THE MODE OF ACTION OF NANAOMYCINS D AND A ON A GRAM-NEGATIVE MARINE BACTERIUM
VIBRIO ALGINOLYTICUS

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Nanaomycin (NNM) D had a higher growth inhibitory activity than NNM-A against a Gram-negative marine bacterium, Vibrio alginolyticus. These quinone antibiotics were reduced by the respiratory chain-linked flavin dehydrogenase of the organism and the reduced forms of NNM were quickly autoxidized by molecular oxygen to produce superoxide radicals ($\text{O}_2^-$). NNM-D was more effective than NNM-A both in the induction of KCN-insensitive oxygen consumption with the intact cells and in the production of $\text{O}_2^-$ by the redox cycling. The growth inhibitory activities of NNM-D and A were partly reduced by raising the superoxide dismutase level of the cells. Thus, the ability to produce $\text{O}_2^-$ at the cell membrane was correlated to the antibacterial activities of NNM-D and A.

Nanaomycins (NNMs) D and A are quinone antibiotics discovered by ŌMURA et al. in 1974-4), produced by Streptomyces rosa var. notoensis and are mainly effective against mycoplasmas, fungi and Gram-positive bacteria. Marumo et al.5) studied the mode of action of NNM-A on Gram-positive bacteria and found that NNM-A inhibits biosyntheses of protein, DNA, RNA and cell-wall peptidoglycan to a similar extent. These inhibitions were considered to be due to its secondary effects and it was presumed to interfere primarily with the function of bacterial cell membrane. Since NNM-D and A are derivatives of 1,4-naphthoquinone, these antibiotics are likely to interact with the respiratory chain of bacteria. We found that NNM-D and A were readily reduced by the respiratory chain-linked flavin dehydrogenases of a Gram-negative marine bacterium, Vibrio alginolyticus and that these antibiotics have a strong growth inhibitory activity against this organism. Furthermore, the reduced antibiotics produced superoxide radicals ($\text{O}_2^-$) during their autoxidation. Therefore, the possible participation of oxygen radicals in the antibacterial activities of these antibiotics was studied.

Materials and Methods

Medium and Growth Conditions
Vibrio alginolyticus 138-2 was shake-cultured at 37°C in a complex medium containing 0.5% Polypeptone, 0.5% yeast extract, 0.4% K$_2$HPO$_4$, 0.2% glucose and 3% NaCl (pH 7.2). The cells were harvested in the late exponential phase of growth by centrifugation, washed twice with a buffer containing 0.5 M NaCl, 10 mM KCl, 10 mM MgSO$_4$ and 20 mM tris-HCl (pH 7.5), and suspended in the same buffer.

Membrane Preparation
The preparation of the membrane fraction from V. alginolyticus was performed by the osmotic

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Enzyme Preparations

The respiratory chain-linked NADH dehydrogenase was extracted from the membrane fraction of *V. alginolyticus* with 1.0% octyl glucoside. The extract contained most of the NADH dehydrogenase and the presence of an appropriate electron acceptor such as menadione or ferricyanide was required for the NADH oxidation. Respiratory chain-linked L-glycerol 3-phosphate dehydrogenase was purified from *V. alginolyticus* as described previously. Superoxide dismutase and xanthine oxidase were obtained from Miles Laboratories and Sigma, respectively.

Spectral Changes of NNM-D during Reduction under Anaerobic Conditions

A Thunberg-type cuvette was used for the reduction under anaerobic conditions. The main compartment contained 10 mM NNM-D, 20 mM tris-HCl (pH 8.5), 0.2 M NaCl and the purified L-glycerol 3-phosphate dehydrogenase in a total volume of 2.0 ml and the side arm contained 12 mg of crystalline disodium DL-glycerol 3-phosphate. The cuvette was evacuated and then filled with N₂ gas. The reference cuvette contained all of the components described above except for NNM-D. The reaction was started by the addition of substrate at 30°C. The absorption spectrum was recorded with a Hitachi 356 dual wavelength spectrophotometer.

Oxygen Consumption

Oxygen consumption was measured with a Yellow Springs Instruments Oxygen Monitor at 30°C. The washed cells of *V. alginolyticus* were suspended in 4.0 ml of the complex medium supplemented with 10 mM KCN at the final concentration of 0.2 mg cell protein/ml. NNM-D or A dissolved in ethanol was added to the reaction mixture and the oxygen consumption was monitored. The saturated concentration of oxygen in the complex medium corresponded to 400 ng atom O/ml at 30°C. The increase in oxygen concentration observed with the addition of NNMs (see Fig. 4) was due to the simultaneous addition of ethanol to the mixture. Ethanol had no effect on the respiration of cells at the concentration of 1% (maximum amount of ethanol added with NNMs).

Enzyme Assays

NADH dehydrogenase was assayed at 30°C in the reaction mixture containing 20 mM tris-HCl (pH 7.5), 0.2 M NaCl, 0.2 mM NADH, enzyme and an electron acceptor in a total volume of 1.0 ml. The reaction was followed by the decrease in absorbance at 340 nm. One unit of activity was expressed in 1.0 μmole of NADH oxidized/minute.

Superoxide dismutase (SOD) assay and the definition of its unit were carried out according to the method of MACORD and FRIDOVICH.

Determination of Superoxide Radicals

Superoxide generation was determined by the adrenochrome assay as described by CADENAS et al. The reaction mixture contained 25 mM tris-HCl (pH 7.5), 0.1 mM NADH, 1.0 mM epinephrine, 10 mM KCN, 18 μg protein of the membrane fraction from *V. alginolyticus* and NNM-D or A in a total volume of 2.0 ml. The reaction was started by the addition of NNMs at 30°C and the absorption change at 485–575 nm was measured with a Hitachi 356 dual-wavelength spectrophotometer. One unit of activity was expressed in 1.0 μmole of adrenochrome formed/minute utilizing the millimolar absorption coefficient of 2.96.

Chemicals

Epinephrine, NADH, xanthine, cytochrome C and n-octyl glucoside were purchased from Sigma. All other reagents were of highest grade available.

Results

Antibacterial Activities of NNM-D and A

As shown in Table 1, NNM-D and A had strong growth inhibitory activities against Gram-negative marine bacteria such as *V. alginolyticus* and *V. parahaemolyticus*. Since marine bacteria require 3.0%
NaCl for optimum growth, the test was conducted in its presence. The activity of NNM-D against Gram-positive bacteria increased in the presence of NaCl and it was comparable to that observed against marine bacteria. Despite of closely related chemical structure of NNM-D and A (see Fig. 3), the former was about 30~100 times more potent than the latter when assayed by agar dilution method in the presence of NaCl. On the other hand, NNM-D and A were less effective against E.coli. Minimal inhibitory concentrations of NNM-D and A against V.alginolyticus correspond to less than 0.17 and about 21 μM, respectively.

**Table 1. Antibacterial activities of NNM-D and A.**

<table>
<thead>
<tr>
<th>Test organism</th>
<th>Medium*</th>
<th>Minimal inhibitory concentration** (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NNM-D</td>
</tr>
<tr>
<td><em>Vibrio alginolyticus 138-2</em></td>
<td>B</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus K-1</em></td>
<td>B</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td><em>Bacillus subtilis PCI 219</em></td>
<td>A</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.05</td>
</tr>
<tr>
<td><em>Staphylococcus aureus FDA 209P</em></td>
<td>A</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.1</td>
</tr>
<tr>
<td><em>Micrococcus luteus PCI 1001</em></td>
<td>A</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.2</td>
</tr>
<tr>
<td><em>Escherichia coli NIHJ (JC-2)</em></td>
<td>A</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>25</td>
</tr>
</tbody>
</table>

* Medium A: 0.5% peptone, 0.5% meat extract, and 1.0% agar (pH 7.0).
  Medium B: medium A supplemented with 3.0% NaCl.
  ** The test was conducted by an agar dilution method (37°C, 20 hours).

NNM-D and A as the Electron Acceptor for the Respiratory Chain-linked NADH Dehydrogenase

Fig. 1 shows the activity of NNM-D and A as electron acceptors for the NADH dehydrogenase extracted from the membrane fraction of *V. alginolyticus*. The reaction followed to the Michaelis-Menten kinetics and the apparent *Km* values of NNM-D and A in the presence of 0.2 mM NADH were calculated to be 12.4 and 19.8 μM, respectively. On the other hand, the apparent *Km* of menadione, that is routinely used as the electron acceptor for this enzyme, was 51 μM under the same conditions. Thus, the affinities of NNMs toward NADH dehydrogenase were higher than that of menadione. As the electron acceptor, NNM-D was better than NNM-A especially at low concentrations.

Fig. 1. NNM-D and A as the electron acceptor for NADH dehydrogenase.

The NADH dehydrogenase extracted from the membrane fraction of *V. alginolyticus* was used as the enzyme and the activity in the presence of varying concentrations of NNM-D or A was assayed as described in Materials and Methods.
Conversion of NNM-D to A under Anaerobic Conditions

Fig. 2 shows spectral changes of NNM-D during the reduction with the l-glycerol 3-phosphate dehydrogenase purified from 
*V. alginolyticus* under anaerobic conditions. Since glycerol 3-phosphate had no appreciable absorption in UV region, spectral changes in the wide range of wavelength could be measured by the use of this enzyme. At an early stage of the reaction, the absorption spectrum of NNM-D (curve 1) changed to curve 2. Then, the absorbance at 525 nm decreased and that at 355 nm increased with the incubation time, and the spectrum finally became as curve 3. When air was introduced to the reaction mixture, the spectrum characteristic of NNM-A (curve 4) appeared. The reduction of NNM-A under anaerobic conditions gave the absorption spectrum identical to curve 3. Thus, it is apparent that the reduced form of NNM-D was converted to NNM-A and then the latter was further reduced under anaerobic conditions as shown in Fig. 3.

The effective conversion of NNM-D to A occurred only in the absence of oxygen and the reduced forms of NNM-D and A could not be spectrophotometrically detected under aerobic conditions, where oxygen consumption was induced indicating that these quinone antibiotics were quickly autoxidized by molecular oxygen.
Induction of Oxygen Consumption and Production of Superoxide Radicals by NNM-D and A

The respiratory rate of intact cells of *V. alginolyticus* in the complex medium was little affected by the addition of NNM-D or A. When the respiration was inhibited by the addition of 10 mM KCN, the presence of NNM-D or A induced oxygen consumption depending on its concentrations. As shown in Fig. 4a and b, the addition of more than 0.15 and 2.5 μM of NNM-D and A, respectively, induced KCN-insensitive oxygen consumption after a brief lag period, which was shortened by increasing the concentrations. The rate of oxygen consumption approached to that in the absence of KCN. The effect of 0.25 μM NNM-D was comparable to that of 10 μM NNM-A, indicating that the former is about 40-times more effective than the latter in terms of concentration. The induction of oxygen consumption occurred at very low concentrations of NNMs as compared with their apparent *Km* values for the NADH dehydrogenase. Thus, the large concentration difference between NNM-D and A observed with the intact cells was not due to the difference in their affinities to the enzyme, but rather seemed to reflect the difference in their accessibilities to the reactive site in the cell membrane.

The rapid autoxidation of reduced NNMs suggests that superoxide radicals were produced during their redox cycling. Fig. 5 shows the production of O$_2^-$ by the addition of NNMs as measured by the adrenochrome assay. The membrane fraction isolated from *V. alginolyticus* was employed in the experiments. Using NADH as a substrate, no measurable amount of O$_2^-$ was produced in the absence of NNMs. The addition of NNM-D or A to the reaction mixture induced the production of adrenochrome, that was completely inhibited in the presence of superoxide dismutase (SOD). The concentrations of NNM-D and A which give a half-maximum velocity were 0.8 and 5.5 μM, respectively. Thus, NNM-D was about 7-times more effective than NNM-A in producing O$_2^-$. The rate of O$_2^-$ production amount-
Effect of SOD Levels of *V. alginolyticus* on the Growth Inhibitory Activities of NNM-D and A

*V. alginolyticus* contains SOD as a scavenger of O$_2^-$ and its contents were influenced by growth conditions. When the cells were shake-cultured in the complex medium and harvested in the late exponential phase of growth, the cells contained 31 units of SOD/mg cell protein. The cells grown without shaking and harvested in the exponential phase of growth contained 9.5 units of SOD/mg cell protein. Employing these cells containing high and low intracellular levels of SOD, the growth inhibitory activities of NNM-D and A were examined in the complex liquid medium. As shown in Fig. 6, the cells having a high level of SOD were more resistant toward both NNM-D and A than those having a low level of SOD. Thus, 1 μM NNM-D or 5 μM NNM-A was sufficient to arrest the growth of the cells having a low SOD level, but not for the cells having a high SOD level. These results suggested a participation of O$_2^-$ in the manifestation of antibacterial activities of NNMs.

**Discussion**

NNM-D and A act as effective electron acceptors for the respiratory chain-linked flavin dehydrogenase of the marine bacterium *V. alginolyticus*. When the respiration of the intact cells is inhibited by 10 mM KCN, the addition of catalytic amount of NNM-D or A induces oxygen consumption (Fig. 4), indicating that these quinone antibiotics are reduced by the flavin dehydrogenase in the respiratory chain
and then autoxidized by molecular oxygen. Furthermore, a large amount of $O_2^-$ is produced during their redox cycling (Fig. 5). Since NNM-D is more potent than NNM-A in the growth inhibitory activity against *V. alginolyticus*, the facts that the former is more effective than the latter both in the accessibility to the respiratory chain of the intact cells (Fig. 4) and in the production of $O_2^-$ (Fig. 5) strongly suggest that the ability to produce $O_2^-$ at the cell membrane is closely related to their antibacterial activities. Indeed, the cells having a lower level of SOD are more sensitive to these antibiotics (Fig. 6).

GOODMAN and HOCHSTEIN\(^{13}\) reported the generation of $O_2^-$ and lipid peroxidation by the redox cycling of adriamycin and daunomycin, quinone anticancer drugs, and these reactions were considered to be the basis for the cardiotoxic effects of these drugs. A general mechanism for microosomal activation of quinone anticancer drugs has been proposed by BACHR et al.\(^{14,15}\), who postulated that the formation of the site-specific free radical intermediate is central to the cytotoxic action of these drugs. Because of the low reactivity of $O_2^-$, $O_2^-$ itself is not considered to be a direct cytotoxic agent. However, since $O_2^-$ is produced by the univalent electron transfer from the reduced quinones or from semiquinone radicals\(^{10,16}\), the induced production of $O_2^-$ by NNMs strongly suggests the formation of semi-quinone radicals. Thus, the antibacterial activities of NNMs may possibly be related to the formation of semiquinone radicals in the cell membrane.

Recently, MOORE and CZERNIAK\(^{17}\) reviewed naturally occurring quinones as potential bioreductive alkylating agents. The intermediate quinone methide is viewed as the cytotoxic species functioning as alkylating agents to give adducts. According to their models, we think NNM-A is an example giving a quinone methide by a non-reductive process (Model 3 in ref. 17). It is worth noting that the reduction of NNM-D, but not NNM-A, gives a quinone methide as shown in Fig. 3. Therefore, NNM-D may have the advantage over NNM-A in producing the quinone methide intermediate in the biological systems. Further experiments are required for the elucidation of this possibility.

References

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