0014-4754/96/060600-05$1.50 + 0.20

Antibacterial effect of 2-hydroxy-*N*-(3,4-dimethyl-5-isoxazolyl)-1,4-naphthoquinone-4-imine on *Staphylococcus aureus*

P. Bogdanov[a], I. Albesa[a,*], N. R. Sperandeo[a], C. Luna[b] and M. M. de Bertorello[a]

[a]*Departamento de Farmacia, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Suc. 16 C.C. 61, 5016 Córdoba (Argentina), Fax +5451 694724*
[b]*Fisiología Vegetal, Facultad de Ciencias Exactas, Universidad Nacional de Córdoba (Argentina)*
Received 19 January 1995; recieved after revision 1 September 1995; accepted 7 September 1995

Abstract. The mechanism by which a new naphthoquinone derivative, the 2-hydroxy-*N*-(3,4-dimethyl-5-isoxazolyl)-1,4-naphthoquinone-4-imine (INQI-E) has antibacterial effect against *Staphylococcus aureus* was studied. The interaction of INQI-E with the bacteria was followed by absorption spectroscopy at 323 and 490 nm. The absorption band of INQI-E at 490 nm undergoes a hypochromic shift with a decrease of intensity. This effect was found to be reversible by oxygenation during the first hours of incubation. The participation of an oxidation–reduction process related to the respiratory chain was demonstrated by oxygen consumption. An increase in O_2 uptake and inhibition of *S. aureus* growth was observed. Experiments with three inhibitors of the respiratory chain demonstrated that the pathway induced by INQI-E was antimycin-resistant and KCN- and salicylhydroxamic acid (SHAM)-sensitive, which suggests that INQI-E is capable of diverting the normal electron flow to an alternate superoxide-producing route. On the other hand, experiments with Tiron, a specific scavenger of superoxide, hindered the effect of INQI-E against *S. aureus*, indicating that the inhibitory growth effect of this quinone-imine is mainly due to the production of the cytotoxic superoxide radical.
Key words. *Staphylococcus aureus*; isoxazolylnaphthoquinone; antibacterial effect; superoxide-producing pathway.

It is well known that an increasing number of infections are produced by penicillin-resistant *Staphylococcus aureus*, which underlines the necessity to develop new antibiotics as alternative therapeutic treatments. In our laboratory we have synthesized a series of isoxazolylnaphthoquinones with important biological properties[1,2]. One member of these naphthoquinone derivatives, the 2-hydroxy-*N*-(3,4-dimethyl-5-isoxazolyl)-1,4-naphthoquinone-4-imine (INQI-E) (fig. 1) showed antibacterial activity against *Staphylococcus aureus* with a minimum inhibitory concentration (MIC) of 16–32 $\mu g \cdot ml^{-1}$ for *S. aureus* ATTC 29213, *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228 and 31 clinical strains producing β-lactamase (5 of them were MRSA)[3,4]. Moreover, successive cultures at subinhibitory doses of INQI-E or 7-day cultures with 150 $\mu g \cdot ml^{-1}$ of INQI-E did not induce resistant mutants and spontaneous resistance was not found[4].

The mechanism of antibacterial and antiprotozoal activities of several naphthoquinones[5,6] and isoxazolylnaphthoquinones[2,7] has been studied, and these compounds were suggested to exert their action by generation of oxyradicals. In fact, Tarlovsky et al.[2] showed that incubation of INQI-E and other isoxazolylnaphthoquinone imines with trypanosomes gave rise to a reversible increase in oxygen consumption and superoxide generation.

On the basis of these observations, it seemed of interest to investigate the antibacterial mechanism of INQI-E on *S. aureus*, in order to see if there is any relationship between the induction of an alternative respiratory pathway and generation of oxyradicals, particularly and cytotoxic superoxide radical.

Materials and methods

2-Hydroxy-*N*-(3,4-dimethyl-5-isoxazolyl)-1,4-naphthoquinone-4-imine (INQI-E) was prepared by reaction of sodium-1,2-naphthoquinone-4-sulphonate and 5-amino-3,4-dimethylisoxazole in alkaline aqueous medium temperature for 30 min[8]. The insoluble product was

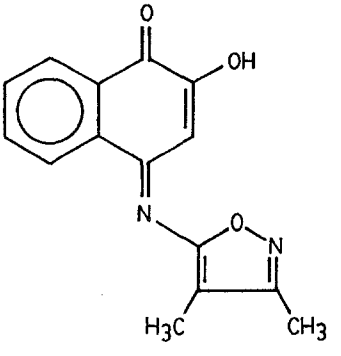

Figure 1. Chemical structure of 2-hydroxy-*N*-(3,4-dimethyl-isoazolyl)-1,4-naphthoquinone-4-imine (INIQ-E).

* Corresponding author.

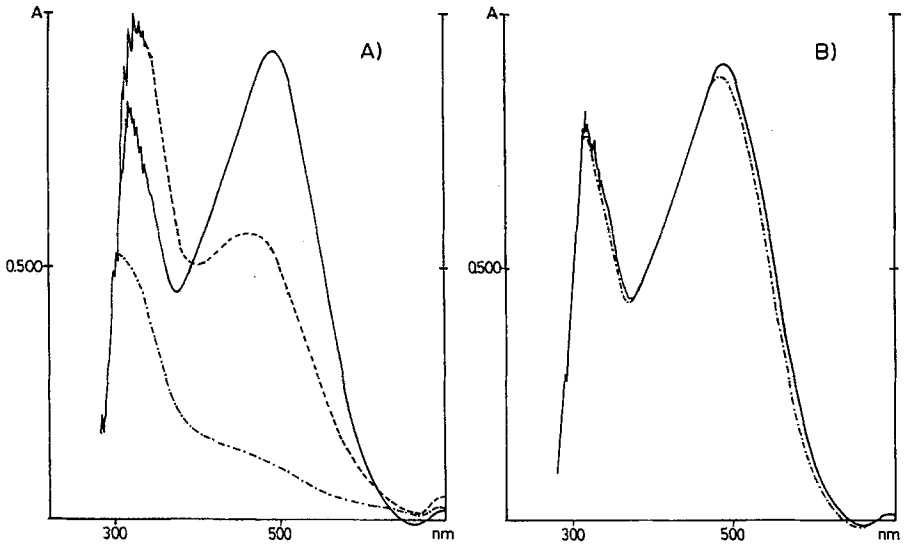

Figure 2. Absorption spectra of INQI-E (50 μg · ml^{-1}) obtained during incubation with *S. aureus* ATCC 29213 (10^9 cfu · ml^{-1}). (*A*) Complete culture: zero time (—), 3 h (– · –) and 24 h (– –). (*B*) Supernatant culture: zero time (—), and 24 h (– –).

crystallized from ethanol, and its purity was determined by conventional methods, including high-pressure liquid chromatography (HPLC); nuclear magnetic resonance (NMR) spectroscopy, mass spectroscopy (MS) and infrared (IR) spectroscopy; and thermal analysis[9,10]. Its m.p. was 214–5 °C; IR (KBr pellet): 3126, 1647, 1307, 1328, 1595 cm^{-1}; MS: *m/e* 268 (M$^+$). *S. aureus* ATCC 29213 (obtained from the American Type Culture Collection) was cultured and maintained on Trypticase soy agar slants at 4 °C.

Oxygen consumption. Oxygen uptake was determined in a Gilson oxygraph (with a Clark-type O_2 electrode based on a design of Delieu and Walker) at 20 °C in a total volume of 2.5 ml. *S. aureus* isolates were grown at 35 °C for 18 h in Mueller–Hinton agar (MHA, Merck). The cultures were resuspended and centrifuged at 12,000 *g* for 20 min at 4 °C. The resulting cell pellets were prepared (10^{11}–10^{12} cfu · ml^{-1}) in Mueller–Hinton broth (MHB, Merck) and added to the oxygraph cell which was air-saturated before the measurements. The capacity of the cyt respiratory pathway, expressed as μM · min^{-1}, was studied without INQI-E or with the addition of 50, 100 and 200 μg · ml^{-1}. In addition, determinations were done in the presence of the following respiratory chain inhibitors (assay concentrations): KCN (1 mM in MHB); antimycin A (10 μM in MHB) and salicylhydroxamic acid (SHAM, 10 mM in MHB). Controls performed without the addition of INQI-E were used as references.

Spectrophotometric measurement. *S. aureus* grown for 18 h MHB at 35 °C was separated into three fractions: A, complete culture (10^9 cfu · ml^{-1}); B, supernatant; and C, suspended bacterial pellet (10^{12} cfu · ml^{-1}); the last two were obtained by centrifugation at 12,000 *g* for 10 min at 4 °C. INQI-E was dissolved in the minimum amount of DMSO and diluted in MHB to give 50 μg · ml^{-1} assay concentration and then added to these fractions. They were then incubated for 20 min and 1, 2, 3 and 24 h. The absorption spectrum of INQI-E in the free cell supernatants of fractions A, B and C was recorded on a Shimadzu UV-260 spectrophotometer. Sodium borohydrure (300 mM) was added to INQI-E in assays without *S. aureus* and then the absorption spectrum was determined.

Effect of free-radical scavengers on *S. aureus* growth. The identity of the oxygen radicals involved in the oxidative injury of *S. aureus* was investigated by means of fairly specific antioxidants. 4,5-Dihydroxy-1,3-benzene-disulfonic acid (Tiron, SIGMA) at 10 and 50 mM (in MHB) and sodium benzoate at 1 mM (in MHB) were used as superoxide and hydroxyl radical scavengers, respectively. Sodium ascorbate at 1 mM (in MHB) was also used as antioxidant. The normal bacterial growth was compared with growth in the presence of: a) 50, 100 and 200 μg · ml^{-1} of INQI-E; b) Tiron, 10 and 50 mM; c) combination of Tiron (50 mM) with 50, 100 and 200 μg · ml^{-1} of INQI-E. The culture growth kinetics were evaluated using two procedures, spectrophotometry at 600 nm and counting of the cfu · ml^{-1} by dilution in Mueller–Hinton agar incubated at 35 °C for 24–48 h.

The experimental results are the mean values of three assays. All of them deviate from the mean by less than 5%.

Hydrogen peroxide was measured spectrophotometrically at 505 nm using aminoantipyrine as reagent according to the horseradish peroxidase reaction[11].

Table 1. Effect of INQI-E (50 μg · ml^{-1}) on oxygen uptake of *S. aureus* at short-time.

Time (min)	Count (cfu · ml^{-1})	Oxygen uptake (μM · min^{-1})		Increase %
		normal[a]	+INQI-E[a]	
4	10^9	7.0 ± 0.9	9.2 ± 0.3	31
	10^{12}	33 ± 1	39 ± 3	18
10	10^9	5.6 ± 0.7	8.1 ± 0.5	45
	10^{12}	29.4 ± 0.7	33 ± 2	12

[a]Mean ± SEM.

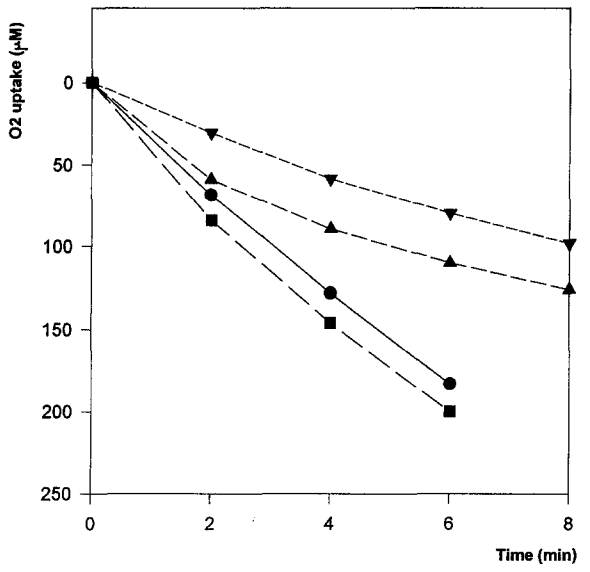

Figure 3. O_2 uptake (μM) of *S. aureus* ATCC 29213 (10^{12} cfu · ml^{-1}) in presence of INQI-E (50 μg · ml^{-1}) and KCN (1 mM). Normal culture (-●-); with INQI-E (-■-); with KCN (-▲-); and with INIQ-E — KCN (-▼-).

Results

The absorption spectrum of INQI-E (50 μg · ml^{-1}) changed markedly when it was incubated with the complete culture of *S. aureus* (10^9 cfu · ml^{-1}). The absorption band at 490 nm was progressively shifted to shorter wavelengths with a decrease in intensity (fig. 2A). This effect is more pronounced in the presence of the bacterial pellet (10^{12} cfu · ml^{-1}), which displaced the 490 nm band to 450 nm in 3 h (data not shown) while the complete culture (10^9 cfu · ml^{-1}) displaced that band to 465 nm in the same period of time. During the first hours of incubation with INQI-E the 490 nm band recovered by oxygenation, but it disappeared irreversibly at 24 h. An irreversible effect was also observed by reaction of INQI-E with sodium borohydrure, which immediately reduced INQI-E to a colorless derivative without absorption at 490 nm. On the other hand, the spectrum was not affected when INQI-E was incubated with culture supernatant (fig. 2B).

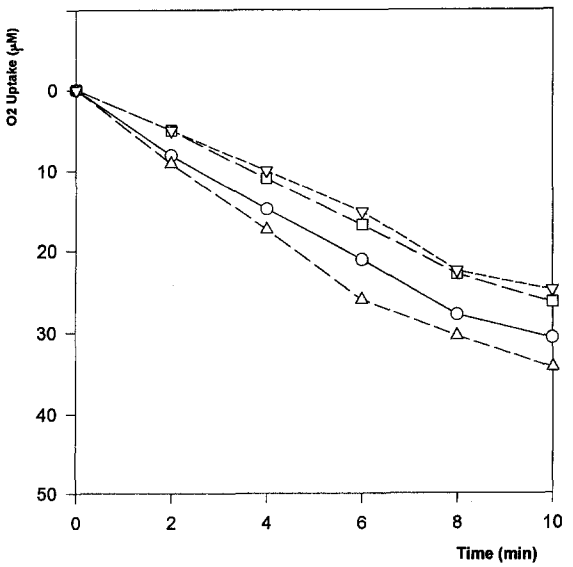

Figure 4. Effect of SHAM (10 mM) and INQI-E (50 μg · ml^{-1}) on O_2 uptake of *S. aureus* ATCC 29213 (10^{11} cfu · ml^{-1}). Normal culture (-□-); in presence of INQI-E (-○-); with SHAM (-△-); and with INQI-E + SHAM (-▽-).

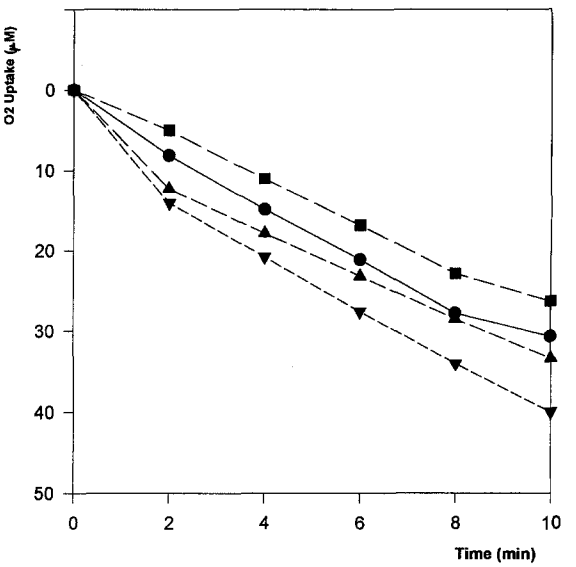

Figure 5. Effect of antimycin A (10 μM) and INQI-E (50 μg · ml^{-1}) on O_2 uptake (μM) of *S. aureus* ATCC 29213. Normal culture (-■-); in presence of INQI-E (-●-); antimycin A (-▲-); and combination INQI-E – antimycin (-▼-).

Respiration measurements show that the oxygen uptake by *S. aureus* increased in the presence of INQI-E (50 μg · ml^{-1}) (table 1), so an induced respiratory pathway becomes apparent. This effect was higher with 10^9 than with 10^{12} cfu · ml^{-1}. Moreover, when INQI-E was incubated with the lower-density culture, the increase in oxygen consumption was higher after 10 min than after 4 min of treatment. This time dependence was not observed with 10^{12} cfu · ml^{-1}.

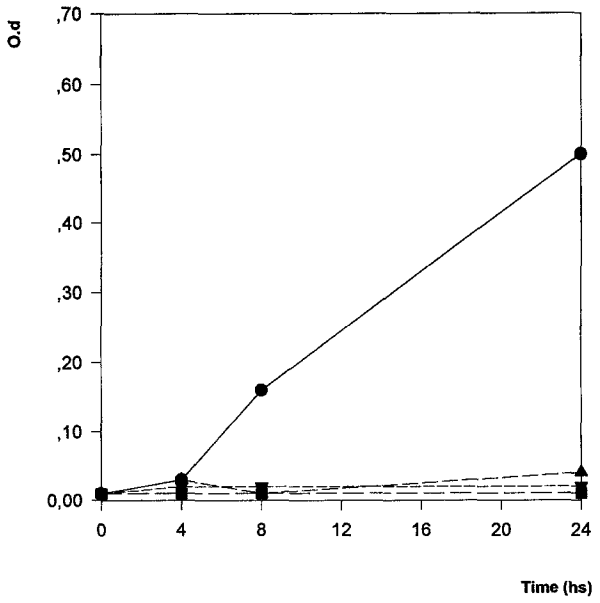

Figure 6. *S. aureus* ATCC 29213 growth using different concentrations of INQI-E. Normal culture (-●-); INQI-E (50 μg · ml^{-1}) (-▼-); INQI-E (100 μg · ml^{-1}) (-▲-); and INQI-E (200 μg · ml^{-1}) (-■-). OD = optical density.

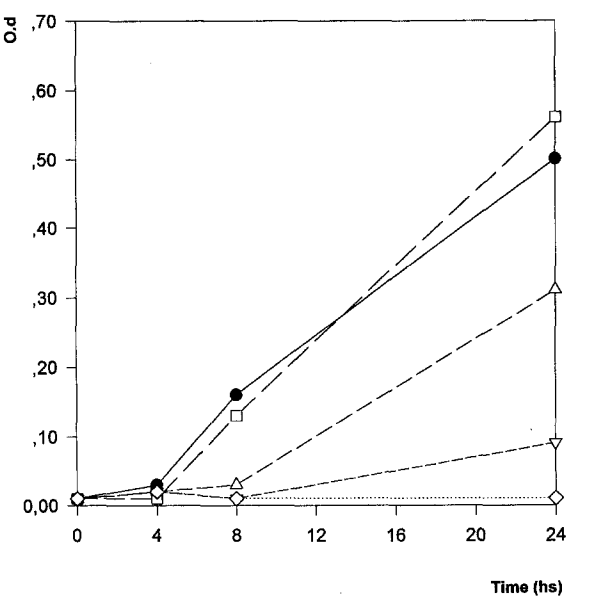

Figure 7. Scavenger effect of Tiron (50 mM) on growth of *S. aureus* ATCC 29213 treated with different concentrations of INQI-E. Normal culture (-●-); Tiron (-□-); Tiron + INQI-E (50 μg · ml^{-1}) (-△-); Tiron + INQI-E (100 μg · ml^{-1}) (-▽-); and Tiron + INQI-E (200 μg · ml^{-1}) (-◇-).

Oxygen consumption experiments in the presence of potassium cyanide (KCN) (1 mM), an inhibitor of the classic route of electron transport, showed that the respiration was partially inhibited. For example, after 6 min of incubation the oxygen uptake diminished by 40% (fig. 3). This effect was higher (61%) when *S. aureus* was incubated, during the same period of time, with a mixture of KCN (1 mM) INQI-E (50 μg · ml^{-1}) which indicates that the respiratory route induced by INQI-E is more sensitive to cyanide than the normal way.

Figures 4 and 5 show the behavior of *S. aureus* (bacterial density 10-fold lower than that employed in figure 3) grown in the presence of the other two respiratory chain inhibitors, salicyl hydroxamic acid (SHAM; 10 mM) and antimycin A (10 μM). As can be seen in figure 4, the addition of SHAM increases the uptake of oxygen. However, the combination INQI-E–SHAM provokes a significant reduction in oxygen consumption. This demonstrates that the induced respiratory pathway is SHAM-sensitive.

In contrast, addition of antimycin A, a classical inhibitor of cytochrome chain respiration, and INQI-E (50 μg · ml^{-1}), increased oxygen uptake, demonstrating that the metabolic route induced by INQI-E is antimycin-resistant (fig. 5).

The effect of free-radical scavengers on *S. aureus* density was determined by spectrophotometry at 600 nm. Bacterial growth in the presence of INQI-E at different concentrations was measured at 0, 4, 8 and 24 h. Figure 6 shows that the initial inoculum density remained almost constant after 24 h with the three concentrations INQI-E (50, 100 and 200 μg · ml^{-1}) assayed. In all cases the observed effect of INQI-E was inhibition of bacterial growth.

Tiron (50 mM), a specific scavenger of superoxide, exerted a protective effect on *S. aureus* growth when 50 μg · ml^{-1} of INQI-E was added (fig. 7), which indicates the presence of superoxide. This scavenger effect diminished in the presence of 100 μg · ml^{-1} of INQI-E, and finally disappeared when 200 μg · ml^{-1}, a concentration six-fold the MIC, was used.

Similar results were obtained when bacterial growth was measured by counting the cfu · ml^{-1} by dilution in Mueller–Hinton agar incubated at 35 °C for 24–48 h (data not shown).

The addition of sodium ascorbate and sodium benzoate did not reduce the antibacterial effect of INQI-E (data not shown), suggesting the absence of hydroxyl radicals.

Hydrogen peroxide accumulation was not detected by the method used here because the *S. aureus* strain contains larger quantities of catalase which destroys the H_2O_2.

Discussion

INQI-E inhibited growth of *S. aureus* and increased O_2 uptake and free radical generation. The interaction of INQI-E with *S. aureus* was demonstrated by spectrophotometry and by experiments with respiratory chain inhibitors and free radical scavengers.

The observed hypsochromic shift of 26 nm for the quinonoid electron-transfer band[8,10] at 490 nm of

INQI-E following contact with *S. aureus* for a few hours indicates that an effective interaction has occurred which produced a structural change in the quinonoid ring, probably due to a redox reaction. After 24 h of incubation, the resulting spectrum did not exhibit any absorption in the visible region which is consistent with the decoloration observed visually, and indicates that INQI-E was irreversibly transformed into a new entity without quinonoid absorption.

On the other hand, the benzenoid electron-transfer band at about 323 nm was not shifted by the presence of *S. aureus*. This is reasonable because it is known that its position is relatively independent of chemical modifications in the quinonoid ring[12]. Experiments with free cell supernatant showed that it was unable to change the absorption spectrum of INQI-E, leading to the conclusion that interaction with the bacteria was essential for its transformation.

Similar spectral changes were observed for INQI-E with eukaryotic cells[2], which was interpreted to mean that INQI-E participates in a redox cycling; the first product formed being a semiquinone radical which subsequently disproportionates or isomerizes to other products. Analogous spectral changes were also associated by Land et al.[13] with the presence of semiquinone free radicals. In consequence, it is possible to conclude that INQI-E was first reduced to a semiquinone radical by *S. aureus*. This semiquinone must be stabilized by intermolecular hydrogen-bonding, like other semiquinone species that arise from hydroxylquinones with the hydroxy substituents α to the carbonyl group[14].

Therefore, as is assumed for quinones[15] and isoxazolynaphthoquinones[7], the reactions involved in the redox cycling would be the following:

$$NAD(P) + Q_1 \rightleftharpoons NAD(P)^+ + Q_1^{\cdot}$$

$$Q_1^{\cdot} + O_2 \rightleftharpoons Q_2 + O_2^{\cdot -}$$

where Q_1 is INQI-E, $Q_1^{\cdot}$ is a semiquinone radical, $O_2^{\cdot -}$ is superoxide anion and Q_2 is the final product.

According to reaction **2**, the oxidation of the semiquinone generates $O_2^{\cdot -}$, with enhancement of oxygen consumption[2]. This was demonstrated by the increased oxygen uptake of INQI-E and by the positive protective effect of Tiron, a specific superoxide scavenger[16–18]. Since it is known that this radical has many deleterious effects, not only on cell physiology[19–20], *S. aureus* growth could be inhibited in the presence of INQI-E due to an increased production of superoxide. This alternative O_2-consuming route which produces superoxide was found to be anitmycin-resistant and KCN- and SHAM-sensitive in a similar way as described for eukaryotic cells[23].

The results obtained in our laboratory demonstrate that the activity exhibited by INQI-E on *S. aureus* both in vitro[4] and in vivo[3] is relevant, particularly regarding its antibacterial effects in the sub-millimolar concentration range.

The fact that it was observed that there is no cross-resistance with methicillin and penicillin and that *S. aureus* strains arising from different samples of abcess, fistulous, otic secretion and blood were inhibited[4], would make INQI-E an alternative compound of clinical interest to treat antibiotic resistant strains of *S. aureus*.

Although doses from 300 to 1500 mg/kg did not produce toxic effects of Balb/c mice, to evaluate its potential as an antistaphylococcal drug, further studies on its toxicity would be necessary.

Acknowledgements. This work was supported by the Consejo Nacional de Investigaciones Cientificas y Técnicas (CONICET) and the Consejo de Investigaciones Científicas y Technológicas de la Provincia de Córdoba (CONICOR) and the Secretaría de Ciencia y Técnica de la Universidad National de Córdoba. The authors also thank Eng. V. Trippi (Facultad de Ciencias Exactas Fisico Puimica y Naturales (U.N.C.)) for the availability of the Gilson oxygraph and helpful discussions.

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