Interaction of Asterriquinone with Deoxyribonucleic Acid *in Vitro*

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The interaction of asterriquinone (ARQ), a novel antitumor agent isolated from *Aspergillus* fungi, with deoxyribonucleic acid (DNA), has been studied. The binding of ARQ *in vitro* with DNA (calf thymus) was ascertained by its behavior in gel filtration using a Sephadex G-25 column at pH 5.4. Some ARQ analogs having no, or less, antitumor activity did not exhibit any evidence of interaction with DNA under the same condition. From the results obtained in this work, the pK_a value of ARQs seemed to be critical between 6 and 7 for their binding to DNA and for exhibition of antitumor activity. Also, ARQ showed serious membrane deformations and an inhibitory effect on the membranous adenosine triphosphatase of Ehrlich carcinoma cells.

Keywords asterriquinone; fungal metabolite; antitumor activity; DNA binder; pK_a value; Na,K-ATPase

In previous works[1,2] we demonstrated that asterriquinone (ARQ), a metabolic product of *Aspergillus terreus* IFO 6123, is potent in inhibiting the growth of transplantable murine tumor cells *in vivo*, and that related inactive metabolites isolated from another strain (*Asp. terreus* var. *africanus* IFO 8835) can be altered to active compounds by chemical modifications. Furthermore, the data obtained in the experiments on structure–activity relationship of ARQ derivatives showed that the presence of free hydroxy groups in the benzoquinone moiety and of *tert-* or iso-pentenyl groups in the indole ring of ARQ is important in order for them to exhibit antitumor activity.[2]

It is known that mitomycin C (MMC) and adriamycin (ADM) suppress deoxyribonucleic acid (DNA) replication by cross-linking or intercalation to the DNA molecule, while both agents affect ribonucleic acid (RNA) and protein syntheses only to a lesser degree.[3]

In this report, we investigated the action mechanism of ARQ in its antitumor activity with reference to its interaction with DNA molecules and the plasma membrane of tumor cells, and obtained information towards the development of more potent ARQ derivatives. The structure–activity relationships are also discussed.

Materials and Methods

Animals Male 5-week-old mice (SPF) of ddY were obtained from the Shizuoka Agricultural Cooperative Association, Hamamatsu, Japan.

Tumors Ehrlich ascites carcinoma cells, which are maintained by i.p. passage in our laboratory, were used.

Chemicals and Drugs ARQ and its analogs (Fig. 1) were prepared from freshly cultured mycelia by the methods reported previously.[4] These compounds were dissolved in water with the aid of dimethylsulfoxide (DMSO) in *in vitro* experiments. MMC (Mitomycin Kyowa-S, Kyowa Hakko Kogyo Co., Tokyo) was dissolved in 0.85% saline. Calf thymus DNA (Type I) was obtained from Sigma Chemicals and all other chemicals were of analytical grade.

Radioactive Chemicals ARQ [3,6-^{14}C] and its analogs, Cl-1 [3,6-^{14}C] and D-1 [3,6-^{14}C],[5] were prepared by cultivating the fungi in feeding of DL-tryptophan [3-^{14}C] as reported previously.[4]

Binding of ARQ and Its Analogs to DNA *in Vitro* Calf thymus DNA (500 μg) and the ^{14}C-labeled compound (50 μg) to be tested were incubated at 37 °C for 60 min in 1 ml of 0.05 M phosphate buffer (pH 7.4) with or without 2 mM $Na_2S_2O_4$. These ^{14}C-labeled compounds were also incubated with DNA in 1 ml of 0.05 M acetate buffer (pH 5.4) instead of phosphate buffer. After the incubation, the mixture was charged on a Sephadex G-25 column (1 × 12 cm) and eluted with the same buffer. Radioactivity and OD_{260} of each 2.5 ml portion of the elute were measured by a liquid scintillation counter and by a spectrophotometer (Model 181, Hitachi, Tokyo), respectively.

Determination of pK_a Value ARQ or its analogs was dissolved in a mixture of 2 ml of DMSO and 8 ml of 0.01 N NaOH, and titrated with 0.01 N HCl. The pK_a value of each tested compound was determined from the titration curve.

Inhibition of ATP Hydrolysis by Microsomal Fraction from Ehrlich Ascites Cells The microsomal fraction was prepared from Ehrlich ascites cells according to the method of Wallach *et al.*,[6] with some modifications, and the activation of Na^+, K^+-adenosine triphosphatase (ATPase) with NaI was carried out by the method described by Nakao *et al.*[7] The effects of ARQ and its analogs on Na^+, K^+- and Mg^{2+}-ATPase were examined as follows; 1 ml of the reaction mixture, which consisted of 0.5 mM ATP (disodium salt), 0.55 mM $MgSO_4$, 0.05 mM EDTA, 100 mM NaCl, 10 mM Tris–HCl (pH 8.6) and microsomal fraction (corresponding to about 40 μg of protein) with or without 5 mM KCl, was incubated at 37 °C for 60 min. It was followed by the addition of 1 ml of 10% trichloroacetic acid, and then centrifuged at 1500 *g* for 10 min after standing in an ice-bath for 10 min. One millilitter of the aliquot from the supernatant was submitted to the determination of liberated inorganic phosphorus by the method of Fiske *et al.*[8]

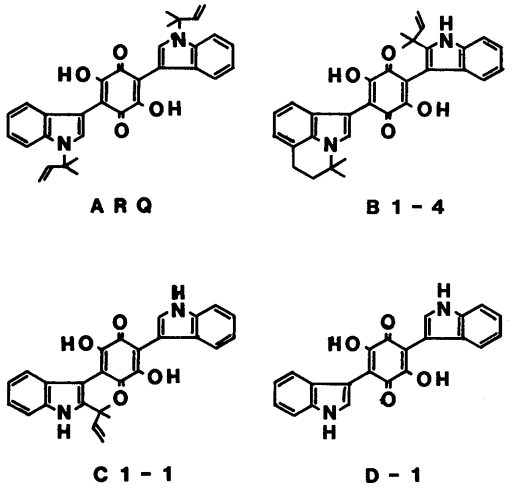

Fig. 1. Structures of Asterriquinone (ARQ) and Its Analogs

Results and Discussion

Interaction of Asterriquinone with Calf Thymus DNA *in Vitro* As shown in Fig. 2, when calf thymus DNA was incubated with ^{14}C-labeled ARQ in phosphate buffer (pH 7.4), the radioactivity was not detected in DNA fractions (fractions no. 2 and 3) regardless of the presence of sodium hydrosulfite leading to the quinol form. Meanwhile, in acetate buffer (pH 5.4), the radioactivity coeluted with DNA fractions. Where ethylacetate or chloroform extraction was

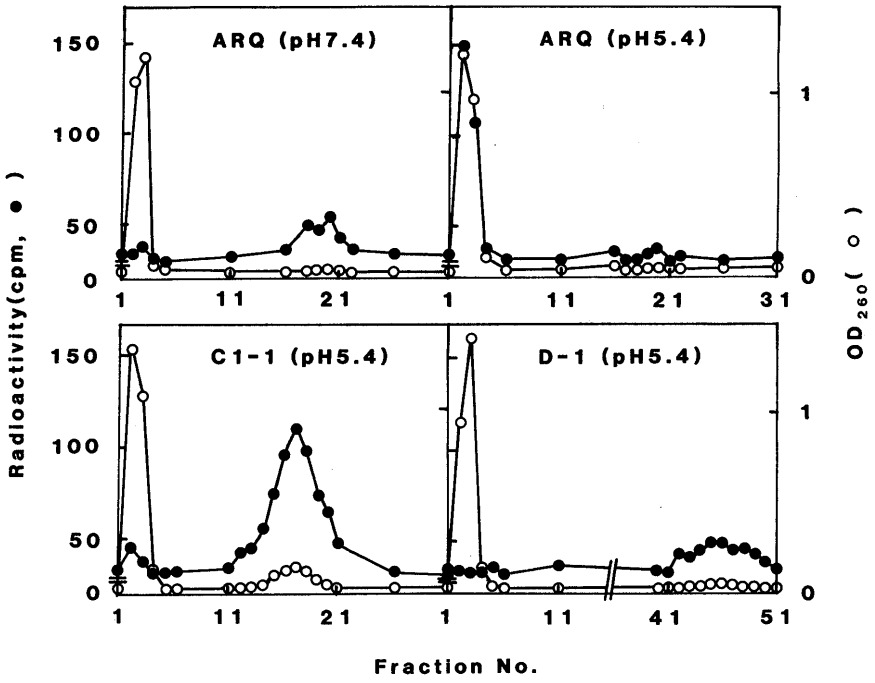

Fig. 2. Profiles of the Binding of ARQ and Its Analogs to Calf Thymus DNA *in Vitro*

DNA was incubated with ^{14}C-labeled ARQ or its analogs at 37 °C for 60 min in 0.05 M phosphate buffer (pH 7.4) or in 0.05 M acetate buffer (pH 5.4), and then applied on a Sephadex G-25 column.

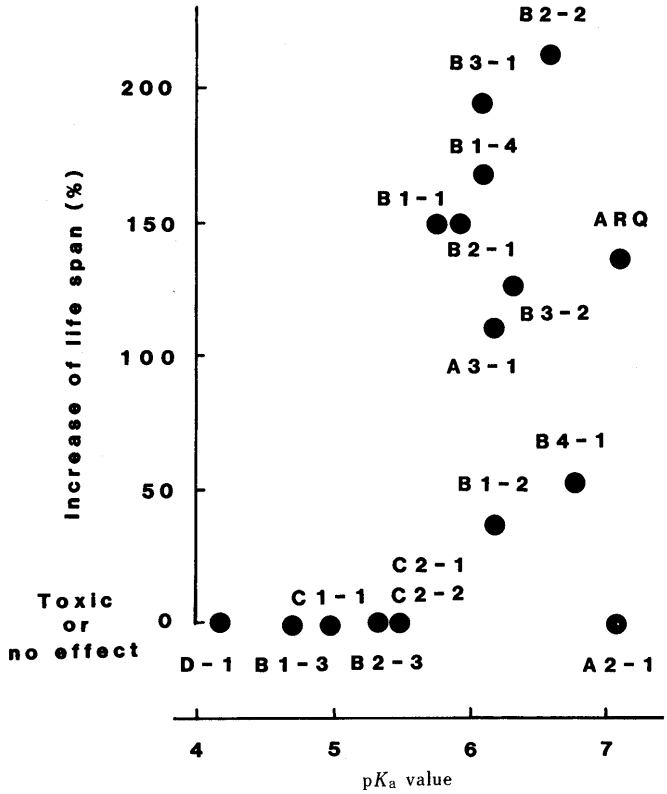

Fig. 3. Correlation between pK_a Value and Antitumor Activity of the Series of ARQ Analogs

Antitumor activity shows an increase of the life span of mice bearing Ehrlich ascites carcinoma reported in ref. 1.

carried out, the radioactivity of ARQ could be removed from the ARQ–DNA complex fraction. On the other hand, radioactivity of the ^{14}C-labeled compounds, Cl-1 and D-1, did not coelute with DNA in either case.

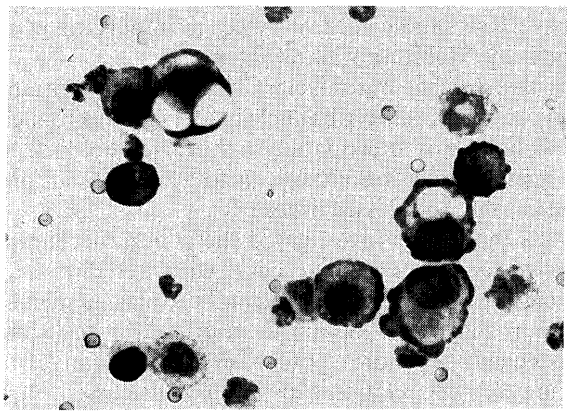

Fig. 4. Microscopical Finding of Ehrlich Ascites Cells Treated with ARQ *in Vivo*

ARQ (120 mg/kg) was administered to mice once intraperitoneally on the 4th d after the implantation of Ehrlich ascites cells (2.5×10^6/head). After 72 h, the peritoneal fluids were withdrawn and examined microscopically after the Wright-Giemsa staining (× 350).

Correlation between pK_a Value and Antitumor Activity of ARQ Analogs From the point of view of a structure–activity relationship, the pK_a value of various ARQ analogs were determined. As shown in Fig. 3, the antitumor activity of ARQ analogs against Ehrlich ascites carcinoma seems to depend on their pK_a values. For exhibiting antitumor activity, the pK_a value of ARQ and its analogs should be, at least, within a range of 6—7.

Inhibitory Effects of ARQ and Its Analogs on ATPase Activity When ARQ was administered intraperitoneally to mice bearing Ehrlich ascites carcinoma, the peritoneal tumor cells resulted in morphological changes with serious vacuolar degeneration and deformation of plasma membrane (Fig. 4). This finding suggested that ARQ acted directly on membranous constituents of tumor cells. Then

TABLE I. Inhibitory Effect of ARQ and Its Analogs on ATP Hydrolysis by NaI-Treated Microsomal Fraction of Ehrlich Ascites Carcinoma Cells

Concentration of chemical (μg/ml)	Addition of potassium ion	Inhibition (%)				
		ARQ	Bl-4	Cl-1	D-1	MMC
5	+	2	22	7	18	2
	−	6	10	9	0	0
10	+	16	63	60	29	2
	−	14	31	28	4	0
25	+	77	93	90	73	·
	−	86	81	79	44	·
50	+	95	100	93	91	·
	−	90	100	85	80	·

the influence of ARQ upon Na^+, K^+- and Mg^{2+}-ATPase activities was examined. Microsomal fraction was prepared from Ehrlich ascites cells as described in Materials and Methods. About 9 mg of protein with the specific activity of Na^+, K^+-ATPase of 7 μmol/mg protein/h was obtained constantly from 6.4×10^8 cells in repeated experiments. As shown in Table I, antitumor active ARQ and Bl-4 markedly inhibited both Na^+, K^+- and Mg^{2+}-ATPase activities at the concentration of 25 μg/ml or more. However, the analogs with less or no antitumor activities, Cl-1 and D-1, also showed a similar inhibitory effect. Meanwhile, MMC did not show any inhibiting capacity against both ATPases activity.

Ehrlich ascites cells (3×10^6 cells) were pretreated with ARQ (50 μg/ml) at 37 °C for 60 min in 100 ml of Eagler's MEM, and then microsomal fraction was prepared as described above. The ATPase activity of ARQ-treated cells was 44% to that of untreated cells.

It has been reported that diketocoriolin B decreases the permeability of cell membrane, most probably by interaction with Na^+, K^+-ATPase system.[9] Such a mechanism was supposed for the effect of ARQ on tumor cells considering the fact that ARQ suppressed the Na^+, K^+-ATPase activity as well as Mg^{2+}-ATPase activity of tumor cells. Although the inhibitory effect of ARQ analogs on ATP hydrolysis by microsomal fraction do not necessarily correlate to their antitumor activity, they may have an ability to injure tumor cells even in the intact Ehrlich carcinoma cells.

The interaction between DNA and carbazilquinone,[10] daunomycin or ADM[11] have already been studied by means of Sephadex gel filtration. ARQ did not interact with calf thymus DNA in phosphate buffer (pH 7.4) even in the presence of sodium hydrosulfite which has been used in the formation of an adduct between MMC and DNA.[3b] On the other hand, in acetate buffer (pH 5.4), ARQ combined with DNA. ARQ could be removed from the formed ARQ–DNA complexes by extraction with ethylacetate or chloroform. Therefore, it is suggested that ARQ–DNA binding is a non-covalent binding. In contrast, some ARQ derivatives, Cl-1 and D-1, which were far less active against Ehrlich carcinoma *in vivo*, showed no complex formation with DNA in the same condition. The incubation of ARQ with calf thymus DNA, poly d(A-T) or poly dG-poly dC in the acetate buffer containing Mg^{2+} resulted in no spectral perturbation, indicating the intercalation such as daunomycin in absorption spectra[12] and circular dichroism (CD) spectra.[13] From these results on the relationship between pK_a and antitumor activities of ARQ analogs, it is suggested that binding of ARQ to DNA is considered to be essential for the formation of hydrogen bonding between undissociated hydroxybenzoquinone moiety and bases of DNA, as denoted by J. D. Watson *et al.*[14] Therefore, the antitumor actvity of ARQ analogs might be critical for the binding to template DNA and a resultant cytostatic effect.

Acknowledgements We are grateful to Dr. Yuzuru Yamamoto and Dr. Shoji Ohkuma for a critical reading of this manuscript. This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare, Japan.

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