*Short Communication*

# AN IN VITRO STUDY OF THE INTERACTION OF SEA-NINE® WITH RAT LIVER MITOCHONDRIA

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**Abstract**—The interactions of the antifouling compound Sea-Nine® with rat liver mitochondria have been studied. The results indicate that low doses of this compound inhibit adenosine 5′-triphosphate (ATP) synthesis. Further investigations indicate that ATP synthesis inhibition should be due to an interaction of Sea-Nine with the succinic dehydrogenase in the mitochondrial respiratory chain.

## INTRODUCTION

The chemical compound Sea-Nine® (4,5-dichloro-2-*n*-octyl-3[2H] isothiazone [SN]), together with other organic compounds, was tested for use as an antifouling compound to replace tin compounds. Although tin compounds are efficient as antifouling agents, it became necessary to replace them with organic compounds because they are very toxic and persist for a long time in the environment. For this reason, the use of organotin compounds in antifouling paints has been restricted in many countries: Regulation 782/2003 of the European Parliament and Council of April 14, 2003 prohibits tributyltin (TBT)-containing paints to be applied to any vessel, as of July 1, 2003, and enforces the complete removal of TBT from ship hulls as of January 1, 2008.

The Sea-Nine compound is effective as an antifouling agent [1,2], but it also is degraded rapidly by microorganisms in the environment [3]. This property reduces the toxicity of SN. However, it has not been assayed quantitatively in animals belonging to a higher order, not only because the measurements require long durations, substantial laboratory space, costs, and so on, but also because the acute effects (short-term effects) are of the same order of magnitude as the decay time concerning SN [3].

Studies of the in vitro toxicity of this compound have been performed on fish cells [4] and the viability of the cells has been evaluated using the Alamar Blue dye. It has been demonstrated that, in many cases, the preferential target of the toxic compounds responsible for acute toxicity are mitochondria [5–8]. Therefore, in this study, we used the mitochondria (from rat liver) to study the molecular effects of SN, because the behavior and the molecular structure of mitochondria is similar in all living species. The results suggest that the preferential target in mitochondria is the respiratory chain. This behavior could explain both the toxicity and the action of SN as an antifouling compound.

## MATERIALS AND METHODS

The mitochondria were prepared from the livers of fasted albino Wistar rats, weighing about 300 g [9]. The mitochondrial protein was determined using the procedure of Lowry et al. [10].

The mitochondrial oxygen consumption was measured using a Clark oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, USA) fitted in a thermostat-controlled, closed chamber equipped with magnetic stirring. The reaction medium (2 ml) was kept thermostated at 25°C during all the experiments (standard in vitro conditions).

The following reducing substrates were used: Succinate (2 mM), glutamate/malate (2 mM), and ascorbate ([1 mM]/[400 μM]) $N,N,N',N'$-tetramethyl-*p*-phenylenediamine (TMPD).

The rate of $Ca^{2+}$ influx/efflux was monitored by means of a selective $Ca^{2+}$ electrode (Beckman Coulter, Fullerton, CA, USA) in a glass vessel with a magnetic stirring at room temperature. The recording apparatus was a Radiometer pH-meter connected to a Texas recorder (Texas Instruments, Dallas, TX, USA).

Following the absorbance decrease, the swelling experiments were performed at 540 nm, in a Jenway 6400 (Felsted, Essex, UK) spectrophotometer, equipped with a stirring apparatus. After the addition of the mitochondria to the resuspending medium (2.5 ml), the instrument was adjusted to zero absorbance.

The ATP synthesis/hydrolysis experiments were performed in a low buffered medium, using a pH electrode, connected to a Radiometer PHM (pH-meter) 84 (Radiometer, Copenhagen, Denmark) in order to monitor the pH changes that accompany the reaction:

$$ADP + Pi + H^+ \leftrightarrow ATP$$

under stirring conditions at room temperature (ADP = adenosine 5′-diphosphate).

All the reagents were of an analytical grade. The reagents carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone, Ruthenium Red, sodium glutamate, sodium malate, Oligomycin, and TMPD were obtained from Sigma (Milan, Italy). Sea Nine®

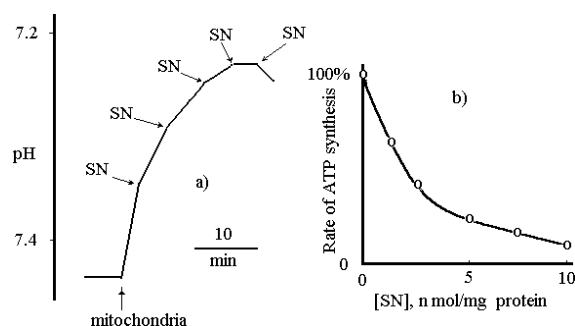* To whom correspondence may be addressed (bragadin@unive.it).

Fig. 1. Adenosine 5′-triphosphate (ATP) synthesis inhibition by Sea-Nine® ([SN], Rohm & Haas, Philadelphia, PA, USA) in rat liver mitochondria. The ATP synthesis is followed by means of a pH electrode, in a low-buffered medium. Medium composition: 0.25 M sucrose, 100 μM Hepes pH 7.4, 200 μM ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, 2 mM MgCl₂, 2 mM ADP, 0.5 mM K₂HPO₄, and 2 mM succinate. The final concentration of mitochondria: 0.5 mg/ml. The addition of μmol amounts of SN, similar to a typical experiment in Figure 1a, induces a decrease in the acidification rate, consequent to the ATP synthesis inhibition (Fig. 1a). It should be noted that, after the complete inhibition of the ATP synthesis, a further addition of SN causes an alkalinization in the medium, thus suggesting (see experiments of Fig. 2) that SN does not inhibit the enzyme ATPase (the enzyme that catalyses the ATP synthesis). The graph (**b**) reports the ATP synthesis inhibition as a function of the SN concentration. The value of 100% corresponds to the rate of ATP synthesis in the absence of SN.



Fig. 2. In vitro adenosine 5′-triphosphate (ATP) hydrolysis in rat liver mitochondria after addition of Sea-Nine® ([SN], Rohm & Haas, Philadelphia, PA, USA) and Oligomycin (specific mitochondrial ATPase inhibitor [the enzyme that catalyses the ATP synthesis]). The medium contained: 0.25 M sucrose, 100 μM Hepes pH 7.4, 200 μM ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, and 2 mM MgCl₂. The mitochondria were added to the medium (4 ml) and the final concentration was 0.5 mg/ml. The addition of 2 mM ATP causes alkalinization with a rate that is not modified by the addition of SN up to 80 μM. The addition of 2 γ Oligomycin, the ATPase inhibitor, induces a complete inhibition of the alkalinization rate.

(4,5-dichloro-2-n-octyl-3-isothiazone) was supplied by Rohm & Haas (Philadelphia, PA, USA).

## RESULTS AND DISCUSSION

In the mitochondria, substrates arising from the Krebs cycle are oxidized by molecular oxygen, and ATP is produced. The oxidation occurs by means of a sequence of cytochromes: The mitochondrial respiratory chain (RC). Coupled to the electron flow in the RC, a proton extrusion occurs and, because the mitochondrial membrane is not permeant to the protons, their extrusion causes a $\Delta$pH (alkaline inside) and a potential difference, $\Delta\Psi$ (negative inside). The sum of the $\Delta$pH and $\Delta\Psi$ was named by Mitchell [11] as the proton motive force (PMF)

$$PMF = \Delta pH + \Delta\psi$$

This is the high-energy intermediate that stores the free energy arising from the substrates oxidation, which subsequently is transferred to the ADP to form ATP.

In order to verify if the mitochondria are a target for the SN, the rate of ATP synthesis in the presence of the SN was measured. Figure 1a shows a typical experiment of the ATP synthesis rate and its inhibition by the SN. The graph in Figure 1b reports the rate of ATP synthesis with increasing amounts of SN and shows that the SN actually inhibits the ATP synthesis at low doses.

Taking into account the mechanism that drives ATP synthesis [11] (see above), the inhibition of the ATP is due to one of the following four causes: Inhibition of the ATPase, uncoupling effect, opening of the permeability transition pore, or inhibition of the respiratory chain.

### Inhibition of the ATPase

The ATPase is the enzyme that catalyses and drives the conversion of the ADP into ATP. The inhibition of this enzyme gives rise to a corresponding inhibition of the ATP synthesis.
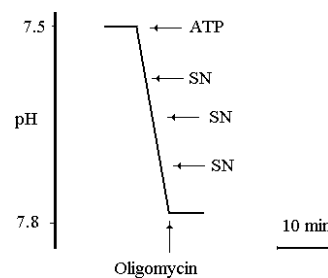
Because ATP hydrolysis, which is catalyzed by the same enzyme (the ATPase), induces acidification of the medium, the measurement of the ATPase inhibition was followed using a pH-meter, in a low-buffered medium. Figure 2 shows an example of this measurement. As a means of comparison, the response of the system to the presence of the potent ATPase inhibitor, Oligomycin, is shown. The addition of amounts of SN, in doses up to more than 100 μM, does not change the rate of ATP hydrolysis. Consequently, we concluded that SN is not an ATPase inhibitor.

### Uncoupling effect

Uncoupling compounds are chemical compounds that permeate the mitochondrial membrane to the protons. Because the PMF results from the impermeability to the protons, the uncouplers cause the inhibition of the ATP synthesis.

The uncouplers are weak acids (or weak bases). For example, 2,4-dinitrophenol, a weak acid ($pk_a = 4.3$, where $k_a$ is the acidity constant and $p$ is the $-\log$), is an uncoupler because it enters as an undissociated compound and tends to accumulate in the matrix, because the driving force is the alkaline-inside pH. Once inside, the phenolate ion is extruded by the negative-inside potential. This extrusion gives rise to a cyclic mechanism and the whole balance is the entry of a proton at any cycle.

This mechanism cannot be proposed as regards SN because SN is not a weak acid (or a weak base). However, it cannot be excluded that SN acts by means of a detergent-like effect [12], causing an enhancement of the permeability to all ions, the protons enclosed. In order to ascertain this possibility, we measured the rate of $Ca^{2+}$ efflux, which previously had accumulated in the energized mitochondria (see *Materials and Methods* section). Since following the accumulation of the $Ca^{2+}$, the $Ca^{2+}$ carrier has been inhibited using Ruthenium Red [9,13]; the rate of $Ca^{2+}$ efflux from the matrix to the resuspending medium is a measure of the membrane leak, or detergent effect induced by the SN. Figure 3 shows a typical experiment concerning $Ca^{2+}$ release after its accumulation in the matrix, by means of ATP. The addition of Ruthenium Red induces only a negligible $Ca^{2+}$ release, and the successive addition of SN induces a significant $Ca^{2+}$ release. Figure 3b reports the rate of $Ca^{2+}$ release at different SN concentrations.
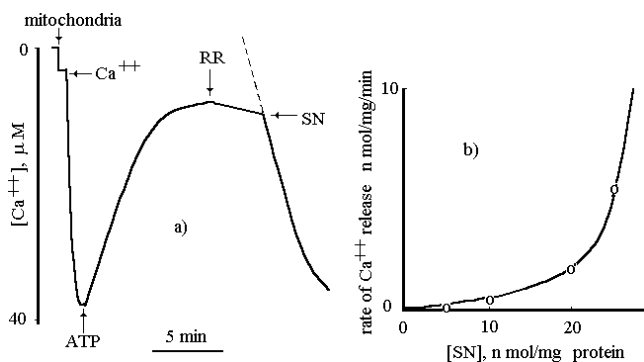
Fig. 3. Sea-Nine®([SN], Rohm & Haas, Philadelphia, PA, USA) induces a Ca$^{2+}$ release in energized rat liver mitochondria (detergent effect). The mitochondria (0.5 mg/ml) were resuspended in a medium containing: 0.25 M sucrose, 10 mM Hepes-Mops pH 7.4, 2 γ cyclosporine, 30 μM CaCl$_2$, and 10 mM sodium acetate. The addition of 2 mM adenosine 5'-triphosphate (ATP) induces Ca$^{2+}$ uptake up to a steady state, which is measured by means of a selective Ca$^{2+}$ electrode. In this situation, the addition of 1 μM Ruthenium Red (RR) inhibits the Ca$^{2+}$ carrier and, subsequently, the addition of SN induces a Ca$^{2+}$ release due to a detergent (or membrane leak mechanism) (**a**). The graph (**b**) reports the initial rate of the Ca$^{2+}$ efflux, induced by the SN, which is the slope in the dotted line (**a**). It should be noted that the effect of the SN is relevant only when the concentration of SN is above 20 n mol/mg (10 μM).

A significant Ca$^{2+}$ efflux is observed only when the concentration of SN is about 10 μM, thus demonstrating that SN induces a membrane leak. This phenomenon, however, cannot be the cause of the inhibition of ATP synthesis (Fig. 1) because this inhibition occurs with lower doses of SN.

*Opening of the permeability transition pore*

In recent years [14], it has been demonstrated that many chemical compounds induce the opening in mitochondria of large-sized membrane pores (permeability transition pores) that allow for the transport through the membrane of solutes of about 1,500 Da (Dalton) in size. Consequently, the opening of the permeability transition pore implies the inhibition of ATP synthesis. The opening of the pore is inhibited by cyclosporine and, as is due to a colloid-osmotic mechanism [14], this is evidenced by the swelling of the mitochondria at 540 nm. Figure 4a shows a typical swelling experiment, induced using SN, and the corresponding graph reports the swelling rate at increasing doses of SN. The graph shows that the swelling occurs when the dose of SN is about 10 μM. Because the swelling is not cyclosporine-sensitive, the interpretation, in this case, was that the swelling was due to a detergent effect and not to the opening of a permeability transition pore. This interpretation is suggested by the failure of the cyclosporine sensitivity, but is supported by a comparison with the graph in Figure 3b that shows a similar behavior. Therefore, we also confirmed by means of these kinds of experiments (swelling experiments) that SN induces a detergent effect, but that this effect is not responsible for the ATP synthesis inhibition.

*Inhibition of the RC*

An inhibition of the RC causes an equivalent ATP-synthesis inhibition. The possibility of an inhibition of the RC by SN was evaluated by means of respiratory rate experiments (i.e., oxygen consumption experiments). Figure 5a shows a typical experiment related to respiratory rate inhibition by SN using succinate as substrate. The graph in Figure 5b reports the con-
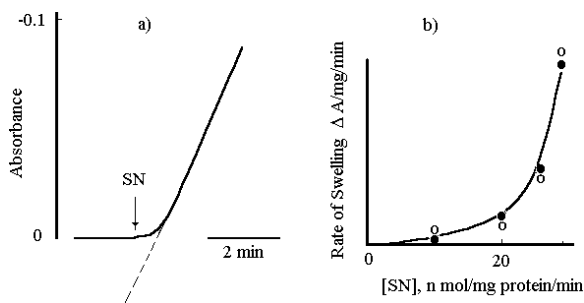


Fig. 4. Swelling of rat liver mitochondria induced by Sea-Nine® ([SN], Rohm & Haas, Philadelphia, PA, USA). The swelling of the mitochondria was monitored following the absorbance quenching at 540 nm. The mitochondria (0.5 mg/ml) were resuspended in a medium containing: 0.25 M sucrose, 10 mM Hepes-Mops, pH 7.4, 200 μM ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, and 2 mM MgCl$_2$. The addition of SN induces swelling. (**a**) An example of a typical experiment; (**b**) graph reporting the rate of swelling, which is the slope in the dotted line in (**a**), in the absence (○), or in the presence (●) of 2 γ cyclosporine.

sequent respiratory rate inhibition at increasing amounts of SN. The graph shows that the dose necessary to induce an inhibition of the respiratory chain is lower than that necessary to induce a detergent effect. Therefore, this fact, together with the comparison between the graph in Figure 5b and the graph in Figure 1b, which reports the ATP synthesis inhibition, suggests that the ATP synthesis inhibition (=toxicity) induced by SN is due to an inhibition of the mitochondrial respiratory chain.

Taking this conclusion into account, the RC was analyzed further using different substrates for the mitochondrial RC: Glutamate/malate, which allows for the functioning of the whole RC and ascorbate/TMPD in order to analyze only the third phosphorylating site. In both cases, we did not find any inhibition of the RC. Therefore, we conclude that, as in the
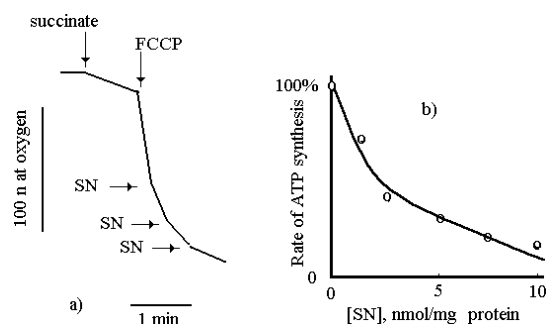


Fig. 5. Mitochondrial respiration and its inhibition by Sea-Nine® ([SN], Rohm & Haas, Philadelphia, PA, USA). The mitochondria (0.5 mg/ml) were resuspended in a medium containing: 0.25 M sucrose, 10 mM Hepes-Mops, 2 mM MgCl$_2$, and 200 μM ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid. The addition of the substrate succinate (2 mM) induced a slow oxygen consumption (state 4 mitochondrial respiration). The addition of 100 nM/mg of the carbonyl cyanide 4-(trifluoro-methoxy)phenylhydrazone uncoupler stimulated the respiratory rate up to a maximum value. In this condition, the effects of the inhibitors of the respiratory chain (RC) can be studied. Successive additions of SN induce a slowing of the RC up to the point where a complete inhibition occurs. The graph (**b**) reports the percentage of RC inhibition induced by the SN. The value of 100% corresponds to the respiratory rate in the presence of the carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone and in the absence of SN.

case of many toxic compounds, the preferential target of SN in the mitochondria is the succinate deydrogenase [15].

An inhibition of the respiratory chain of mitochondria has been observed with many toxic compounds; however, only in some cases this interaction has been pointed out as the prevailing effect obtained with the minimal doses of compounds.

The 2,2'-3,3'-2,2'-4,4' and 2,2'-5,5' tetrachloro biphenyls (TCBs), as in the case of SN, are inhibitors of the succinate dehydrogenase, and complex I and cytochrome oxidase were unaffected [16]. Other authors [17] found that not only the respiratory chain, but also the phosphorylation system is inhibited with 20 n mol TCB/mg protein, while the same dose increases the membrane leak.

A similar behavior has been observed with DDE, the major metabolite of DDT. The authors [18] find that the prevailing effect (the effect with the lowest dose, 80 n mol/mg protein) is the inhibition of the succinate dehydrogenase. At higher doses, the compound uncouples the oxidative phosphorylation, although the ATPase activity is unaffected.

In the case of DDT, the effect is controversial: An inhibition of the respiratory chain with about 90 n mol/mg protein has been observed in the ubiquinol-cytochrome c segment [19], and Oyama et al. [20] and Byzkowsky and Tuczkiewicz [21] found that the prevailing action is an uncoupling effect.

It is worth remarking, however, that many toxic compounds of chemical and pharmacological interest inhibit complex II in mitochondria [22–28]. These compounds are very different in formula and structure and, because complex II is a very complicated system [29], a structural explanation of the mechanism of inhibitory effects never has been proposed. For this reason, even if a structure for complex II has been proposed [29], in the case of SN we are not able to explain the mechanism of inhibition of SN on complex II.

A comparison with other antifouling compounds such as trialkiltin compounds shows that the tributyltin (TBT), which was the most extensively used, is much more toxic than SN (the dosage of TBT necessary to inhibit ATP synthesis is about 1 n mol/mg [30], and the dose of SN necessary to counteract the ATP synthesis is about 20 n mol/mg; Fig.1) and points to different action mechanisms.

In the case of TBT and other trialkyltin and trialkyllead compounds, the proposed mechanism is that the compounds are $Cl^-/OH^-$ electroneutral exchangers in the mitochondrial membrane [30,31].

More recently, a new model has been proposed [32,33] to explain the toxicity in mitochondria and other membranes. The authors propose that trialkyltin and trialkyllead compounds are uncouplers of the oxidative phosphorylation. This is the consequence of a cyclic mechanism in which the compounds enter as trialkylSn$^+$ (or trialkylPb$^+$) and are extruded as trialkyl-SnOH (or trialkylPbOH).

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