Comparative Biological Activity of Nogalamycin and Its Analogs

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SUMMARY

Nogalamycin, a cytotoxic antibiotic, inhibited DNA-directed RNA synthesis by binding to adenine or thymine of the DNA primer. Partial structures of nogalamycin and its derivatives are shown. All compounds were highly cytotoxic to KB and L1210 cells in culture; all, except nogalarene, inhibited uridine incorporation into RNA more than thymidine or valine incorporation into DNA or protein, respectively. However, nogalarene inhibited all three macromolecule syntheses equally. All the derivatives bound to DNA with resulting increase in the $T_m$ ($\Delta T_m$) of DNA. At equimolar concentrations of the compounds, the $\Delta T_m$ with nogalamycin was twice that obtained with the other compounds. The base specificity of the compounds in binding to DNA is reported. The results indicate that: (a) the absence of the nogalose side chain markedly affected binding of the compounds to DNA and also their cytotoxicity, and (b) the oxygen attached to carbon 7 was involved in determining the specificity of binding to bases in DNA. The antileukemic activity in vivo of these compounds is reported.

INTRODUCTION

Nogalamycin is a cytotoxic antibiotic which inhibits DNA-directed RNA synthesis by binding to the DNA primer (3). However, unlike actinomycin D, which is believed to bind to the deoxyguanylate residues in DNA (8), nogalamycin is postulated to act by binding to the deoxyadenylate or thymidylate residues (3). Nogalamycin completely inhibited the hydrocortisone-induced increase in tryptophan pyrrolase activity, but it did not affect the substrate-induced increase (9). These results indicated that nogalamycin interferes primarily with RNA, including mRNA synthesis. Arrighi (1) reported that the nucleolar RNA synthesis was more susceptible to nogalamycin and actinomycin than chromosomal RNA synthesis.

In this paper we compare the biological activities of several nogalamycin analogs. The findings suggest that nogalamycin binds to DNA at 2 different sites and that the oxygen attached to carbon 7 of the tetracyclic ring influences the specificity of binding of the analogs to the bases in DNA. A part of this paper has previously been presented (2).

MATERIALS AND METHODS

The inhibition, by nogalamycin and its analogs, of cell growth was determined by the method of Smith et al. (13) and Buskirk (4), respectively. The method used to determine the incorporation of radioactive precursors into DNA, RNA, and protein has been described previously (3).

Calf thymus DNA was obtained from Calbiochem, Los Angeles, Calif. Escherichia coli DNA was extracted from cells in the log phase by the method of Marmur (10). Apurinic DNA was prepared from calf thymus DNA by the method of Tamm et al. (15). The melting temperature ($T_m$) of DNA was determined with a recording thermospectrophotometer (Gilford Instruments Laboratories Inc., Oberlin, Ohio). The difference spectra were measured in the Cary spectrophotometer, in specially designed cuvets described by Trowne and Rabin (16), by the method of Goldberg et al. (8). E. coli RNA polymerase was isolated and the assay was done by the method of Chamberlin and Berg (7). The ATP-14C (47 mCi/mmole) and GTP-14C (153 mCi/mmole) were obtained from Schwarz BioResearch Inc., Orangeburg, N. Y. The poly dAT and poly dL:dc were obtained from Biopolymers Laboratory of General Biochemicals, Inc., Chagrin Falls, Ohio. The thymidine-methyl-3H (2 Ci/mmole), uridine-3H (15 Ci/mmole), DL-valine-114C (20.4 mCi/mmole) and L-proline-114C (>180 mCi/mmole) were obtained from New England Nuclear Co., Boston, Mass. The E. coli cell-free protein-synthesizing system (S-30) was prepared by the method of Nirenberg and Matthaei (11). For this purpose, E. coli B cells harvested in middle log phase were obtained from General Biochemicals, Inc. For assay of radioactivity incorporated into protein in the cell-free systems, the reaction was stopped with 5 ml 10% TCA plus bovine serum albumin (0.5 mg/ml). The precipitated protein was washed twice with 10% TCA, heated for 20 min at 70°C with 0.5 N perchloric acid, and washed twice more with 10% TCA, once with ethanol-ether, and finally with ether. The dry material was dissolved in formic acid and counted in a scintillation counter.

The method described by the Cancer Chemotherapy National Service Center, National Cancer Institute (6), was used in determining the effect of the agents on the survival of leukemic (L1210) mice. The parent line was obtained from Dr. I. Wodinsky (Arthur D. Little Co., Cambridge, Mass.).

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2The abbreviation used is: TCA, trichloroacetic acid.
Mass.) and was maintained by weekly i.p. inoculation of ascitic cells into female BDF1 (C57BL/6 X DBA/2) mice (19 ± 2 g). The therapy schedule is described under Table 6.

The different nogalamycin analogs were prepared by Dr. Paul F. Wiley of our laboratories and their structures are shown in Chart 1 (18).

![Chart 1. Structure of nogalamycin and analogs.](image)

Nogalamycin: R = nogalosyl = –CH₃O
O-Methylnogalarol: R = CH₃
7-Deoxynogalarol: no OR on carbon 7 of Ring A
Nogalamycin N-oxide: The N in nogalamycin has 2 CH₃ groups attached. In nogalamycin N-oxide the N also has an O attached.
Nogalarene: Ring A becomes aromatic and the OH on carbon 9 and the OR on carbon 7 are absent.

This is a schematic representation in which the amino sugar moiety (C₈H₁₇NO₈) is attached to 2 adjacent positions of the 1, 2, 3, 4 group carbons and 2 hydroxyls are present in 2 positions of the 1, 4, 6, 11 group carbons (P. F. Wiley, personal communication; Ref. 18).

All experiments were done in duplicate; several experiments were repeated and were found to be reproducible. The highest and lowest value in any particular experiment did not deviate from the mean by more than 10%.

RESULTS

Inhibition of Growth and of Macromolecule Syntheses. The incorporation of appropriate precursors into RNA, DNA, and protein was inhibited to different extents by the drugs and are compared in Table 1. The results show that nogalamycin and all its derivatives, except nogalarene, inhibited the incorporation of precursor into RNA much more than into DNA or protein. Nogalarene inhibited precursor incorporation into all 3 macromolecules about equally. The levels needed for 50% inhibition of RNA synthesis and growth are compared in Table 2. The results indicate that: (a) nogalamycin was the most inhibitory of all the compounds tested; (b) the order of inhibition of RNA synthesis was nogalamycin > O-methylnogalarol > 7-deoxynogalarol > nogalor > nogalarene > nogalamycin N-oxide. The order of inhibition of growth was nogalamycin > O-methylnogalarol > nogalor > nogalarene > nogalamycin N-oxide. The inhibition of RNA synthesis was measured after 2 hr incubation of cells with drug while growth inhibition was measured after 3 days of incubation of cells with the drug.

Binding of Drug to DNA and Inhibition of DNA-directed RNA Synthesis. Nogalamycin is postulated to act by binding to the dA or dT moiety of DNA, thus inhibiting DNA-directed RNA synthesis. The effect of varying proportions of drug to DNA on the increase in "Tm" (ΔTₘ) is shown in Chart 2, a and b. The results indicate that the order of increase in Tₘ of both calf thymus and E. coli DNA was nogalamycin > nogalor, O-methylnogalarol, 7-deoxynogalarol > nogalarene or nogalamycin N-oxide. The very low degree of binding of nogalamycin N-oxide to DNA (as indicated by ΔTₘ) could be due to the decreased basicity of the compound as compared to nogalamycin, but this consideration does not apply to the other compounds which are equally as basic as nogalamycin. The greater increase in ΔTₘ caused by nogalamycin as compared to its derivatives could be due to either (a) more nogalamycin binding to DNA and all the drugs binding in a similar manner, (b)
nogalamycin binding to DNA differently from its derivatives, or (c) a combination of these. An attempt to distinguish between these possibilities was made on the basis of the fact that the binding of nogalamycin to DNA results in decreased absorption by the drug. This decrease is proportional to the amount of drug bound to DNA. The decrease in absorption caused by the binding of different drugs to calf thymus DNA is compared in Chart 3. The results show that 1 μmole of each of the 3 drugs was bound per 31 260 units calf thymus DNA. This result might indicate that nogalamycin stabilizes DNA more than the other drugs by binding in a different manner. A good dose-response curve was not obtained with 7-deoxynogalarol. Nogalarene behaved differently from nogalamycin as seen by the slope of the curve. Nogalarene also precipitated DNA at about 1 μmole drug/10 A260 units calf thymus DNA.

Addition of apurinic DNA, unlike DNA, does not cause marked spectral changes in the absorption of nogalamycin (3). The decrease in absorption when different drugs combined with apurinic DNA or DNA are compared in Table 3. The results indicate that apurinic DNA caused little spectral change in the absorption of nogalamycin, nogalarene, and O-methylnogalarol. 7-Deoxynogalarol behaved differently from the above 3 drugs. Apurinic DNA apparently was bound to a marked degree by 7-deoxynogalarol. This might indicate that 7-deoxynogalarol has a base specificity in binding to DNA which is different from nogalamycin.

Effect on E. coli RNA Polymerase. When E. coli RNA polymerase was used with poly dAT as primer, nogalamycin caused 99% inhibition of RNA synthesis compared to only 3% inhibition when poly dG:dC was used as the primer (3). Therefore, the inhibition of RNA synthesis by the different drugs was compared with poly dAT as the primer. The results are shown in Chart 4. The amounts of drug needed for 50% inhibition of RNA synthesis were as follows (mμmole/ml): nogalamycin, 0.85; O-methylnogalarol and nogalarene, 14.5; 7-deoxynogalarol, 39; and nogalarol, 51. Except for nogalarene, the order of inhibitory activity was...
Table 2). The inhibition of RNA synthesis primed by poly dAT incorporation into RNA in intact KB cells was measured (see Table 4). The method is described in detail under Table 4. Nogalarol, and O-methylnogalarol inhibited RNA synthesis caused 80% inhibition of poly dAT primed RNA synthesis. In accordance with previous reports (7), 7-deoxynogalarol did not show such base specificity. Poly dI:dC was then determined at drug concentrations which with nogalamycin and O-methylnogalarol. The results given in Table 4 indicate that nogalamycin, nogalarene, and O-methyl-nogalarol inhibited RNA synthesis primed by poly dAT to a much greater extent than when poly dI:dC was used as the primer. Nogalarene and 7-deoxynogalarol did not show such base specificity.

Inhibition of Polypeptide Synthesis in a Cell-free System. Since nogalarene inhibited protein synthesis markedly in intact cells, its activity in a cell-free system was compared with nogalamycin and O-methyl-nogalarol. The results given in Table 5 show that nogalamycin did not inhibit protein synthesis in intact cells or polypeptide synthesis in the cell-free system. Nogalarene inhibited protein synthesis in intact cells and polypeptide synthesis in the cell-free system while O-methyl-nogalarol was active only in the cell-free system. Also, polyproline synthesis directed by poly C was more sensitive to the compounds than polyphenylalanine synthesis directed by poly U. Similar differential sensitivity has been reported by Vazquez (17) with chloramphenicol, erythromycin, and several other antibiotics.

Antileukemic Activity of the Drugs. The acute toxicity and antileukemic activity of the drugs are compared in Table 6. Nogalamycin was the most toxic of all the compounds tested. However, O-methyl-nogalarol which was 0.1 as cytotoxic as nogalamycin was more active as an antileukemic agent in mice than nogalamycin. Nogalamycin N-oxide and 7-deoxynogalarol were inactive.

Antibacterial Activity of Nogalamycin Analogs. The antibacterial activities of several of the nogalamycin analogs are compared in Table 7. The results indicate that, like nogalamycin, the analogs were active against gram-positive bacteria.

Chart 4. Inhibition of E. coli RNA polymerase activity primed by poly dAT. The method is described in detail under Table 4.
Table 6
Antileukemic activity of nogalamycin and its derivatives

<table>
<thead>
<tr>
<th>Drug</th>
<th>Acute LD&lt;sub&gt;50&lt;/sub&gt; (mg/kg)</th>
<th>L1210 ID&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</th>
<th>Antileukemic activity in mice&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Dose (mg/kg)</th>
<th>Increase in life-span (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nogalamycin</td>
<td>8.8</td>
<td>0.036</td>
<td></td>
<td>0.5</td>
<td>26 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>32 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>8 (toxic)</td>
</tr>
<tr>
<td>Nogalarol</td>
<td>25</td>
<td>0.62</td>
<td></td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30</td>
<td>32 ± 4</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>60</td>
<td>21</td>
</tr>
<tr>
<td>O-Methynogalarol</td>
<td>88.4</td>
<td>0.3</td>
<td></td>
<td>25</td>
<td>42 ± 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>40</td>
<td>48 ± 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60</td>
<td>Toxic</td>
</tr>
<tr>
<td>7-Deoxynogalarol</td>
<td>178</td>
<td>0.3</td>
<td></td>
<td>25</td>
<td>17 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>10 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>75</td>
<td>Toxic</td>
</tr>
<tr>
<td>Nogalamycin N-oxide</td>
<td>0.98</td>
<td></td>
<td></td>
<td>12.5</td>
<td>8</td>
</tr>
<tr>
<td>Nogalarene</td>
<td></td>
<td>3.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Solutions of all drugs, except nogalamycin N-oxide, were injected i.p. in determining antileukemic activity and the acute LD<sub>50</sub>. Nogalamycin N-oxide was injected as a homogenized suspension in 0.25% aqueous methyl cellulose.

<sup>b</sup>The dose for 50% inhibition of growth of L1210 cells in culture was determined by Mr. H. H. Buskirk of our laboratories. The L1210 cells and drug were inoculated into tubes and the cells were counted 3 days later.

<sup>c</sup>Drugs were administered i.p., once daily for 7 days starting 24 hr after 10<sup>6</sup> L1210 cells were implanted. The control mice died after 7 ± 0.65 days. The percentage of increase in life-span was calculated according to protocols established by the Cancer Chemotherapy National Service Center (5). In order to be considered active, a compound must increase the life-span of leukemic animals by 25%. Ten mice were used at each dosage level. The results under % increase in life-span are the average of 2 or 3 separate experiments with the average deviations noted.

Table 7
Antibacterial activity of nogalamycin analogs

Antibacterial activity in vitro was determined by the method of Smith et al. (12).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Minimum inhibitory concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td>Nogalamycin</td>
<td>0.39</td>
</tr>
<tr>
<td>Nogalarol</td>
<td>6.25</td>
</tr>
<tr>
<td>O-Methynogalarol</td>
<td>6.25</td>
</tr>
<tr>
<td>Nogalarene</td>
<td>1.56</td>
</tr>
</tbody>
</table>

bacteria and not against most gram-negative bacteria. However, in contrast to nogalamycin, nogalarol and O-methyl-nogalarol were active against Klebsiella pneumoniae.

DISCUSSION

The results indicate that nogalamycin and several of its derivatives inhibited RNA synthesis more than DNA or protein synthesis. This conclusion is based upon the assumption that precursor incorporation is an effective measure of synthesis. However, changes in pool size or in degradation rates could have been affected by drug administration.

The ability of the drugs to inhibit RNA synthesis in intact cells and in the cell-free RNA polymerase system and to increase the T<sub>m</sub> of DNA are arranged in order of decreasing activity: (a) RNA synthesis: nogalamycin > O-methyl-nogalarol > 7-deoxynogalarol > nogalarol > nogalarene > nogalamycin N-oxide; (b) RNA polymerase: nogalamycin > O-methyl-nogalarol > nogalarol > 7-deoxynogalarol > nogalarene; (c) increase T<sub>m</sub> of DNA: nogalamycin > nogalarol, O-methyl-nogalarol, 7-deoxynogalarol > nogalarene > nogalamycin N-oxide. The order of activities are approximately the same in all cases except for the position of nogalarene in the RNA polymerase system. This result suggests that the inhibition of RNA synthesis in intact cells and in the RNA polymerase system results from the binding of drug to DNA. However, the order of growth inhibition, namely nogalamycin > O-methyl-nogalarol > nogalarol > nogalamycin N-oxide > 7-deoxynogalarol > nogalarene, is very different from the order of inhibition of RNA synthesis. The inhibition of RNA synthesis in intact cells is measured after a 2-hr incubation of cells with drug while the inhibition of growth was measured after 3 days incubation with drug. It is possible that while RNA synthesis may be the prime site of inhibition, in long-term incubation other inhibitory effects of the drugs are superimposed on RNA inhibition leading to inhibition of growth. This might result in difference in the order of arrangement of the drugs for inhibition of RNA synthesis and of growth.

Although similar amounts of nogalamycin, nogalarol, and O-methyl-nogalarol were bound per mg DNA, nogalamycin stabilized DNA (as seen by increase in T<sub>m</sub>) to a much greater extent than the other 2 compounds. Nogalamycin differs from nogalarol and O-methyl-nogalarol in possessing the sugar (nogalose) side chain. This might indicate that, in addition to the hydroxyl groups on the chromophore, nogalose is involved in the binding of the nogalamycin to DNA resulting in greater stabilization of the DNA helix. Nogalose by itself does not bind to DNA. In case of daunomycin, the hydroxyl groups on the chromophore as well as the amino groups on the sugar (daunosamine) have been implicated in the linkage between daunomycin and DNA (5). A similar situation may exist for nogalamycin; namely, there may be 2 binding sites between DNA and the antibiotic. The characteristics of the binding between DNA and nogalamycin indicate that, like proflavin, the antibiotic or at least its chromophores may intercalate between adjacent base pairs of helical DNA (3). Since the ring system of nogalamycin is larger than proflavin, it is possible that the nonintercalated portion of the antibiotic molecule may project into the minor groove of DNA and cause steric interference of the RNA polymerase. This type of binding has been suggested for daunomycin to explain the selective inhibition of RNA synthesis (5).

Unlike nogalamycin or nogalarol or O-methyl-nogalarol, 7-deoxynogalarol does not have any oxygen attached to carbon 7. Unlike the former 3 compounds, 7-deoxynogalarol
Activity of Nogalamycin Analogs

binds markedly to apurinic DNA and equally strongly to poly dAT and poly dI:dC. Nogalamycin, nogalarol, and O-methylignogalarol bind preferentially to poly dAT. This change in the specificity of binding to polynucleotides could be either due to the absence of the nonbonding electrons on the oxygen attached to carbon 7 or change in the conformation of the 4th ring due to removal of the OH or OR groups attached to carbon 7. Evidence cited earlier suggests that the nogalose side chain may bind to DNA. However, this binding does not affect the specificity of binding towards poly dAT and poly dI:dC.

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