density of 1.6×10^6 cells/ml. The cells, after exposure to the indicated amounts of agents for 30 min or 60 min, were incubated with ^{3}H-CR (2 μCi/ml, 0.45 μM) at 37° for 60 min.

Table IX. Reversal of HQ II Inhibition by Deoxyribonucleosides

XdR added to medium[a] (mM)				Incubation time (hr)	^{3}H-TdR incorporated to TCA-insolubles[b] (cpm/cells in 1 ml of culture)		
TdR	CdR	AdR	GdR		None	HQ II (100 μg/ml)	% of control
0.02	—	—	—	2	410	189	46.1
				4	1093	198	18.2
0.02	0.1	—	—	2	446	187	41.9
				4	1411	202	14.3
0.02	0.1	7.0	—	2	134	114	85.4
				4	259	136	52.5
0.02	0.1	7.0	2.0	2	147	188	127.9
				4	286	409	142.9
				24	1411	1552	110.0

a) Eagle's MEM containing 20% fetal calf serum *b*) ^{3}H-TdR, 1 μCi/ml of culture

Discussion

HP is an anticancer Schiff base which has been exploited in this institute and its activity needed 3 functional groups as described in the previous paper.[3] HQ II, HP analogue, has the 3 functional groups in the chemical structure and metal chelate-forming activity too. Furthermore, HQ II is more stable and more anticancer active than HP.[4]

From the attemps to clear a feature of mechanism of action of HQ II on cancer cells, following were found out. Firstfy, it was confirmed that HQ II caused cell injury in medium containing no serum.[4] Under the condition, the nucleic acid synthesis was inhibited and the effect of HQ II was appeared to prefer on direct cell injury.[4] It is of interest that in the presence of appropriate amounts of serum (horse, bovine, calf and fetal calf) the cell injury by HQ II is not seen no longer. Serum concentration which requires for compensating the inhibitory effect of HQ II is up to 10% in the case of AH 13 cells and at least 0.1% v/v in the case of EA cells. However, even in the presence of 10% or more of serum HQ II caused inhibition of the DNA synthesis. Besides, bovine serum albumin (Armour) does give disappearance of the direct cell injury effect of HQ II. Secondly, the growth of AH 13 cells in culture were inhibited by adding HQ II (0.14 mM) to the culture medium (Eagle's minimum essential medium supplemented with 20% fetal calf serum) and the cellular DNA synthesis

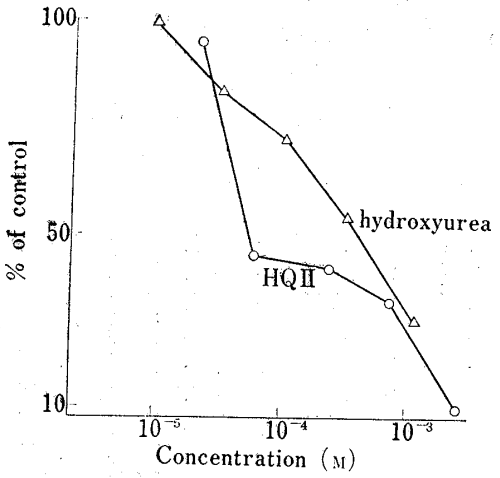

Fig. 7. Effect of HQ II and Hydroxyurea on the Reduction of ^{14}C-CDP by a Cell-free Extract of AH 13 Cells

AH 13 extract (350 μg of protein) was incubated for 60 min at 37° in a total volume of 125 μl with 4.2 mM Mg acetate, 6.2 mM DTE, 8.3 mM NaF, 4.4 mM ATP, 8.3 mM K phosphate (pH 7.0) and 0.4 mM ^{14}C-CDP (0.1 μCi). Activity was measured as the CdR recovered after hydrolysis.

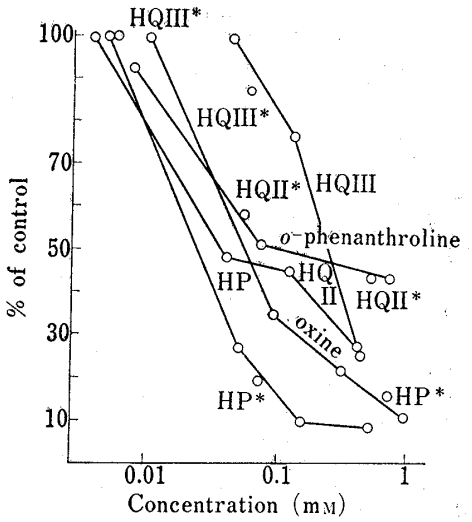

Fig. 8. Effect of Several Agents upon the Reduction of ^{14}C-CDP by a Cell-free Extract of AH 13 Cells

* no *n*-hexyl group

was selectively inhibited with no effect on the RNA and protein syntheses. It should here be noted that in the marked contrast the cellular RNA synthesis in procaryotic *Bacillus subtilis* was preferentially inhibited by HQ II.[4] Reversal of HQ II effect on the cellular DNA synthesis and the cell proliferation by washing out the agent with the fresh medium or by adding $FeCl_3$ or $FeSO_4$ with its appropriate amount to the culture medium may suggest that the inhibitory effect of HQ II is ascribed to depriving Fe^{2+} or Fe^{3+} from the culture medium or from the cells, in which Fe is dynamic equilibrium with extracellular Fe, resulting in disruption of the function(s) of Fe-requiring enzyme(s) involved in DNA synthesis. All tested ions including Cu^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+} and Zn^{2+} were not effective to restore the inhibitory effect of HQ II. Recently, Robbins, *et al.*[10] have suggested that there might be a shift in Fe salts during the cell cycle in cultured HeLa cells. They have also reported that addition of Fe-chelating agent, Desferrioxamine or Desferal to culture medium causes the selective inhibition of Fe uptake into the cells and the selective suppression of DNA synthesis. HQ II resembles these chelators with respect to Fe-chelating ability. Thirdly, 0.27 mM HQ II did not yield any effect on uptake of ^{3}H-CR to the acid-solubles and its incorporation to RNA but the conversion of it into ^{3}H-CdR nucleotides and its incorporation to DNA was moderately inhibited. These data and the resumption of the DNA synthesis in the cultured cells by the addition of certain amounts of deoxyribonucleosides suggest that the reductase may be the site of action of HQ II. The activity of the reductase of the cell-free extracts was also interfered by HQ II and its related compounds (Fig. 8).

It is known that Fe has a role in the enzymatic reduction of ribonucleotides by the reductases of *Escherichia coli*[11] and mammalian cells.[9] An Fe-chelate-forming and anticancer agent, 5-hydroxy-2-formylpyridine thiosemicarbazone (NSC-107, 392, 5-HP), inhibits the reductase activity and its inhibition is partially reversed by Fe ion. Therefore, it is thought that 5-HP has share in the chelating ability and interferes with the enzyme system by forming

10) *a*) E. Robbins and T. Pederson, *Proc. Natl. Acad. Sci. U.S.*, **66**, 1244 (1970); *b*) E. Robbins, J. Fant, and W. Norton, *ibid.*, **69**, 3708 (1972).
11) N.C. Brown, R. Eliasson, P. Reichard, and L. Thelander, *European J. Biochem.*, **9**, 512 (1969).

chelate with Fe which is necessary for the enzyme activity.[12] Fe chelate-forming HQ II may be thought to have the same behavior as 5-HP. As presented in Fig. 8, among several tested compounds relating with HQ II, 2-(2-hydroxyphenyliminomethyl)phenol (HP*), HP, 2-(2-hydroxyphenyl)-8-quinolinol-4-carboxylic acid (HQ II*) and 8-hydroxyquinoline (oxine) prevented severely ^{3}H-CdR production, while 2-(2-hydroxy-5-*n*-hexylphenyl)quinoline-4-carboxylic acid (HQ III)[4] with less metal chelate-forming ability was shown to be a weak inhibitor of the reductase.

It was observed that HP, HP*, HQ II* and oxine exhibited prominent inhibition of the cell growth of AH 13 cells in culture. It seemed that the inhibition of the cell proliferation and the reductase activity by HQ II-related compounds without-*n*-hexyl group and HP* indicated no efficiency of hexyl group *in vitro*- or in culture-experiments.

Finally, it may be summarized as that cell membrane injury and on the other hand selective inhibition of DNA synthesis resulting from interaction with extra and/or intracellular Fe (in which ribonucleotide reductase may be involved.) would be the mechanism of action of HQ II.

The other role of Fe in DNA synthesis requires further examinations.

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12) E.C. Moore, B.A. Booth, and A.C. Sartorelli, *Cancer Res.*, **31**, 235 (1971).