QUINOCARCIN, A NOVEL ANTITUMOR ANTIBIOTIC 3. MODE OF ACTION

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Quinocarcin is a colorless antibiotic that was discovered in the culture broths of *Streptomyces melanovinaceus*.[1] The structures of quinocarcin and quinocarcinol (an inactive homologue) were elucidated by us[2] and by HIRAYAMA *et al.*[3] as shown in Fig. 1. Quinocarcin consists of the novel skeleton, 8,11-iminoazepinoisoquinoline and it showed antibacterial activity and antitumor activity.[1] The mode of action was investigated, and the results are presented in this publication.

Quinocarcin strongly inhibited incorporation of [3H]thymidine into *Bacillus subtilis in vivo*. Inhibition of DNA synthesis in *B. subtilis* was found to be due to inhibition of DNA polymerase reaction and cleavage of double stranded DNA. Cleavage of DNA by quinocarcin was inhibited by the addition of radical scavengers, superoxide dismutase and catalase suggesting that it is caused by generation of oxygen and/or hydroxyl free radicals. DNA polymerase I from *Escherichia coli* was blocked by quinocarcin.

Effect of Quinocarcin on the Synthesis of Macromolecules in *B. subtilis*

Quinocarcin exhibited a rather narrow antibacterial spectrum. However, it consists of the novel skeleton, 8,11-iminoazepinoisoquinoline which has been discovered in nature for the first
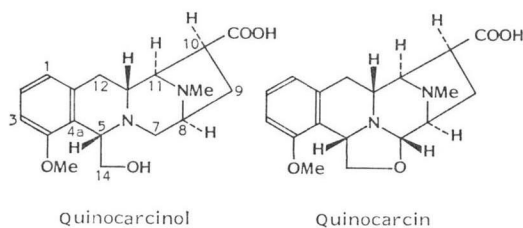
time.[1,2] Thus it is of interest to study the effect of quinocarcin on the synthesis of macromolecules. The effects of quinocarcin on the syntheses of DNA, RNA and protein were followed by measuring the incorporation of labeled [*methyl*-3H]thymidine, [2-14C]uracil and [4,5-3H]-L-leucine into acid-insoluble precipitates. After the addition of radioactive precursors, 0.5 ml samples were removed at intervals and poured into 2.5 ml of ice-cold 5% trichloroacetic acid and placed for one hour in the ice bath. They were filtered through HA Millipore filters (0.45 μm) and washed with 15 ml of cold 5% trichloroacetic acid. The filters were dried and counted in vials containing toluene scintillation fluid consisting of 4 g 2,5-diphenyloxazole and 0.1 g 2,2'-p-phenylene-bis-(5-phenyloxazole) per liter of toluene.

As shown in Fig. 2, inhibition of DNA, RNA and protein syntheses was observed at the high concentration of quinocarcin (100 μg/ml). At the concentration of 25 μg per ml, inhibition of RNA and protein syntheses was slight and detected only after 20 minutes. However, DNA synthesis was blocked completely at 5 minutes. These results indicate that quinocarcin primarily inhibits DNA synthesis and subsequently affected RNA and protein syntheses.

Interaction of Quinocarcin and Quinocarcinol with DNA Molecules

As shown in the previous section quinocarcin was found to be a potent inhibitor of DNA synthesis at the concentrations without affecting on protein and RNA syntheses. Thus, interaction of quinocarcin with DNA was examined further in order to find how the antibiotic affects DNA molecules. The effects of quinocarcinol, which is an inactive homologue of quinocarcin, were also examined to determine the structure-activity relationship.

Phage PM2 DNA was purchased from Boehringer Mannheim, Germany and showed three bands on agarose gel electrophoresis. According to AAIJ and BORST,[4] the fastest moving band corresponded to the native form of covalently closed circular (ccc) DNA, the intermediate band to the linear form and the most slowly moving one the open circular (oc) form was illustrated in Fig. 3, lane A. The reaction mixture consisted of PM2 DNA and various amounts of quinocarcin in 20 mM Tris-HCl

Fig. 1. Structures of quinocarcinol and quinocarcin.

Quinocarcinol   Quinocarcin

Fig. 2.   Effects of quinocarcin on macromolecular synthesis in *Bacillus subtilis*.

When the density of *B. subtilis* in the medium reached $OD_{660nm}=0.1$, labeled precursors ([³H]thymidine, [¹⁴C]uracil or [³H]leu) were added.   After incubating them for 15 minutes at 37°C, the drug was added and incorporation of radioactive precursors into the acid-insoluble fraction was measured. Numbers in Fig. indicate amounts of the drug added (μg/ml).
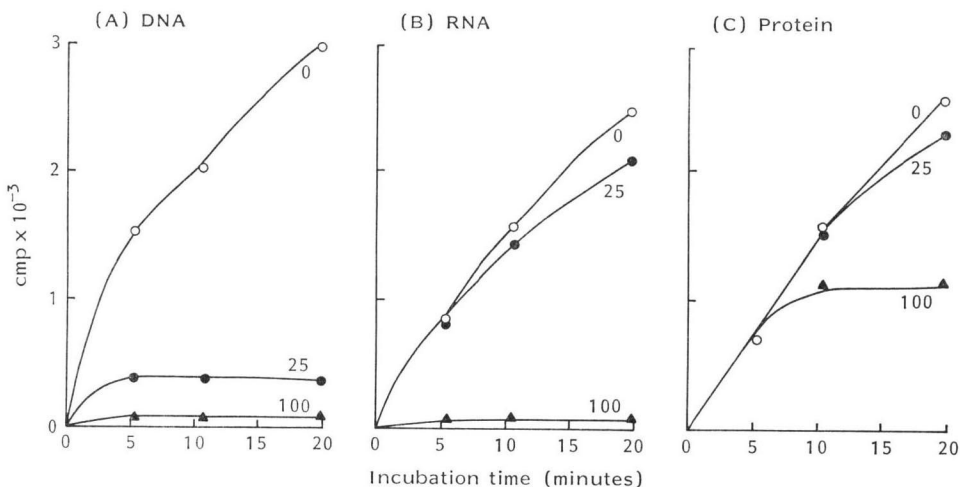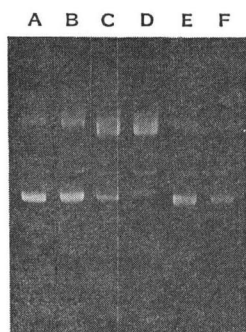

Fig. 3.   Interaction of quinocarcin and quinocarcinol with PM2 DNA.

A: Drug free control,   B: 0.1 mM quinocarcin, C: 1.0 mM quinocarcin,   D: 10 mM quinocarcin, E: 1.0 mM quinocarcinol, F: 10 mM quinocarcinol.
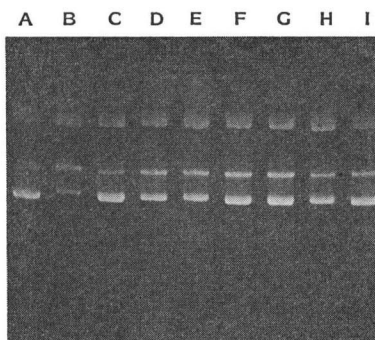

Fig. 4.   Effects of metal ions on interaction of quinocarcin and PM2 DNA.

A: Drug free control, B: 1.0 mM quinocarcin, C: 0.1 mM quinocarcin, D: 0.1 mM quinocarcin + 0.05 mM Fe^{2+}, E: 0.1 mM quinocarcin + 0.1 mM Fe^{2+}, F: 0.1 mM quinocarcin + 0.05 mM Cu^{2+}, G: 0.1 mM quinocarcin + 0.1 mM Cu^{2+}, H: 0.1 mM Fe^{2+}, I: 0.1 mM Cu^{2+}.

buffer (pH 8.0).   The reaction was carried out for one hour at 37°C and stopped by the addition of bromophenol blue and sucrose to make 0.01% and 10%, respectively.   The reaction was followed by agarose gel (0.8%) electrophoresis as described by YAGI *et al.*[5]

As shown in Fig. 3 (lanes B~D), quinocarcin caused reduction in the amount of cccDNA and an increase in ocDNA, while quinocarcinol did not cause any changes in the mobility of DNA or in the amounts of DNA subfractions (Fig. 3, lanes E, F).   Thus it was shown that the opening

of the aminoacetal ring abolished quinocarcin's ability to cleave DNA, which reflected its inability to exhibit biological activities.[1,2]   The DNA cleaving activity of quinocarcin was stimulated in the presence of a reducing agent, dithiothreitol (DTT) as shown in Fig. 6, lane D, while no changes were observed in the presence of metal ions as seen in Fig. 4, lanes A~K.

Fig. 5. Effects of radical scavengers on interaction of quinocarcin with PM2 DNA.

A: Drug free control, B: 1.0 mM quinocarcin, C: 10 mM quinocarcin, D: 1.0 mM quinocarcin + 1.0 mM β-carotene, E: 1.0 mM quinocarcin + 1.0 mM α-tocopherol, F: 1.0 mM quinocarcin + 10% MeOH, G: 1.0 mM quinocarcin + t-BuOH, H: 10 mM quinocarcin + 1.0 mM β-carotene, I: 10 mM quinocarcin + 1 mM α-tocopherol.
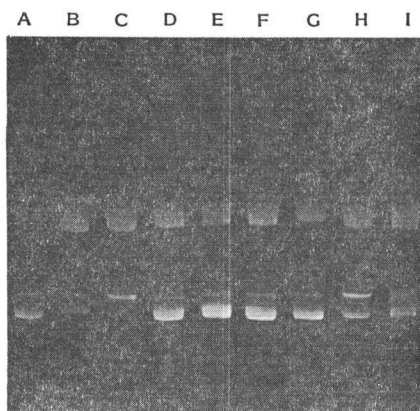

Fig. 6. Effect of radical scavenging enzymes on interaction of quinocarcin with PM2 DNA.

A: Drug free control, B: 1.0 mM quinocarcin, C: 10 mM quinocarcin, D: 1.0 mM quinocarcin + 0.1 mM dithiothreitol, E: 1.0 mM quinocarcin + 10 μg/ml of superoxide dismutase, F: 10 mM quinocarcin + 10 μg/ml of superoxide dismutase, G: 1.0 mM quinocarcin + 10 μg/ml of superoxide dismutase + 0.1 mM dithiothreitol, H: 1.0 mM quinocarcin + 10 μg/ml of catalase.

Scavengers for hydroxyl radicals, methanol and tertiary butanol, inhibited DNA cleavage by quinocarcin. Scavengers for singlet oxygen radicals, β-carotene and α-tocopherol, also inhibited the action of quinocarcin (Fig. 5, lanes A ~ H). Furthermore, catalase and superoxide

Table 1. Reduction of neotetrazolium by quinocarcin.

Quinocarcin (mM)	Superoxide dismutase (μg/ml)	ΔOD_{500}/minute
A)		
—	—	0.008
1	—	0.010
2	—	0.019
5	—	0.036
2	10	0.000
B)		
—	—	0.000
5	5	0.010
5	10	0.000

A) With 0.1 mM dithiothreitol.
B) Without dithiothreitol.

dismutase (SOD) inhibited DNA cleavage by quinocarcin (Fig. 6, lanes A ~ H). These findings are supported by the fact that the hydroxyl radical is generated from the reaction of superoxide with hydrogen peroxide.[6,7,8] and are similar to quinone antitumor agents.[9~13]

Generation of Superoxide Radicals by Quinocarcin

As shown above, quinocarcin seems to be a generator of oxygen free radicals. In order to clarify this property, stimulation of reduction of neotetrazolium during the autooxidation of DTT in combination with quinocarcin was measured according to the method by MISHRA.[14] The reduction was initiated after the addition of 0.5 mM DTT to a mixture containing 0.12 mM neotetrazolium, 0.8% Triton X-100 and various amounts of quinocarcin in 0.5 ml. The initial neotetrazolium reduction rate was measured at 500 nm and 25°C. SOD was added at a concentration of 5 μg/ml. The reduction, measured as the change in OD_{500} per minute, was increased as the level of quinocarcin was raised as shown in Table 1. On the other hand, SOD completely suppressed the increase in the reduction rate. These results clearly indicate that quinocarcin is involved in the generation of free radicals.

It has been known that antitumor agents with quinone moieties are able to cleave DNA under reduced conditions and the reduced quinone moiety is required for cleaving DNA in the presence of small amounts of metal ions. However, as seen from Fig. 1, quinocarcin does not consist of a quinone moiety. Ferrous ion and other metal ions had no effect on the cleaving

Fig. 7. Effect of quinocarcin on DNA polymerase I.
● Without preincubation, △ preincubation with DNA, ▲ preincubation with DNA polymerase I.
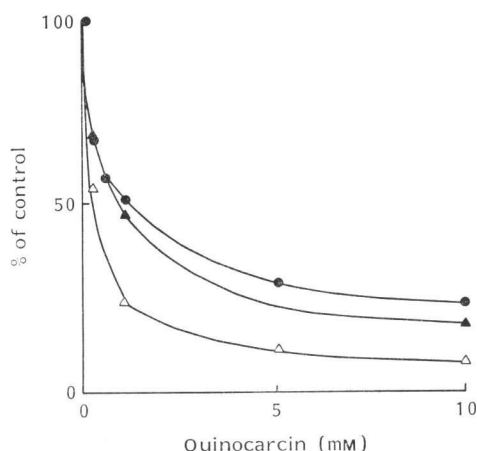
activity of quinocarcin. Thus the mechanisms proposed for antitumor agents with quinone moieties cannot be applied to quinocarcin and it is important to elucidate how quinocarcin could produce oxygen radicals. Studies along this line are in progress. Nevertheless, similarly to quinone antitumor agents, quinocarcin may be a site-specific catalyst for superoxide generation and the antitumor activity may depend on this activity. As described above, quinocarcin differs from quinone antibiotics and may provide a novel mechanism to generate oxygen free radicals through the involvement of aminoacetal structure.

Effect of Quinocarcin on DNA Polymerase

The effect of quinocarcin on DNA polymerase was examined using a commercial DNA polymerase (DNA polymerase I from *E. coli*) purchased from Boehringer-Mannheim-Yamanouchi (Japan). Tests were made according to the method described by JOVIN et al.[15] The reaction mixture consisted of various amounts of quinocarcin, activated calf thymus DNA (100 μg/ml), DNA polymerase I (2.5 units/ml), 0.017 mM (0.25 μCi/ml) [³H]dATP, 0.035 mM each of dGTP, dCTP and dTTP, 1 mM 2-mercaptoethanol and 6.7 mM MgCl₂ in 66.7 mM potassium phosphate buffer, pH 7.4 (final volume 0.3 ml). After the incubation of the reaction mixture for 30 minutes at 37°C, it was poured into 1 ml of ice-cold 10% trichloroacetic acid containing 0.1 M sodium pyrophosphate and placed in the

ice bath for 15 minutes. Each was filtered through HA Millipore filters (0.45 μm) and washed with 15 ml of cold 5% trichloroacetic acid containing 0.1 M sodium pyrophosphate. The filters were dried and counted in vials containing toluene scintillation fluid as described in the previous section. The activated calf thymus DNA was prepared according to APOSHIAN and KORNBERG[16] and was used as the template DNA. As shown in Fig. 7, quinocarcin inhibited the synthesis of DNA by DNA polymerase. The preincubation of the enzyme with quinocarcin for one hour at 37°C did not cause reduction of DNA synthesis compared with that of the control without quinocarcin, while the preincubation of the template DNA with quinocarcin caused about a two fold reduction of DNA synthesis compared with the control. These results suggest that quinocarcin inhibits DNA synthesis through the interaction with the template DNA.

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