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2-(2-Hydroxy-5-*n*-hexylphenyl)-8-quinolinol-4-carboxylic Acid and Its Related Compounds—Anticancer and Other Biological Activities[1]

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2-(2-Hydroxy-5-*n*-hexylphenyl)-8-quinolinol-4-carboxylic acid (HQ II), as an analogue of 2-(2-hydroxyphenyliminomethyl)-4-*n*-hexylphenol (HP) which exhibits anticancer activity, was prepared by the method of Doebner synthesis. HQ II, having the ability to form metal complexes with several metallic ions as well as HP, inhibited prominently the growth of Ehrlich ascites carcinoma and ascites hepatoma AH 13 in animals. However, it was not effective against L1210, solid tumors of both Sarcoma 180 and Ehrlich carcinoma and Yoshida sarcoma.

From the results of preliminary examinations which were carried out to obtain some information concerning the mode of action of HQ II on cancer cells, it was suggested that the cell membrane injury and the inhibition of nucleic acid synthesis might be contributable to the HQ II effect on the susceptive cancer cells.

4-*n*-Hexyl-6-(2-hydroxyphenyliminomethyl)resorcinol (HR) and 2-(2-hydroxyphenyliminomethyl)-4-*n*-hexylphenol (HP) have been shown to exhibit anticancer effect against some kinds of rodent tumors *in vivo*.[3] The data obtained in structure-activity studies have revealed the necessity of azomethine bond which links two phenyl rings, of both hydroxy groups which are situated in *ortho* position of each of both phenyl rings, and of alkyl radical (carbon chain length, 6 to 8) in the molecule.[3]

HR and HP are unstable in aqueous solutions,[4] and the candidate hydrolysis products of HR, 2,4-dihydroxy-5-*n*-hexylbenzaldehyde and *o*-aminophenol, are far less effective than the original compound.[3a] Therefore, it is reasonable to assume that the compounds, which have the three functional groups in HP structure and are not labile against hydrolysis, may exhibit prominent anticancer activity.

Then, as an attempt in this line of works, 2-phenylquinoline structure was designed, a series of quinoline derivatives including 2-(2-hydroxy-5-*n*-hexylphenyl)-8-quinolinol (HQ I) and 2-(2-hydroxy-5-*n*-hexylphenyl)-8-quinolinol-4-carboxylic acid (HQ II) were prepared, and their anticancer activities were tested in comparison with that of HP. Additionally, some other biological properties of these compounds were investigated for the purpose of obtaining some information about the mode of action on cancer cells.

This paper is the presentation in a program to characterize the structure-activity relationships of quinoline derivatives.

Results

Preparation of 2-Phenylquinolines

As representative examples of preparation, the preparative procedures of HQ I and II were described below. Doebner synthesis by equimolar *o*-aminophenol and 5-*n*-hexylsalicyl-

1) This work was partly presented at the 34th Annual Meeting of Hokuriku Branch, Pharmaceutical Society of Japan, June, 1972. The work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education.

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

3) *a*) T. Ujiie, *Ann. Rep. Cancer Inst. Kanazawa*, **1**, 109 (1967); *b*) T. Ujiie, *Chem. Pharm. Bull.* (Tokyo), **16**, 165 (1968).

4) T. Ujiie, *Chem. Pharm. Bull.* (Tokyo), **16**, 2488 (1968).

aldehyde with pyruvic acid did produce but little HQ II. However, the reaction of O-benzylated *o*-aminophenol and O-benzylated 5-*n*-hexylsalicylaldehyde with pyruvic acid gave O,O'-dibenzylated HQ II (V) with 20% yield, and its debenzylation with HBr afforded HQ II with the yield of 89%. Heating of V in quinoline in the presence of Cu-chromite catalyst or heating in nitrobenzene yielded O,O'-dibenzylated HQ I (VII), following debenzylation afforded HQ I. Other 2-phenylquinolines were also prepared by the method described above with some modifications. The chemical structures of these newly prepared compounds were confirmed by chromatography, elemental analyses and spectral data (see Experimental).

Metal Chelation of HQ I and II

Spectrometrical analyses have revealed that HP forms chelate compounds with several metallic ions including Cu^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Ag^{1+}, Pb^{2+} and Zn^{2+}.[4] Similarly, HQ I and II were shown to have interactions with these ions. Moreover, HQ I and II as well as HP did form chelate compounds with Cu^{2+} (1: 1), as shown in Fig. 1. The data suggest that HQ I and II are likely to have a spatial structure being similar to that of HP. In contrast, no interaction with metallic ions except Fe^{3+} was observed in the case of 2-(2-hydroxy-5-*n*-hexylphenyl)quinoline-4-carboxylic acid (HQ III).

Anticancer Effect of Several 2-Phenylquinolines

***In Vitro* Experiments**——For examining anticancer activities of the compounds against Ehrlich ascites carcinoma (EA) cells *in vitro*, 7-day-old tumor cells were suspended to a cell density of 10^7 cells per ml of PBS containing 100 μg of an agent. After 1 hr-incubation at 37°, the response to the cancer cells was examined by measuring the extent of cell death, which was followed by exclusion of dyestuff, nigrosine. As a result, 2-phenylquinolines having *n*-hexyl moiety were toxic against the cells, and the treatment with HQ I, II and III caused 20% or more cell killing without cell lysis. On the contrary, no significant response was observed in the cases of 2-phenylquinolines without *n*-hexyl moiety and of 8-hydroxyquinoline (oxine). While, it was shown that HP had a more potent action (47% cell killing) than HQ I, II and III.

***In Vivo* Experiments**——For examining *in vivo* effect of these compounds, 5-day-old tumor bearing mice were administered by the stated dose of the compound to be tested. Pertioneal fluids were quantitatively collected 24 hr later, and total number of the cancer cells and the viability were measured. As indicated in Table I, HQ II was more active than

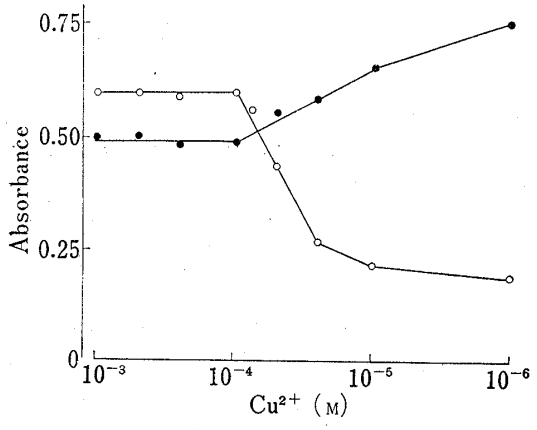

Fig. 1. Complex Formation of HQ II with Various Concentrations of Cu^{2+}

—●—: A_{384nm}, λ_{max} of HQ II
—○—: A_{414nm}, λ_{max} of Cu-HQ II complex

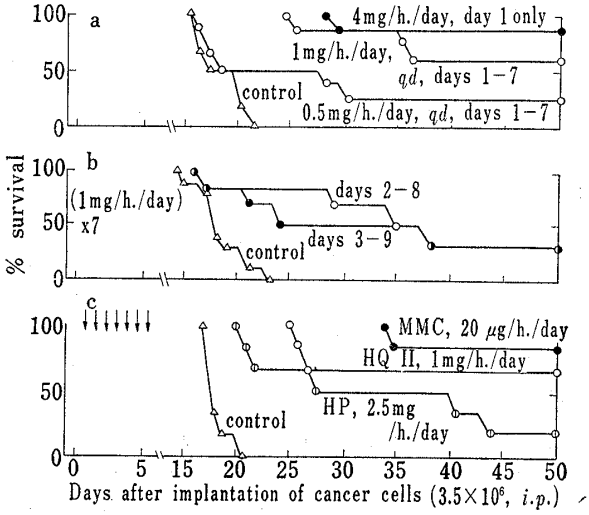

Fig. 2. Effect of HQ II on the Survival Time of Mice Bearing Ehrlich Ascites Carcinoma Cells

h.=head

Table I. Toxicity of HQ II to Ehrlich Ascites Carcinoma *in Vivo*

Compound	Dose mg/head, *i.p.*	Total cells/head ($\times 10^{-6}$)		Viability (%)
HQ II	1.0	212	(10)[a]	98.1
	2.0	53	(14)	82.4
HQ III	10.0	108	(4)	96.9
HP	2.5	215	(8)	98.7
	5.0	161	(5)	97.6
MMC	0.02	229	(5)	98.4
	0.04	182	(5)	96.4
Oxine	0.5	234	(6)	97.0
CMC (control)		232	(12)	97.2

a) number in parenthesis indicates that of mice used

HQ III, HP and Mitomycin C (MMC) in causing the cell growth inhibition. Unsuccess of preparation of suspension of HQ I for administration was forced to give up testing of its effect.

Effect of HQ II on Life Prolongation of Animals Bearing Tumor Cells

a) Ehrlich Ascites Carcinoma——HQ II treatment caused a prolongation of survival time of dd mice bearing EA cells. As presented in Fig. 2a, 7 consecutive intraperitoneal administration of HQ II (1.0 mg/head/day), being initiated 24 hr after tumor cell implantation (3.5×10^6 cells/head, *i.p.*), gave the 60—70% survivors of longer than 50 days in the treated mice, while the untreated control mice were all died within 25 days after tumor implantation. 90% or more survivors were expected in mice treated with single injection of 4.0 mg/head at Day 1. Even when initial treatment was started 48 hr or 72 hr after tumor implantation, the prolongation of life-span was apparently observed as shown in Fig. 2b. Comparative experiments with HQ II, HP and MMC under these optimum doses revealed that the activity of HQ II was over that of HP, but was less effective than that of MMC (Fig. 2c). Oral administration of HQ II instead of peritoneal route resulted in no effectiveness (data not presented). It must be noted here that in the experiments with HQ III, which has no obvious chelating activity, 2-(2-hydroxyphenyl)-8-quinolinol-4-carboxylic acid (III), 2-phenylquinoline-4-carboxylic acid (cinchophene) and oxine, no apparent increase of survival days occurred even at sub-toxic doses (data not presented).

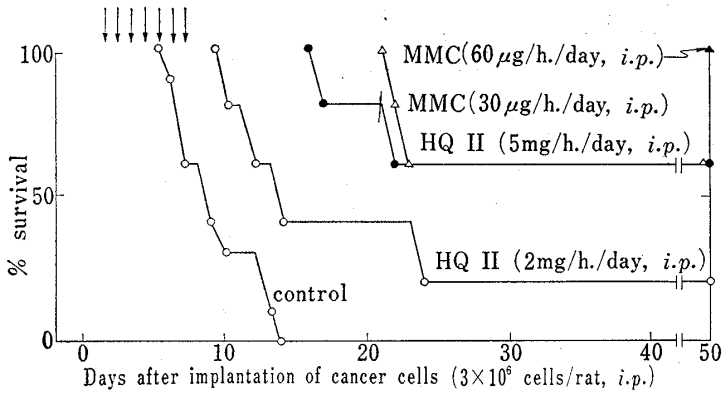

Fig. 3. Effect of HQ II on the Survival Time of Rats with Ascites Hepatoma AH 13

h.=head

b) Ascites Hepatoma AH 13——From these results, HQ II was selectively employed for further evaluation of the anticancer activity. Fig. 3 shows the results of growth inhibition of ascites hepatoma AH 13 (AH 13) in Donryu rats treated with HQ II. The treatment with HQ II was initiated the next day after tumor cell implantation (3.0×10^6 cells/head), being administered *i.p.* daily 7 successive days. At a dose of 5.0 mg/head/day, 60% of the HQ II-treated rats were survived longer than 50 days after implantation, while rats receiving 0.5% carboxymethyl cellulose (CMC) alone, a vehicle of HQ II, died with a mean time of 10 days. With HP at a dose of 5.0 mg/head/day, similar results as shown in HQ II were obtained. For positive control, the treatment with MMC had a 100% survival rate.

c) **Mouse Lymphoid Leukemia L1210**——There was no delay in the death of BDF_1 mice inoculated with 1.5×10^5 cells/head intraperitoneally and treated with HQ II (1.0 mg/head/day, *i.p.*, *qd*, Day 1—7), when compared with controls (mean survival day, 8.5).

Effect of HQ II on the Growth of Subcutaneous Tumor

a) **Sarcoma 180 Solid Form**——Mice bearing subcutaneous tumor of Sarcoma 180 (S180) did not respond to intraperioneal and oral administration of HQ II, whereas, 65—75% growth inhibition occurred with *i.p.* or oral administration of 6-mercaptopurine (6-MP). Intraperitoneal treatment with MMC gave a result of 37.5% inhibition of the tumor growth.

b) **Ehrlich Carcinoma Solid Form**——In contrast to the effect of HQ II on the ascites form of Ehrlich carcinoma, the solid form implanted subcutaneously into left groin of mice were scarcely affected by HQ II (data not presented).

Although HQ II was not effective against L1210 and both solid tumors of S180 and Ehrlich carcinoma, it did prominently inhibit the growth of EA and AH 13. Therefore, further structure-activity studies in this line were thought to be warranted.

Then, for the purpose of obtaining some information about the action mechanism of HQ II on cancer cells, the effect of HQ II on nucleic acid synthesis in EA cells, on rabbit erythrocytes and on growth profiles and macromolecular synthesis in *Bacillus subtilis* were preliminarily examined.

Effect of HQ II on Nucleic Acid Synthesis in Ehrlich Carcinoma Cells *in Vitro*

Fig. 4 represents the data showing the effects of HQ II, 2-(2-(5-nitro-2-furyl)vinyl)-8-(β-(N,N-diethylamino)ethoxy)quinoline (NQ-DEAO),[5] L-arabinosyl cytosine (Ara-C) and MMC on nucleic acid synthesis in the cancer cells *in vitro*. At the concentrations which did not cause obvious cell death, HQ II inhibited equally the incorporation of ^{14}C-thymidine and ^{3}H-uridine into trichloroacetic acid (TCA)-insolubles of the cells. The same inhibitory effect as HQ II but at smaller amounts was observed by NQ-DEAO, which has been reported

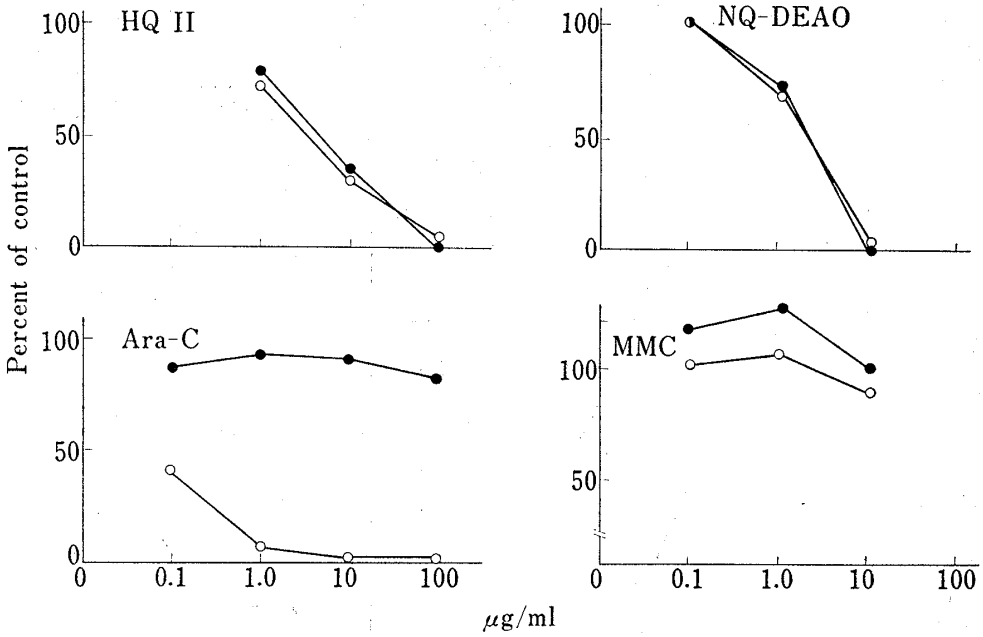

Fig. 4. Effects of Various Compounds on Incorporation of ^{14}C-Thymidine (○) and ^{3}H-Uridine (●) into Acid-insolubles in Ehrlich Ascites Carcinoma Cells *in Vitro*

5) T. Ujiie, *Chem Pharm. Bull.* (Tokyo), **22**, 2470 (1974).
6) T. Ujiie, Reported at the Annual Meeting of Hokuriku Branch, Pharmaceutical Society of Japan, Kanazawa, October, 1969.

by the author to have anticancer activity[5] and to cause the selective inhibition of DNA synthesis in *Escherichia coli* B (*E. coli*).[6] In the case of MMC, no prominent effect was produced, presumably, because of necessity of more longer incubation period to bring about any influences. While, thymidine incorporation was predominantly suppressed by Ara-C.

Effect of HQ II on Rabbit Erythrocytes *in Vitro*

To investigate influences of 2-phenylquinolines on cell membranes, some experiments were made with rabbit erythrocytes. Incubation for 30 min of the erythrocyte suspension (1.7×10^8 cells/ml of PBS) containing HQ II at 50 μg/ml gave 13.2% hemolysis, at 100 μg/ml gave 85.4% hemolysis. While, HQ III, which is less active than HQ II in anticancer activity, gave 81.5% hemolysis at the concentration of 20 μg/ml (Table II).

Table II. Hemolytic Activity of HQ II on Rabbit Erythrocytes

Compound	Concentration μg/ml	Hemolysis (%) 1.7×10^8 cells/ml
HQ II	0	2.4
	25	4.7
	50	13.2
	100	85.4
Cinchophene	100	5.2
X	100	5.2
HQ I	20	87.5
	100	100
HQ III	20	81.5
	100	98.1
HP	5	4.9
	10	9.7
	25	100
	50	98.0
	100	97.4
SDC[a]	250	6.6
	500	94.0

a) sodium desoxycholate

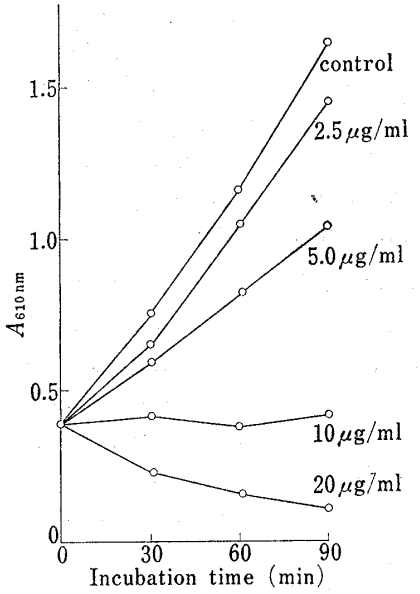

Fig. 5. Effect of HQ II on Growth of *B. subtilis*

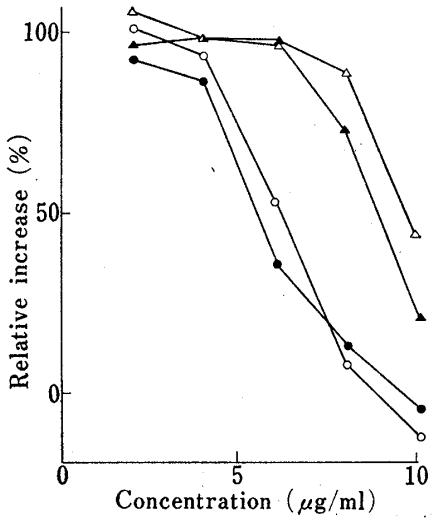

Fig. 6. Effects of HQ II on Nucleic Acids and Protein Syntheses in *B. subtilis*

○—○: viability ●—●: RNA
△—△: DNA ▲—▲: protein

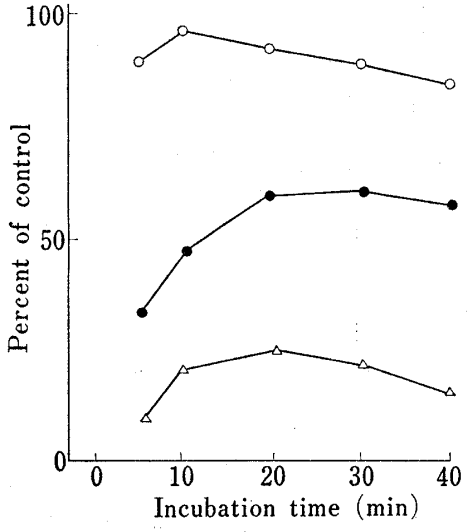

Fig. 7. Effects of HQ II on Nucleic Acids and Protein Syntheses in *B. subtilis*

—○—: DNA (TdR)
—●—: protein (Leu)
—△—: RNA (UR)

Effect of HQ II on *Bacillus subtilis*

On antimicrobial effects of the series of compounds, all compounds tested were not inhibitory against the cell growth of *E. coli* excepting for oxine, which inhibited the growth at 50 μg/ml. While, among 2-phenylquinolines tested HQ II only was shown to be inhibitory against *Bacillus subtilis* W168 (*B. subtilis*), and the cell lysis was observed at 20 μg/ml of HQ II (Fig. 5). Then, to determine whether or not the antibacterial activity of HQ II is related with the syntheses of macromolecular materials, the amounts of desoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein in growing bacteria were assayed by the modified Schmidt-Thannhauser's method. As a result, RNA synthesis was preferentially inhibited in contrast to the results obtained in the experiment with the cancer cells (Fig. 6). The study of the incorporation of radioactive precursors (^{14}C-thymidine, ^{14}C-uracil and ^{14}C-L-leucine) indicated that uracil uptake was affected to a greater extent than thymidine and leucine incorporation by 8.0 μg of HQ II per ml (Fig. 7). The result is in accordance with the above described data at the point of the preferential inhibition of RNA synthesis. In addition, RNA synthesis catalyzed by RNA polymerase of *E. coli* was severely inhibited by HQ II at 0.11 mM (Table III). It suggests that RNA synthesis inhibition by HQ II might depend on blockage of RNA polymerase(s).

TABLE III. Effect of HQ II on RNA Synthesis by RNA Polymerase of *E. coli*[a)]

Concentration (mM)	^{3}H-UMP incorporated[b)] nmole/10 min	% inhibition
0	4.76	0
0.01	4.83	−1.5
0.11	0.84	82.4
0.27	0.19	96.0
1.11	0.03	99.4

a) data presented by Dr. A. Matsukage of this institute *b*) 0.25 ml per tube of reaction mixture contains Tris-HCl (pH 7.8 at 37°), 30 μmole; Mg acetate, 1.25 μ mole; Mn sulfate, 0.5 μmole; β-mercaptoethanol, 1.25 μmole; ATP, GTP, CTP, 0.02 μmole each; ^{3}H-UTP (5000 cpm per nmole), 0.02 μmole; DNA (T_4 phage), 15 μg; RNA polymerase (5300 units per mg protein), 9.4 μg. The reaction mixture was incubated at 37° for 10 min, and then 0.25 ml of 4 mg per ml bovine serum albumin solution and 0.5 ml of ice chilled 10% TCA were added to the reaction mixture. After maturation, precipitate was washed by cold 5% TCA (2 ml×3 times), dissolved in 0.4 ml of 2N NH_4OH and transferred into a vial. 10 ml of a PPO- dioxane scintillation mixture was added and the radioactivity was measured (A. Matsukage, *Molec. gen. Genet.*, **118**, 11 (1972)).

Discussion

HQ II, which is similar to HP in respect of having the three functional groups in its chemical structure and of forming metallic complexes with several metallic salts, but which is dissimilar to HP in respect that it can exist in stable state in aqueous solution, is effective in prolongation of life-span of animals inoculated with EA and AH 13 cells. The results of anticancer experiments with EA indicate that HQ II is more active than HP. However, HQ II was not found to be effective in growth inhibition of L1210, both solid tumors of EA and S180 and Yoshida sarcoma (YS) in animals.

From the data of preliminary experiments performed to explore the action mechanism of anticancer activity of HQ II, it was firstly suggested that cell membranes were a site of action of HQ II, considering the results of hemolysis tests and dye exclusion tests. Secondly, nucleic acid synthesis machinery may be the next site of HQ II attack. Now, L1210 which is inert to HQ II *in vivo* is sensitive *in vitro* to the drug in that the nucleic acid synthesis is inhibited and the dye-staining reaction become positive. The discrepancy between *in vitro* and *in vivo* data awaits further study for explanation. Since only slight inhibition of cancer growth was observed by the treatment with HQ III which did not interact with metallic

salts except Fe salts, it was assumed that metal complex-forming ability of HQ II would be associated with the anticancer activity.

Recently, Hart, *et al.*[7] have reported that tumor affinitive gallium salts inhibit the tumor growth of Walker 256 carcinosarcoma and some other kinds of tumors in animals. Therefore, it was expected that co-administration of HQ II, which reacts with Ga^{3+} to form metal complexes, and $Ga(NO_3)_3$ might give a synergistic anticancer effect. However, in this line of experiments no effectiveness was observed. It is natural to suppose that the anticancer activity of HQ II may partly depend on its surfactant activity. Indeed, a surface active agent, sodium dodecyl sulfate, was also found to act on the cancer (EA) growth, resulting in life prolongation of the cancer bearing animals.

Finally, the preferential inhibition by HQ II of RNA synthesis in *B. subtilis* prompted further examinations of effect of HQ II on nucleic acid synthesis in cancer cells.

The detailed investigations about the mode of action of HQ II presented results indicating that HQ II did selectively inhibit DNA synthesis in AH 13 cells in their primary culture. In the following paper these results will be dealt with.

Experimental

Preparation of Chemicals[8]

2-(2-Benzyloxyphenyl)-8-benzyloxyquinoline-4-carboxylic Acid (I)——To a mixture of O-benzylsalicylaldehyde (7.1 g) and pyruvic acid (2.93 g) in abs. EtOH (30 ml) was added, under stirring at room temperature, O-benzyl-*o*-aminophenol (6.4 g) in abs. EtOH (40 ml) during 30 min. After more 2 hr's stirring, refluxing was continued for 16 hr long. Distillation *in vacuo* gave a reddish brown oily residue, treatment of which with $(C_2H_5)_2O$ and dil. NaOH, and then neutralization of an alkaline solution with AcOH gave yellowish orange powders. Crystallization from C_6H_6 gave yellow needles (4.5 g), mp 187—188°. Yield, 28.2%. UV λ_{max}^{EtOH} nm (log ε): 265 (4.39), 332 (3.90). *Anal.* Calcd. for $C_{30}H_{23}O_4N$: C, 78.07; H, 5.02; N, 3.04. Found: C, 77.66; H, 4.90; N, 3.15.

2-(2-Benzyloxyphenyl)-8-benzyloxyquinoline (II)——a) A mixture of I (5.0 g) and Cu-chromite catalyst (1.0 g) in anhydrous quinoline (15 ml) was heated at 230° for 30 min. After cooling, addition of C_6H_6 (100 ml) to the reaction mixture, filtration, removal of quinoline with 10% HCl and evaporation of C_6H_6 yielded a viscous liquid, which was subjected to chromatography on an Al_2O_3 (50 g) column using C_6H_6 as the solvent to give orange plates (3.6 g), mp 113—114°. Yield, 80%. UV λ_{max}^{EtOH} nm (log ε): 248 (4.39), 263 (4.47), 293 (3.95), 318 (3.96). *Anal.* Calcd. for $C_{29}H_{23}O_2N$: C, 83.43; H, 5.55; N, 3.36. Found: C, 83.59; H, 5.72; N, 3.18.

b) I (5.0 g) dissolved in anhydrous nitrobenzene (50 ml) was refluxed for 2 hr on an oil bath. The reaction mixture was evaporated to dryness *in vacuo*, and the residue dissolved in $CHCl_3$, after removal of alkali-solubles, afforded orange plates (3.7 g), mp 113—114°. Yield, 82%.

2-(2-Hydroxyphenyl)-8-quinolinol-4-carboxylic Acid (III)——A mixture of I (3.0 g), Ac_2O (6 ml), AcOH (8 ml) and 47% HBr (8 ml) was heated under refluxing for 5 hr. After cooling and evaporation *in vacuo*, the residue was dissolved in dil. ammonia. Neutralization of the filtrate with AcOH gave a yellow precipitate. Crystallization from AcOH gave fine yellow plates (1.6 g), mp>290°. Yield, 87%. UV λ_{max}^{EtOH} nm (log ε): 278 (4.62), 318 (4.06). *Anal.* Calcd. for $C_{16}H_{11}O_4N$: C, 68.32; H, 3.94; N, 4.98. Found: C, 68.23; H, 3.94; N, 4.93.

2-(2-Hydroxyphenyl)-8-quinolinol (IV)——Alternative addition of II (5.0 g) in anhydrous $(C_2H_5)_2O$ (100 ml) and metallic sodium (3.0 g, in small pieces) to liquid ammonia (300 ml) maintaining at −50° was made under stirring during 2 hr. After further 2 hr's stirring and evaporation of ammonia, the residue was extracted with $(C_2H_5)_2O$ and H_2O, and acidification of water layer with AcOH afforded pale brown powders (2.8 g), which were subjected to chromatography on a Polyamide (30 g) column (2.0×25 cm) using EtOH as the solvent to give reddish orange needles (2.1 g), mp 218—221° (from EtOH or C_6H_6), (lit. mp 219—221°).[9] Yield, 74%. UV λ_{max}^{EtOH} nm (log ε): 269 (sh) (4.57), 276 (4.62), 319 (3.97), 340 (3.97). *Anal.* Calcd. for $C_{15}H_{11}O_2N$: C, 75.93; H, 4.67; N, 5.90. Found: C, 76.17; H, 4.76; N, 6.25.

2-(2-Benzyloxy-5-*n*-hexylphenyl)-8-benzyloxyquinoline-4-carboxylic Acid (V)——After stirring a mixture of 2-benzyloxy-5-*n*-hexylbenzaldehyde (55 g), pyruvic acid (20 g) and abs. EtOH (200 ml) at room temperature for 2 hr, to the mixture was under stirring added dropwisely O-benzyl-*o*-aminophenol (37 g) in abs. EtOH (200 ml) during 2 hr. The reaction mixture was made additional 10 hr's stirring and subsequently

7) M.M. Hart and R.H. Adamson, *Proc. Natl. Acad. Sci. U.S.*, **68**, 1623 (1971).
8) Melting points were uncorrected.
9) J.L. Towle, *Iowa State Coll. J. Sci.*, **26**, 308 (1952) [*C.A.*, **47**, 8063*c* (1953)].

refluxing for 30 hr. Evaporation *in vacuo* to dryness, extraction of the residue with dil. NaOH and $(C_2H_5)_2O$, and acidified water layer gave oily precipitates, which were digested with $(C_2H_5)_2O$ to give $(C_2H_5)_2O$-insoluble pale yellow prisms (2.3 g) (from EtOH), mp 247° (V′) and $(C_2H_5)_2O$-soluble pale yellow wax like needles (11.2 g), mp 182—183° (V). V: Yield, 11.1%. UV λ_{max}^{EtOH} nm (log ε): 264 (4.30), 333 (3.78). *Anal.* Calcd. for $C_{36}H_{35}O_4N$: C, 79.24; H, 6.47; N, 2.57. Found: C, 79.47; H, 6.46; N, 3.11. V′: UV λ_{max}^{EtOH} nm: 247, 321. IR ν_{max}^{KBr} cm^{-1}: 3,465, 2,440, 1710, 1605, 1515, 1380, 1255, 1205, 1175, 1005, 825, 760, 715, 705. *Anal.* Found: C, 73.88; H, 5.19; N, 4.92.

2-(2-Hydroxy-5-*n*-hexylphenyl)-8-quinolinol-4-carboxylic Acid (VI, HQ II)——A mixture of V (3.0 g), Ac_2O (6 ml), AcOH (8 ml) and 47% HBr (10 ml) was heated under refluxing for 5 hr. The reaction mixture was evaporated *in vacuo* to afford an orange product, which was crystallized from AcOH to give fine yellow needles (1.8 g), mp 235—238°. Yield, 89%. UV λ_{max}^{EtOH} nm (log ε): 278.5 (4.52), 334 (3.85), 360 (3.83). *Anal.* Calcd. for $C_{22}H_{23}O_4N$: C, 72.31; H, 6.34; N, 3.83. Found: C, 71.94; H, 6.38; N, 3.92.

2-(2-Benzyloxy-5-*n*-hexylphenyl)-8-benzyloxyquinoline (VII)——Decarboxylation of V was made by heating the mixture of V (5.0 g) dissolved in anhydrous quinoline (15 ml) and Cu-chromite catalyst (1.0 g) at 230° for 1 hr. Addition of C_6H_6 (100 ml) to the reaction mixture, removal of insolubles by filtration and of quinoline by extraction with 10% HCl and evaporation of C_6H_6 gave a dark brown oily product, which was chromatographed on an Al_2O_3 column (2.0 × 20 cm) using C_6H_6 as the elution solvent to give pale yellow needles (2.8 g) (from pet. ether), mp 68—71°. Yield, 76%.

2-(2-Hydroxy-5-*n*-hexylphenyl)-8-quinolinol (VIII, HQ I)——VII (5.0 g) in anhydrous $(C_2H_5)_2O$ (50 ml) was added under stirring to well cooled-liquid ammonia (400 ml). To the mixture metallic sodium (total amount, 3.0 g) in small pieces was added at −60° under stirring during 2.5 hr. After evaporation of ammonia, the residue was extracted with $(C_2H_5)_2O$ and H_2O. Acidified water layer was extracted with $(C_2H_5)_2O$. Evaporation of $(C_2H_5)_2O$ afforded a dark brown viscous oil (2.95 g). Crystallization from ligroin gave fine yellow needles (2.5 g), mp 154—155°. Yield, 78%. UV λ_{max}^{EtOH} nm (log ε): 269(sh) (4.64), 278 (4.69), 325 (4.07), 345 (4.09). *Anal.* Calcd. for $C_{21}H_{23}O_2N$: C, 78.47; H, 7.21; N, 4.36. Found: C, 78.48; H, 7.19; N, 4.51.

2-(2-Benzyloxyphenyl)quinoline-4-carboxylic Acid (IX)——A mixture of O-benzylsalicylaldehyde (22 g) and pyruvic acid (20 g) in abs. EtOH (100 ml) was stirred for 2 hr at room temperature. To the reaction mixture was added dropwisely aniline (9.3 g) dissolved in abs. EtOH (250 ml) during 1 hr. After refluxing for 24 hr, the mixture was cooled, and the deposited product was collected and treated with 0.2 N NaOH (1 liter) to give alkali-insoluble yellow needles (3.5 g) (from AcOH), mp 193—194° (IX′) and an alkaline solution, of which neutralization with AcOH gave white needles (10.2 g) (from EtOH), mp 225—226° (IX). IX: Yield, 28.8%. UV λ_{max}^{EtOH} nm (log ε): 249 (4.37), 334 (3.65). *Anal.* Calcd. for $C_{23}H_{17}O_3N$: C, 77.73; H, 4.82; N, 3.94. Found: C, 78.11; H, 4.81; N, 3.74. IX′ (1-phenyl-3-phenylimino-5-O-benzyloxy-2-ketopyrolidine)[10]: UV λ_{max}^{EtOH} nm (log ε): 258 (4.35). *Anal.* Calcd. for $C_{29}H_{24}O_2N_2$: C, 80.53; H, 5.59; N, 6.48. Found: C, 80.81; H, 5.68; N, 6.28.

2-(2-Hydroxyphenyl)quinoline-4-carboxylic Acid (X)——After heating under refluxing a mixture of IX (3.0 g), Ac_2O (8 ml) and 47% HBr (6 ml) for 3 hr, evaporation of the reaction mixture to dryness *in vacuo* gave a yellowish orange product, which was dissolved in dil. ammonia to remove a little amount of insolubles. Neutralization of ammonia solution with AcOH gave an orange material, which was crystallized from AcOH to afford orange red needles (1.75 g), mp 246—248° (decomp.). Yield, 78%. UV λ_{max}^{EtOH} nm (log ε): 265 (4.57), 348 (4.08). *Anal.* Calcd. for $C_{16}H_{11}O_3N$: C, 72.44; H, 4.18; N, 5.28. Found: C, 72.07; H, 4.21; N, 5.09.

2-(2-Benzyloxy-5-*n*-hexylphenyl)quinoline-4-carboxylic Acid (XI)——After stirring a mixture of 2-O-benzyloxy-5-*n*-hexylbenzaldehyde (30 g) and pyruvic acid (8.8 g) in abs. EtOH (70 ml) for 2 hr, to the mixture was added dropwisely an alcoholic (40 ml) solution of aniline (9.3 g) during 30 min followed refluxing for 24 hr. Evaporation of the reddish orange solution *in vacuo* gave a viscous oil, which was subjected to extraction with $(C_2H_5)_2O$ and dil. NaOH. The alkaline solution was neutralized with AcOH and the precipitate was crystallized from EtOH to give white needles (3.9 g), mp 184—186°. Yield, 8.8%. UV λ_{max}^{EtOH} nm (log ε): 214 (4.71), 255 (4.40), 325 (3.97). *Anal.* calcd. for $C_{29}H_{29}O_3N$: C, 79.24; H, 6.65; N, 3.19. Found: C, 79.43; H, 6.68; N, 3.04.

2-(2-Hydroxy-5-*n*-hexylphenyl)quinoline-4-carboxylic Acid (XII, HQ III)——A mixture of XI (2.2 g), Ac_2O (6 ml), AcOH (10 ml) and 47% HBr (10 ml) was refluxed for 6 hr. After cooling, the reaction mixture was condensed under reduced pressure. The filtrate was neutralized with AcOH and the reddish brown precipitate collected was crystallized from AcOH to afford dark reddish brown rods (1.45 g), mp 190—191°. Yield, 83%. UV λ_{max}^{EtOH} nm (log ε): 222 (4.57), 266 (4.60), 354 (4.07). *Anal.* Calcd. for $C_{22}H_{23}O_3N$: C, 75.62; H, 6.63; N, 4.01. Found: C, 75.54; H, 6.70; N, 3.66.

Complex Formation of HQ I, II, and III with Several Metallic Salts

Metallic Salts——$FeCl_3 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, $Ni(NO_3)_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, $Co(AcO)_2 \cdot H_2O$, $CdSO_4 \cdot H_2O$, $ZnCl_2$, $MnCl_2 \cdot 4H_2O$, $AgNO_3$, $CaCl_2$, $MgCl_2 \cdot 6H_2O$, $Ba(NO_3)_2$, $Pb(AcO)_2 \cdot 2H_2O$, $Ga(NO_3)_3 \cdot xH_2O$.

10) R.E. Lutz, P.S. Bailey, M.T. Clark, J.F. Codington, A.J. Deinet, J.A. Freek, G.H. Harnest, N.H. Leake, T.A. Martin, R.J. Rowlett, Jr., J.M. Salsbury, N.H. Shearea, Jr., J.D. Smith, and J.W. Wilson, III, *J. Am. Chem. Soc.*, **68**, 1813 (1946).

Method——a) To 10 ml of an ethanolic solution of 100 μM HQ I (II, III) was added 0.1 ml of 10 mM aqueous solution of metallic salts (in the case of Ga^{3+}, 0.1 ml of 500 μg/ml of Ga salt). After standing at room temperature for 30 min, absorption spectrum ranging from 300 nm to 600 nm was measured by Hitachi spectrophotometer model 124 equipped with autorecorder.

b) 10 ml of 100 μM HQ I or HQ II in EtOH was mixed with 0.1 ml of various concentrations of $CuSO_4$ solution, followed measurement of absorption spectra at wave length ranging 300 nm to 500 nm.

Anticancer Experiments

Tumor and Host——Experiments with EA and S180 were performed on male albino mice of ddYs strain, weighing 20—22 g. L1210 was maintained by weekly transplanting to BDF_1 female mice weighing 20—22 g. As hosts of AH 13 and YS, Donryu rats, weighing 100—120 g, were used.

Toxicity——Acute toxicity of the compounds was estimated from the number of 10 day-survivors among the animals treated with single *i.p.* injection.[3]

***In Vitro* Effect on EA Cells**——Peritoneal fluid was withdrawn from a mouse bearing 7-day-old tumor and centrifuged at 700 rpm for 3 min. The packed cells, after washing 2 times with Hanks' solution (pH 7.4), were suspended in the solution to contain 10^7 cells/ml. The cell suspension (1.0 ml), after adding 10 μl of dimethyl sulfoxide (DMSO) solution of the compound to be tested at 0 time, was incubated under mild agitation at 37°. At certain times later, an aliquot of the suspension was pipetted out and cell death was observed by a hemocytometer after staining with nigrosine solution (0.2% in PBS) or with trypan blue solution (0.5% in PBS).

***In Vivo* Effect on EA Cells**——Tumor Implantation: 0.2 ml of the tumor cell suspension, which was prepared by the same method described above, containing 3.5×10^6 cells, was *i.p.* or *s.c.* inoculated into each mouse.

Test Compounds: Quinoline derivatives to be tested and 6-MP (Kohjin Co., Ltd.) were suspended in sterilized 0.5% CMC. MMC (Sankyo Co., Ltd.) was dissolved in PSS.

Toxic Effects on the Tumor Cells: Chemicals were administered by *i.p.* injection at Day 4 after the tumor implantation (Day 0). After 24 hr, the peritoneal fluid was quantitatively collected and total number of the cells per each animal, the cell viability and the cell morphological change were examined.

Effect on Life-Span Prolongation of Mice Bearing the Tumor Cells——Drugs were administered by *i.p.* injection once daily for 7 consecutive days beginning 24 hr after tumor implantation. The change in body weight from onset to termination of the treatment (Day 0 to Day 8) was used as an indication of drug toxicity. Evaluation of efficacy was based on the survival time of experimental animals and the number of mice surviving over 50 days. On the 50th day mice still alive were sacrificed and autopsied.

Effects on L1210, S180, AH 13 and YS in Animals——1.5×10^5 cells of L1210, 3×10^6 cells of AH 13 and YS were *i.p.* inoculated into each animal. 3×10^6 cells of S180 were implanted *s.c.* in the left groin of each mouse. The compounds to be tested were administered *i.p.* or orally once daily for 7 consecutive days beginning 24 hr after implantation. With the solid tumor system, anticancer activity of the compounds was evaluated by ratios of weights of tumors resected at Day 12 of control and the treated mice. With the ascites tumor systems, the same evaluation method as that employed for EA was adopted.

Determination of the Cellular Nucleic Acid Synthesis in EA Cells

Seven-day-old tumor cells were washed 2 times with Eagle's minimum essential medium (EM) at 4° by centrifugation at $60 \times g$ for 5 min and suspended in EM to contain 10^6 cells per ml. The cells were inoculated with 2 μM thymidine-^{14}C (0.1 μCi/ml) and 0.2 μM uridine-^{3}H (1.0 μCi/ml) for determination of the rates of DNA and RNA syntheses, respectively. Radioactive compounds were purchased from Daiichi Pure Chemicals Co., Ltd. After the preincubation of the cell suspension with the compound to be tested for 30 min at 37°, the cells were incubated with a radioactive precursor for 30 min, and the incubation was terminated by placing a tube in an ice bath. The radioactive medium was removed by washing 2 times with cold PBS. After treatment of the cells with cold 5% TCA and standing for 30 min in cold, the pellet was washed 2 times with cold 5% TCA and EtOH: $(C_2H_5)_2O$ (3 : 1 v/v), respectively. The residue was suspended in Hyamine hydroxide, and its radioactivity was determined with a modified Bray's solution in a Beckman liquid scintillation system LS-200B.

Hemolytic Effect on Rabbit Erythrocytes

The erythrocytes collected from defibrinated blood of healthy rabbit were suspended in PBS at 3% (v/v) concentration after washing 2 times with PBS. To 1.0 ml of the erythrocyte suspension was added 10 μl of DMSO solution of the compound to be tested, and then the suspension was incubated at 37° for 30 min. The incubation was stopped by ice cooling. After adding 4 ml of cold PBS, the mixture was centrifuged, and the absorbance at 550 nm of the supernatant was measured. As 100% hemolysis control, the supernatant of the suspension treated with 4 ml of H_2O was adopted.

Effect of Several Quinolines on Bacterial Growth

Bacteria——*E. coli* B and *B. subtilis* W168 were employed.

Minimum Growth Inhibitory Concentration——Ordinary broth (pH 7.4) inoculated with bacteria was cultured at 37° overnight. An aliquot of 10^4 dilution of the culture was inoculated to the broth, which contained appropriate amounts of the compound to be tested, and incubation was made at 37° for 48 hr.

Effect of HQ II on Growth of *B. subtilis*

The cells of *B. subtilis* were inoculated into Penassay broth (Difco) supplemented with 0.5% glucose and incubated under shaking at 37° overnight. The culture was 10-fold diluted with the fresh medium, followed by incubation for 2 hr. The cells were harvested by centrifugation and after washing 2 times with the medium, resuspended in the fresh medium to give the cell suspension ($A=0.30$ unit at 610 nm). After preincubation at 37° for 30 min, a certain amount of HQ II was added to the cell suspension, which was incubated at 37° under mechanical shaking. Cell growth was followed by observing its absorbance at 610 nm using Hitachi spectrophotometer model 101.

Determination of Nucleic Acids and Protein Syntheses in *B. subtilis*

a) After 30 min-incubation of 40 ml of the cell culture in the presence of HQ II in an appropriate concentration, the culture was rapidly cooled in ice water. The cells harvested by centrifugation were treated with cold 5% perchloric acid (PCA) with 30 min-standing, and the sediment was washed 2 times with cold 5% PCA and then once with EtOH: $(C_2H_5)_2O$ (3 : 1 v/v). The pellet obtained was dissolved in 0.5 N KOH and incubated at 37° for 15 hr. After neutralization with cold 6 N HCl, addition of equal volume of cold 10% PCA and subsequent standing for 30 min, the supermatant obtained by centrifugation was subjected to analysis of RNA. The sediment dissolved in 5% PCA was heated at 90° for 30 min, then cooled, and the supernatant was supplied for DNA assay. The sediment in turn was solubilized in 0.5 N KOH by way of incubation at 37° overnight. The supernatant was supplied as protein fraction. DNA was determined by the Burton's,[11] RNA was by orcinol[12] and protein was by the Lowry's method,[13] respectively. As the references, calf thymus DNA (Sigma) for DNA, yeast RNA (Sigma) for RNA and albumin standard solution (Daiichi Pure Chemicals Co., Ltd.) for protein were used, respectively.

b) Isotope Incorporation Experiments: Radioactive precursors, thymidine-2-^{14}C (50 mCi/mmole), uracil-2-^{14}C (21 mCi/mmole) and L-leucine-^{14}C(U) (198 mCi/mmole) were all supplied from Daiichi Pure Chemicals. 0.1 μCi/ml of thymidine, uracil and leucine were added to the culture at fintl concentrations of 2 μM, 4.8 μM and 0.25 mM, respectively. The isotopes were added at the mid exponential phase of the growth to the control cultures or the cultures which had received drugs 1 min previously. 1 ml of the culture was removed at subsequent time intervals and added to 1 ml of cold 10% TCA. For measurements of RNA, the precipitate was filtered on a glass membrane filter (Gelman Instrument Co., type A) with suction and washed 3 times with 5 ml of 5% TCA and then 2 times with 5 ml of EtOH. The dried filter was put into 10 ml of a PPO-toluene scintillation mixture in a vial. For measurements of DNA incorporation, the sample in cold 5% TCA was centrifuged, and the sediment, after washing with cold 5% TCA, was incubated with 1 N KOH at 37° for 12 hr to hydrolyze RNA. The sample was neutralized with 6 N HCl, and DNA was precipitated with cold 5% TCA. The precipitate was deposited on a glass membrane filter and washed 3 times with 5% TCA and then 2 times with EtOH. The filter was transferred to a vial containing a scintillation fluid. For protein, the precipitate in cold 5% TCA was heated at 90° for 30 min, then after cooling, a deposited material was filtered. The filter was washed 3 times with a 1% TCA–Casamino acids (Difco) solution and then 2 times with 70% EtOH. The radioactivity was determined using Beckman liquid scintillation system LS-200B.

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11) K. Burton, *Biochem. J.*, 62, 315 (1956).
12) A.H. Brown, *Arch. Biochem.*, 11, 269 (1946).
13) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, 193, 265 (1951).