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An ''*In Vitro*'' System Simulates in Membranes the Antibacterial Mechanism Postulated for the Action of Isoxazolylnaphtoquinoneimine in *Staphylococcus aureus*

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The 2-hydroxy-N-(3,4-dimethyl-5-isoxazolyl)-1,4-naphthoquinone-4-imine (Q_1) revealed good activity against *Staphylococcus aureus.* Q_1 in contact with the bacteria experimented reduction evidenced by changes in its spectrum of absorption simultaneously with loss of colour. During the first 4 hours of incubation, oxygenation restored the original spectrum. Treatment with sodium borohydrure reduces irreversibly Q_1. Redox-reaction "*in vitro*" was detected between Q_1 and NADH in the presence of diaphorase. The environment of the probable site of action of Q_1 was simulated using an artificial membrane system, instead of *S. aureus* membranes. Q_1 interacts with lisophosphatidylcholine micelles following a cooperative binding model. The kinetics of Q_1-reduction was increased by lipid micelles incorporated with the antibacterial compound. © 1997 Academic Press

***Key Words: Staphylococcus aureus;* isoxazolylnaphthoquinoneimine; NADH-reduction; antibacterial action; antibiotic-membrane interaction.**

Naphthoquinones have been extensively studied by their broad spectrum of biological activities, which ranges from antineoplastics (1) to antibacterials (2, 3) and antiprotozoals (4, 5). Anti-toxoplasma activity "*in vitro*" and "*in vivo*" on inoculated mices was also demonstrated and protective effect of **Atovaquone,** a hydroxynaphthoquinone, was observed against *Pneumocystis carinii* (6, 7).

Isoxazolylnaphtoquinoneimines are a class of compounds obtained by synthesis in our laboratory (8, 9). The biological activity of some members of this family was studied against *Trypanosoma cruzi,* the ethiologic agent of Chagas disease, with interesting results (10, 11).

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The antistaphylococcal activity of isoxazolylnaphtoquinoneimines "*in vitro*" and "*in vivo*" has been previously reported by us (12, 13, 14). The 2-hydroxy-N-(3,4-dimethyl-5-isoxazolyl)-1,4-naphtoquinone-4-imine (Q_1) showed antistaphylococcal activity against gram positive bacteria, particularly against β- lactamase-positive *S. aureus* strains. The effective minimum inhibitory concentration (MIC) was within 16 - 64 μg ml^{-1}. Q_1 diminished bloodstream infection of mice injected i.m. with *S. aureus;* septicaemia decayed significantly when the antibiotic was applied and a significant protection was afforded.

A previous report (14) demonstrated that Q_1 is involved affecting the respiratory chain process, increasing the oxygen consumption by an alternative pathway with generation of the superoxide radical anion (O_2^-) but to date little is known about isoxazolylnaphtoquinone-imines action on bacterial membranes. Therefore the present paper studies a possible Q_1/NADH redox-reaction as part of its molecular mechanism of action as antibiotic agent. Assays of Q_1 partition into lipid micelles were performed with an artificial membrane system, considering that it is known that the respiratory chain is performed in the bacterial membrane; NADH and diaphorase were used for the redox reaction.

MATERIALS AND METHODS

Materials. Disodium salt of β-nicotinamide adenine dinucleotide in the reduced form (NADH); NADH-deshidrogenase (diaphorase)from *Clostridium kluyveri;* buffer salts; sodium borohydrure and 1-α- lysophosphatidylcholine (LPC) were purchased from Sigma Chemical Company, St. Louis, Mo. USA.

Antibiotic. 2-Hydroxy-N-(3,4-dimethyl-5-isoxazolyl)-1,4-naphtoquinone-4-imine (Q_1) (Fig. 1) was prepared by reaction of sodium-1,2-naphtoquinone-4-sulphonate and 5-amino-3,4-dimethylisoxazole in alkaline aqueous solution at room temperature for 30 min. The insoluble product was crystallized from ethanol, and its purity was determined by conventional methods, including high-pressure liquid chromatography (HPLC), nuclear magnetic resonance (NMR) spec-

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troscopy, mass spectroscopy (MS), infrarred (IR) spectroscopy; and thermal analysis (8, 15, 16). Its m.p. was 214-5°C; IR (KBr pellet): 3126, 1647, 1307, 1328, 1595 cm^{-1}; MS: *m/e* 268 (M^+). This substance was used as freshly made solutions in DMSO/0.5 M Tris-HCl buffer pH 7.4 in concentration of 40 μM.

Micelles preparation. The micelles were prepared by drying appropriate aliquots of the phospholipid (LPC) from stock solutions, resuspended in 20 mM Tris-HCl pH 7.4 buffer and sonicated until complete clarification. The final LPC concentration in the micellar assay was from 0.125 to 20 mM.

Preparation and partition of Q_1 in the lipidic phase. The mixture was performed as follow: The desired amount of freshly-prepared Q_1 in DMSO/Tris-HCl buffer 20 mM pH 7.4 was added to micelles. Different amount of LPC were dried and mixed with Q_1 in constant concentration. The molar ratio LPC/Q_1 was varied in a widely range from 3.12 to 500.00. Spectrophotometric measurements were recorded on a Gilford spectrophotometer Response II, with thermostatized cuvette cell holder at constant temperature of 30°C. The absorption spectrum of the antibiotic alone and that of the antibiotic-lipid mixtures was determined and then the maximum absorbance for each molar ratio LPC/Q_1 was measured.

"In vitro" reduction of Q_1. The assays were performed in soluble phase and in micelles. NADH 200 μM, diaphorase 0.01 international unit and Q_1 in DMSO/TRIS-HCl pH 7.4 were employed in the reaction.

Kinetic of NADH-reaction was monitored by changes in its absorption spectrum and calculated from the decrease of absorbance at 340 nm. Q_1 reduction was followed by the shift and decrease of the 490 nm band of its absorption spectrum.

The reactions were initiated by two triggering ways, one of them by adding Q_1 and diaphorase to NADH and the other one, by adding NADH and diaphorase to Q_1. Both experiments were performed in order to discard the cross reaction between Q_1 and NADH without diaphorase and between NADH and diaphorase.

One unit of diaphorase was defined as the amount able to oxidize 1 mol of NADH at pH 7.4 in a min at 25°C.

Reduction of Q_1 by Staphylococcus aureus. Suspensions of 10^9 c.f.u/mL of *S.aureus* were incubated with Q_1 at 37°C for 4 and 24 hours; the absorption spectrum of Q_1 were performed to check any change due to bacteria presence. Oxygen flow was applied to cultures with Q_1 and the absorption spectrum were runned before and after treatment. In addition, Q_1 was treated with 1% of sodium borohydrure to know if this reducing agent was able to produce on Q_1 absorption spectrum the same effect that *S.aureus.*

RESULTS

The Q_1 compound (Fig. 1) in aqueous phase showed an absorption spectrum with three bands: the first at 490- 500 nm corresponding to the π-π* transition of quinonoid ring, the second centered at 292 nm attributed to bencenoid ring and the third at around 250 nm due to isoxazol ring (Fig. 2). The absorption spectrum of Q_1 changed in presence of a suspension of *S.aureus*, as important decrease in the intensity of 490 nm band was observed, which was accompanied by colour loss (Fig. 2). During the first 4 hours of incubation the colour and the original spectrum of Q_1 could be restored by oxygenation but at longer periods a irreversible change was observed. Reduction of Q_1 with sodium borohydrure caused a decrease in intensity of 490 nm band spectrum, similar to the effect produced by the interaction of Q_1 with the bacteria.

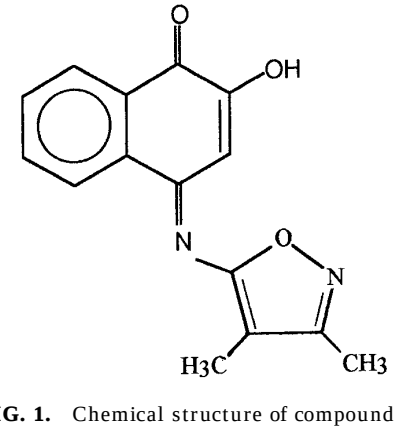

FIG. 1. Chemical structure of compound Q_1.

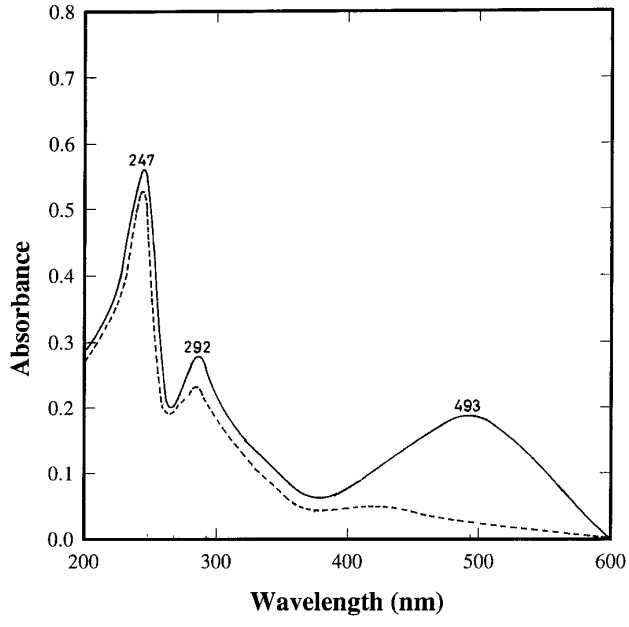

FIG. 2. Absorption spectrum of Q_1 (DMSO/0.5 M buffer Tris-HCl, pH 7.4) in the oxidized (—) or in the reduced form (---) in the presence of *S. aureus* ATCC 29213.

The partition of the antibiotic into LPC micelles brought a shift of the 490 nm band to longer wavelengths (518 nm), as it is shown in Fig. 3. This change in the absorption spectrum caused by the **LPC-Q_1** interaction was employed as a measure of the extent of Q_1 partition into the micellar phase and to test the model of the antibiotic interaction with the lipid interface, as it is summarized in Table 1 and inset Fig. 3.

The absorption spectrum of NADH presents bands at 340 nm and 260 nm (Fig. 4). Incubation of NADH with the NADH-dehydrogenase, showed a constant absorbance value at 340 nm during all the kinetic assay. This stable absorption indicated the absence of a redox-reaction. When the experiments were carried out with different NADH-dehydrogenase concentrations, the intensity of the 340 nm band remained unchanged (data not shown). However, in presence of Q_1 the coupled

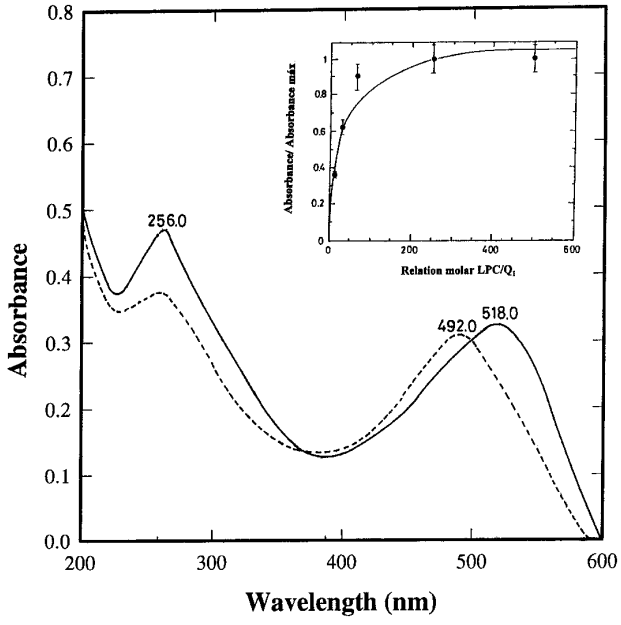

FIG. 3. Partition of Q_1 in LPC micelles. Absorption spectrum in aqueous phase (---) and in LPC micelles (—). (Inset) Binding curve of Q_1 at a different molar relation of LPC/Q_1.

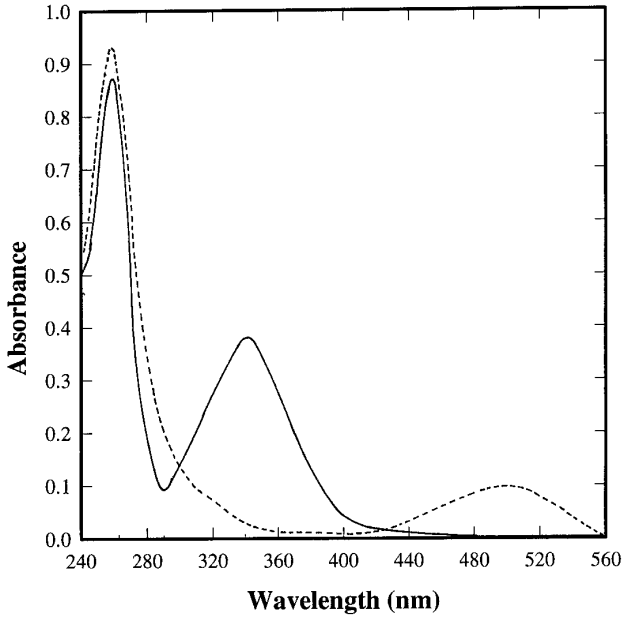

FIG. 4. Absorption spectrum of NADH alone (—) or in the presence of diaphorase and Q_1 in aqueous phase (---).

reaction NADH/NADH-dehydrogenase in aqueous phase was evidenced by the changes in the absorption spectrum, mainly by the disappearance of the 340 nm band of the NADH and by the decrease of the 490 nm band of Q_1. The absorption spectrum of the redox-reaction products showed the isoxazol band at 256 nm due to the electronic transition π-π* (Fig. 4).

The absorption spectrum of NADH in presence of LPC micelles exhibited a similar behavior to that observed in aqueous phase. The band of Q_1 at 256 nm remained unchanged, the shoulder at 300 nm was ascribable to the bencenoid transition and the band of quinonoid ring transition π-π* shifed from 490 nm to 515 nm with notorious decrease due to the Q_1 reduction (Fig. 5).

The kinetic of reaction between Q_1 and NADH in the NADH-containing micelles was close to twice compared with the value obtained in a micellar free system (Fig. 6). This was evidenced by a faster disappearance of the 340 nm band of NADH when a lipidic phase was present (Fig. 6). The slope of the reaction was higher in

TABLE 1

Partition of Q_1 in Phospholipid Micelles (LPC)

Number	Ratio LPC/Q_1	Absorbance at 518 nm	λ of maximum absorbance	Absorbance maximum
0	0	0,2535	492,5	0,2842
1	3,12	0,2925	493,5	0,3169
2	6,25	0,2980	499,5	0,3146
3	9,37	0,3099	499	0,3228
4	12,50	0,3357	504,5	0,3448
5	25,00	0,3694	511,5	0,3719
6	62,50	0,3902	519,5	0,3904
7	93,75	0,3882	524	0,3893
8	125,00	0,3806	522	0,3904
9	250,00	0,3870	523	0,3896
10	500,00	0,3853	524	0,3882

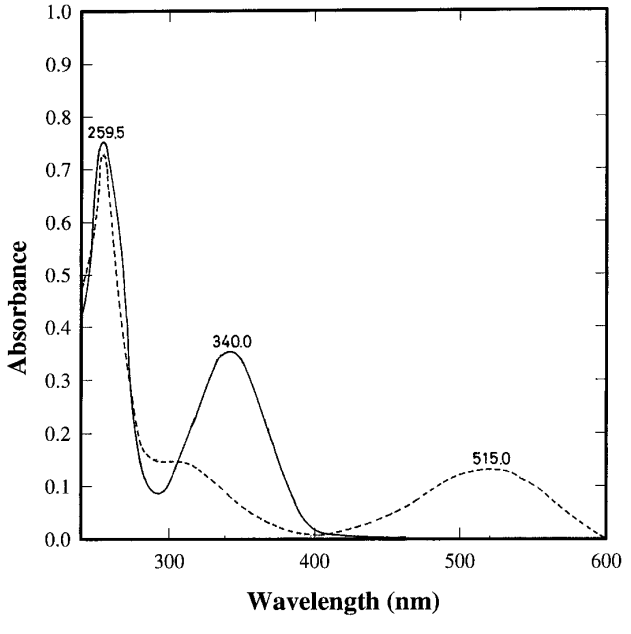

FIG. 5. Absorption spectrum of NADH at zero time (—) or when the reaction has been allowed to proceed in micellar phase (---).

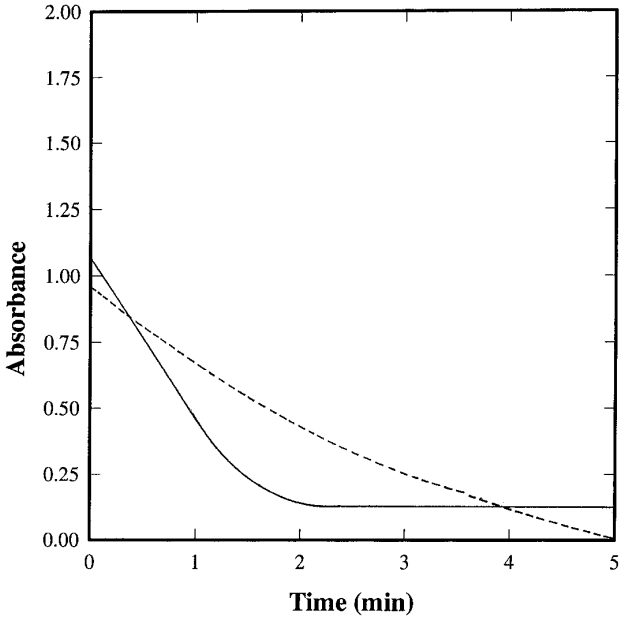

FIG. 6. Velocity of NADH reduction followed by the decrease in the absorbance of the band at 340 nm in the presence of a micellar phase (—) or in aqueous phase (---).

micellar phase (−0.5423±0.0031) than in aqueous phase (−0.2453±0.0040). The kinetic analysis showed that the slope was independent of the NADH concentration in the range studied (Table 2).

The compromise between extintion molar coefficient sensibility of Q_1 and the concentration was checked before the binding experiments were performed, it was observed that the range of solubility of Q_1 in aqueous solution was poor. For this reason it was appropriated to follow the extent of binding to micelles at different lipid/Q_1 molar ratio keeping a constant concentration of the antibiotic. The binding of Q_1 to the micelles can be analyzed in terms of a modified form of the Langmuir isotherm according to

$$A_{518}/A_{máx} = \frac{(LPC/Q_1)^n}{(K + LPC/Q_1)^n}, \qquad [1]$$

where **LPC/Q_1** is the molar ratio between LPC and Q_1, **n** is the cooperativity coefficient and **K** is the apparent partition constant. A value of **n** near two (n = 1.94±0.03) was obtained, with a **K** of **85**. The exact amount of adsorbed molecules was not know, consequently the binding curve was made using molar ratio and not real concentration. The partition of Q_1 was followed by changes in the absorbance at 518 nm with respect to the maximum change obtained at saturating lipid/Q_1 ratio.

DISCUSSION

The isoxazolylnaphthoquinoneimine Q_1 was able to interact with a lipid interface as expected by its amphiphilic character. A **n** value greater than 1 obtained in the binding experiments indicated that Q_1 binds to the micelle in a complex manner. When this analysis was applied, the value of K obtained means, in binding terms, that the lipid/antibiotic ratio at which the half amount of Q_1 is found in the lipid phase was approximately 85/1.

The classic method of measure NADH oxydation by the decrease of its absorption spectrum at 340 nm was usefull to detect the reaction between Q_1 and NADH. In general, this test can be applied to demonstrate that the redox-reaction occurs with the respiratory chain intermediates. In this study the decrease of 490 nm band of Q_1 was also used to confirm its reduction associated with the NADH oxydation. The bathocromic shift of the 490 nm band of Q_1 to 518 nm in the lipid phase was probably due to the sensitivity of the quinonoid ring to the polarity of the medium; because the π-π* quinonoid band position varied with the environmental composition.

Inhibition of *S.aureus* growth induced by Q_1 was well determinated before (13) and it is probably due to both reduction and oxydation reactions occured in the microorganism. The results obtained indicated that there was dependence of diaphorase and NADH in the redox process with the antibiotic. It is possible to associate the redox-reaction "*in vitro*" between NADH and Q_1 with the interaction bacteria-Q_1.

It is probable that Q_1 introduces itself into the proton motive quinone cycle, which interacts with various redox carriers producing its antibiotic effect (10, 17). Q_1 could interact with ubiquinol-pool playing the role of a hidrogen carrier between cytochromes, whose display alternative paths branched at the ubiquinone (UQ) step of the respiratory chain. This last have very different kinetic behavior in response to the modification of the quinone pool (18). The alternative pathway generated by Q_1 at the ubiquinone step of the respiratory chain shows a fast kinetic behavior when the redox-reaction take places in the more amphiphilic and organized medium such us the micellar phase.

The higher reduction rates obtained with the presence of artificial membrane system compared with those obtained in aqueous solution indicated that the partition of Q_1 into the lipid membrane increased the redox-reaction with the help of diaphorase and NADH.

Boveris et al. (1978) found that intermediates of

TABLE 2

Slope of Redox-Reaction with LPC at Two NADH Concentrations

NADH (μM)	Slope (−k)	±SEM
400	0,6220	6,4. 10^{-3}
200	0,5423	3,1. 10^{-3}

partial reduction of oxygen, O_2^- and H_2O_2 are generated by side reactions of physiological electron transfer in membrane-bound multienzyme redox systems (17). The antitrypanosomal and antiprotozoo activities of Q_1 on *T.cruzi* were associated to a NADH oxydation and O_2^- increased generation, as consequence of which epimastigotes dead. It has been suggested that the redox-reaction occurs with protons translocation from NADH to Q_1; this compound can be introduced itself in the proton motive quinone cycle interacting with various redox carriers. Q_1 plays the role of a ubiquinol like molecule as hydrogen carrier before cytochromes (5, 19).

NADH-reactions associated to redox cycling were also observed for Q_1 interacting with eukariotic cells and it was assumed that isoxazolylnaphtoquinones can form semiquinone radicals whose disproportionates or isomerizes to other products (10). The reactions comprised in the redox cycling would be as follow (20, 14):

$$NADH + Q_1 \rightarrow Q_1^- + NAD^+$$

$$Q_1^- + O_2 \rightarrow Q_2 + O_2^-,$$

where $\mathbf{Q}_1^-$ is a semiquinone radical, $\mathbf{O}_2^-$ is superoxide anion and $\mathbf{Q}_2$ is the final product.

A previous report of our group, showed that oxygen consumption of *S.aureus* increased in presence of Q_1 leading to a pathway with more anion superoxide generation. This was demonstrated by scavenger of O_2^-, which restrained the antibiotic effect (14). The generation of superoxide anion is responsible of the oxidative injury in the bacterial cells, since it is known that this radical has many deleterious effects (21).

It is possible to conclude from the results obtained using an artificial membrane system that exists an effective partition of the antibiotic into the lipid interface that facilitates a further interaction with the respiratory chain and the NADH redox reaction. These results are in agreement with previous studies (5, 18, 22) that showed at NADH is a dynamic molecule incorporated in the bacterial membrane, able to interact with diverse macromolecules. In *S. aureus* the partition of Q_1 into membranes facilitates the introduction in the ubiquinone step of the respiratory chain, deriving it to an alternative pathway which provokes O_2^- increase and antibacterial effect.

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