MECHANISM OF ACTION OF LACTOQUINOMYCIN A WITH SPECIAL REFERENCE TO THE RADICAL FORMATION

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Lactoquinomycin A (LQM-A), an antibiotic containing a quinone moiety in the molecule, inhibited biosyntheses of DNA, RNA and protein to a similar extent in doxorubicin-resistant mouse leukemia L5178Y cells at concentrations higher than 0.08 μg/ml. The antibiotic caused cell death in a short period of incubation and the degree of cell death correlated with that of the inhibition of macromolecular syntheses, suggesting that the inhibition of macromolecular syntheses was not a primary effect of LQM-A. LQM-A served as a good electron acceptor, when cytochrome c reductase was used as a quinone reductase. The treatment of the cells with LQM-A significantly reduced cellular NADH and ATP levels. The generation of superoxide radical by LQM-A in cell lysate was observed by reduction of nitro blue tetrazolium, and the production of hydroxyl radical was confirmed by electron spin resonance. The importance of radical formation for the cytotoxicity of LQM-A is discussed.

Lactoquinomycin A (LQM-A), a potent antitumor antibiotic, was isolated from *Streptomyces tanashiensis* IM8442T by our screening method using drug-resistant tumor cells. The production, purification, structure assignment and some biological activity were described in previous papers[1,2]. LQM-A was effective against Ehrlich carcinoma in mice, and strongly inhibited several cultured tumor cells *in vitro*. Especially, doxorubicin-resistant murine leukemia L5178Y cells were more significantly inhibited by LQM-A than the parental cells. In this publication we report the mechanism of action of LQM-A from the viewpoint of radical formation.

Materials and Methods

Cell Culture

Doxorubicin-resistant mouse leukemia L5178Y cells (L5178Y/ADM)[3] were grown in RPMI 1640 medium, supplemented with 10% horse serum, benzylpenicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified atmosphere containing 5% CO_2 at 37°C.

Incorporation of Radioactive Precursors

The cell suspension in the medium (2.0×10^5/ml) was distributed into wells of a microplate (Nunc, Denmark), and incubated with various concentrations of LQM-A for 2 hours. Then [^{3}H]thymidine, [^{3}H]uridine or [^{3}H]alanine was added to the cell suspension at the final concentration of 0.5, 1.0, or 4.0 μCi/ml, respectively. After additional incubation for 30 minutes, the cells were harvested on a glass fiber filter, and washed with phosphate-buffered saline (PBS) and 5% TCA. The radioactivity was determined in a liquid scintillation counter.

Cell Viability

The cells (2.0×10^5/ml) were incubated in the conditions described above for 2.5 hours with various concentrations of LQM-A and the viable cell number was determined by trypan blue dye exclusion.

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NADH Consumption by Cytochrome c Reductase

A reaction mixture containing 0.01 mM electron acceptor (LQM-A or other quinones), 0.15 mM NADH, 0.1 M potassium phosphate buffer (pH 7.0), 1 mM EDTA and 17 μg/ml cytochrome c reductase (Sigma, from porcine heart, type I) was incubated at 30°C in an unsealed cuvette and the decrease in absorbance at 340 nm was monitored.

Cellular NADH Level

Cellular NADH level was determined by the cycling spectrophotometric assay of BERNOFSKY and SWAN[4] with slight modifications. Twenty million cells per data point were harvested by centrifugation, washed with PBS, and treated with 300 μl of ice-cold 0.5 N KOH in 50% ethanol to extract NADH in cells. After heating at 90°C for 5 minutes, the extracts were neutralized with 300 μl of 0.5 M triethanolamine in 0.4 M H_3PO_4 plus 0.3 N HCl. NADH in the extracts was converted to NAD^+ prior to assay by addition of 30 μmol acetaldehyde and 3 U of alcohol dehydrogenase (Oriental Yeast Co., Tokyo, from yeast) and incubated at 37°C for 30 minutes. The extracts were then acidified with ice-cold 2 M *m*-phosphoric acid, centrifuged to remove protein, and neutralized with 2 N KOH in 0.3 M *N*-morpholinopropanesulfonic acid. Amount of NAD^+ was determined by measuring the rate of increase of absorbance at 570 nm due to reduction of thiazolyl blue in the cycling system containing 400 μl of the cell extracts, 0.1 N Tris-HCl (pH 7.0), 5 mM lactic acid, 0.8 mM phenazine ethyl sulfate, 0.2 mM thiazolyl blue and 10 U/ml lactic dehydrogenase (Sigma, from rabbit muscle, type XI). The reaction was carried out at 37°C in a cuvette containing 1.2 ml total volume.

Cellular ATP Level

The level of cellular ATP was measured by HPLC. Cells (2.0×10^5/ml) were incubated with LQM-A or dinitrophenol (DNP) for indicated time, and then harvested and washed with PBS. Nucleotide extraction was carried out by the method of DICK and WRIGHT[5]. Nucleotides in the extract were separated by HPLC on ODS (Nucleosil 5 C_{18}) column, and detected at 260 nm. 0.2 M potassium phosphate buffer (pH 7.0) containing 20% methanol and 10 mM tetra-*n*-butylammonium bromide was employed as mobile phase.

Determination of Superoxide Radicals

Superoxide radical generation was determined by the nitro blue tetrazolium (NBT) assay. Cells (2.0×10^8) were homogenized in 0.1 M potassium phosphate buffer (pH 7.0) containing 0.15 M KCl and 1 mM EDTA, and centrifuged at $1,000\times g$ for 20 minutes and the supernatant was used as cell lysate. The reaction mixture (1.5 ml) containing the cell lysate (0.74 mg protein/ml), 0.05% NBT and various concentrations of LQM-A was incubated in the presence or absence of superoxide dismutase (SOD, Sigma, from bovine erythrocytes; 130 μg/ml) at 37°C for 30 minutes, followed by addition of 2 ml of 1 N HCl to the solution, and was centrifuged at $1,000\times g$ for 20 minutes. The residue was washed with 1 N HCl and dissolved in 1.5 ml of hot pyridine. The absorbance of the supernatant was measured at 515 nm.

Detection of Free Radical by Electron Spin Resonance (ESR)

ESR spectra were obtained at room temp using a Jeol JES-FE 3X spectrometer. The reaction mixture contained 170 μg/ml cytochrome c reductase, 0.5 mg/ml LQM-A, 0.5 mM NADH, 270 mM 5,5-dimethylpyrroline-*N*-oxide (DMPO) and 0.2 M potassium phosphate buffer (pH 7.0). Hyperfine coupling constants were determined by comparison to the spectrum of an aqueous solution of FREMY's salt ($a^N=13.0$ G).

Radioactive Compounds

[*methyl*-^{3}H]Thymidine (25 Ci/mmol) was purchased from Amersham Japan, Tokyo. [5,6-^{3}H]-Uridine (45.9 Ci/mmol) and L-[3-^{3}H]alanine (82.7 Ci/mmol) were products of New England Nuclear, Boston, Mass.

Results

Effects on Cell Viability and Macromolecular Biosyntheses

LQM-A was found to kill L5178Y/ADM cells after 2.5 hours incubation at concentrations more than 0.08 μg/ml, in culture by the method employed.

The effects of LQM-A on nucleic acids and protein syntheses were examined by incorporation of [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]alanine into TCA-insoluble fraction of L5178Y/ADM cells. The antibiotic inhibited biosyntheses of DNA, RNA and protein to a similar extent, and these inhibitions were also similar to that of cell viability examined by trypan blue dye exclusion method (Fig. 1). The results suggest that the inhibition of macromolecular syntheses is not the primary effect of LQM-A.

Ability as an Electron Acceptor of Cytochrome c Reductase

The ability of LQM-A or other quinone compounds to serve as a substrate (an electron acceptor) of cytochrome c reductase was determined by NADH consumption. As shown in Table 1, LQM-A was proved to be the best substrate. Menadion (vitamin K_3) was also a good electron acceptor. On the contrary, coenzyme Q_{10}, which is an intrinsic substrate of cytochrome c reductase, was a weak acceptor. This may imply that LQM-A works as a substrate instead of coenzyme Q_{10} in respiration chain and blocks ATP synthesis. Lactoquinomycin B (LQM-B) was also a poor electron acceptor. LQM-B displayed weaker cytotoxicity compared with LQM-A. The 50%-growth inhibitory concentration of LQM-A for L5178Y/ADM cells was 6 ng/ml, while that of LQM-B was 210 ng/ml[1,6]. The cytotoxic activity of LQM-A and -B seemed to correlate to their ability as an electron acceptor.

Table 1. Ability as an electron acceptor of cytochrome c reductase.

Acceptor	NADH consumption (nmol/mg protein/minute)
Lactoquinomycin A	206
Lactoquinomycin B	7
Menadion (vitamin K_3)	120
Coenzyme Q_{10}	10

Fig. 1. Effects of lactoquinomycin A (LQM-A) on the incorporation of precursors and cell viability.

○ [^{3}H]Thymidine, □ [^{3}H]uridine, △ [^{3}H]alanine, ● viability.

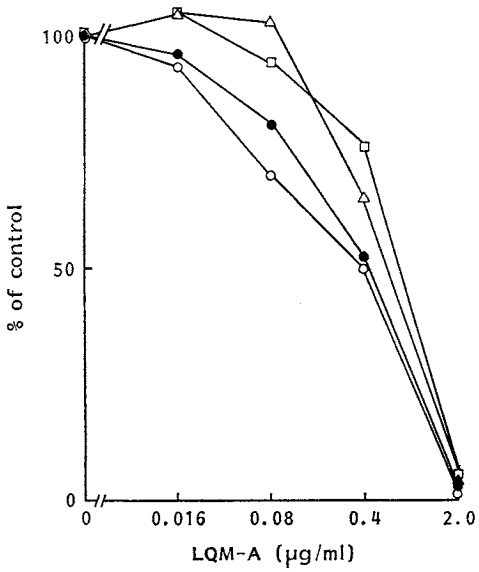
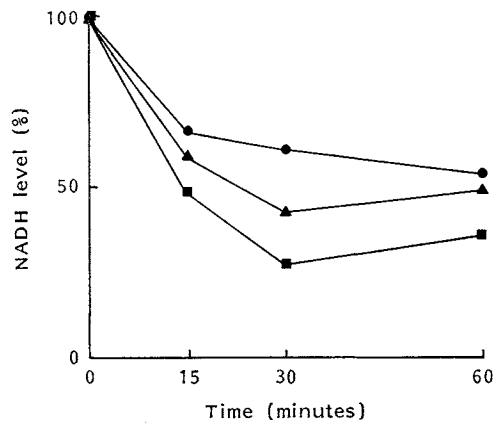


Fig. 2. Effect of lactoquinomycin A (LQM-A) on cellular NADH level (100% NADH: 4.27 nmol/10^7 cells).

● LQM-A 0.5 μg/ml, ▲ LQM-A 1.0 μg/ml, ■ LQM-A 2.0 μg/ml.



Effect on Cellular NADH Level

When the cells were treated with LQM-A, cellular NADH level rapidly decreased depending on the antibiotic concentrations (Fig. 2). At a concentration of 2.0 μg/ml, 50% depletion of NADH occurred with 15 minutes exposure. These results imply that LQM-A is substantially reduced in the cells by NADH dehydrogenase.

Effect on Cellular ATP Level

Cellular ATP level was determined using HPLC after treatment with LQM-A. Forty % decrease in ATP pool was observed after 1 hour treatment with 2.0 μg/ml of LQM-A (Fig. 3). However the depletion was a lesser extent compared with that of NADH. Since NADH is required for synthesis of ATP, the decrease in ATP level may be due to NADH depletion. DNP at 1.0 mM also decreased ATP content to similar level to that with 2.0 μg/ml of LQM-A. However, the mode of cytocidal activity between LQM-A and DNP was different; LQM-A at 2.0 μg/ml exerted killing activity after 1 hour treatment, while DNP at 1.0 mM did not cause cell death even after 3 hours incubation (data not shown). These observations suggest that the decrease in ATP level by LQM-A does not fully explain its cytotoxicity.

Generation of Superoxide Radicals

The generation of superoxide radicals by LQM-A in cell lysate was examined by measuring reduction of NBT. As shown in Fig. 4, superoxide radical was generated by LQM-A depending on the concentrations, and it was completely inhibited by addition of 130 μg/ml of SOD.

Detection of Hydroxyl Radical

Because no ESR signals were detected without DMPO, we added DMPO to the reaction mixture as a spin trap reagent, and the signal of radical produced by LQM-A was observed (Fig. 5). The splitting constants for the DMPO : adduct are: $a^N = a^H = 14.9$ G, which are characteristic of

Fig. 3. Effect of lactoquinomycin A (LQM-A) on cellular ATP level (100% ATP: 506 pmol/10^5 cells).
▲ LQM-A 1.0 μg/ml, ■ LQM-A 2.0 μg/ml, ● DNP 1.0 mM.

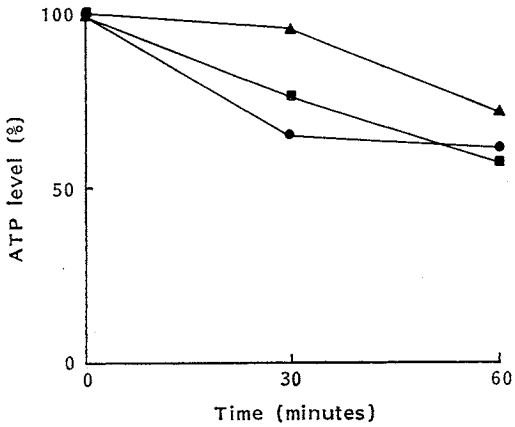


Fig. 4. Superoxide radical generation by lactoquinomycin A (LQM-A).
○ −SOD, ● +SOD (130 μg/ml).

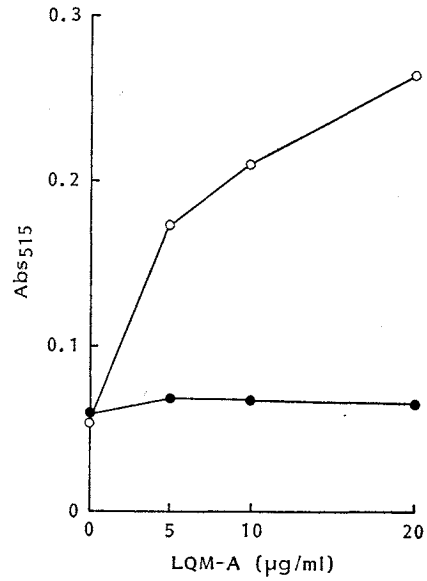
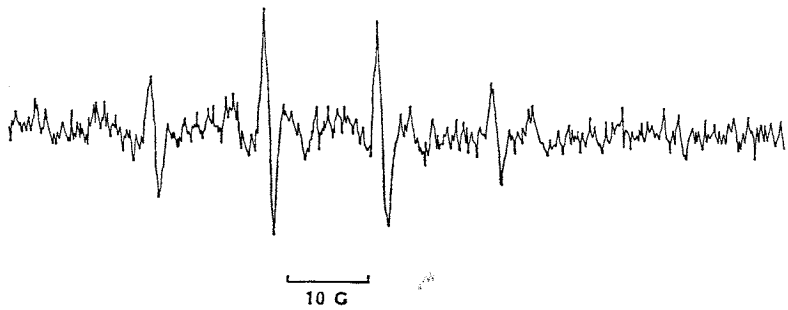


Fig. 5. ESR spectrum of the radical produced by lactoquinomycin A.



DMPO : OH adduct[7], indicating the generation of the hydroxyl radical. When DT-diaphorase was used as a quinone reductase instead of cytochrome c reductase, generation of radical was not detected (data not shown), implying the significance of one electron reduction in the generation of radical.

Discussion

The mechanism of action of LQM-A was studied. First, the effects of macromolecular biosyntheses were investigated. Although LQM-A inhibited biosyntheses of DNA, RNA and protein, these inhibitions were similar to that of cell viability (Fig. 1). Thus the inhibitions of macromolecular biosyntheses seem to be the secondary action of LQM-A.

Secondly, ability as an electron acceptor was examined. Since LQM-A has a quinone moiety in its structure, the reduction of a quinone by quinone reductase was expected. When cytochrome c reductase was used as quinone reductase, LQM-A proved to serve as a good electron acceptor (Table 1). By contrast, LQM-B, structurally related to LQM-A, was a poor acceptor. As for cytotoxicity, LQM-A showed 35-fold higher activity against L5178Y/ADM cells than LQM-B. These results suggest that there is some correlation between the ability as an electron acceptor and the cytotoxicity.

Quinone reductases are known to catalyze one-electron reduction or two-electron reduction[8]. Cytochrome c reductase (NADH: (acceptor) oxidoreductase) converts quinone to semiquinone radical by one-electron transfer, while DT-diaphorase (NAD(P)H: (quinone-acceptor) oxidoreductase) converts quinone to hydroquinone by two-electron transfer. Semiquinone radical rapidly reduces dioxygen to form superoxide radical and regenerates the quinone. In this way large amounts of superoxide radicals are generated during coupled respiration; superoxide radicals and resulting active oxygens (hydrogen peroxide, hydroxyl radical and singlet oxygen) cause severe damages to cells. On the other hand hydroquinone is thought to be detoxified by glucuronate conjugation or sulfonate conjugation. Thus, semiquinone radical formation is crucial for cytotoxicity of quinone.

The above schemes seem to apply to LQM-A. LQM-A radical is generated during reduction of the quinone moiety by quinone reductase like cytochrome c reductase. This radical readily reacts with dioxygen, and produces superoxide radical, resulting in other active oxygens. The consequences of LQM-A reduction are thought to be the cause of cytotoxicity, and are also thought to be responsible for: (1) depletion of NADH with subsequent depletion of mitochondrial ATP (Figs. 2 and 3), (2) superoxide radical and hydroxyl radical formation (Figs. 4 and 5).

The following changes in cells were known to be caused by active oxygen radicals: (1) DNA strand scission by hydroxyl radical[9,10], (2) peroxidative damage caused by active oxygens such as lipid peroxidation[7] or oxidation of protein sulfhydryl groups, (3) depletion of cellular reduced glutathione[11], (4) perturbation of calcium-ion homeostasis[12].

LQM-A radical and resulting active oxygens were thought to affect a wide variety of cellular constituents. Which effect is a critical event in the development of LQM-A cytotoxicity remains, however, to be elucidated.

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