Lethality of Nogalamycin, Nogalamycin Analogs, and Adriamycin to Cells in Different Cell Cycle Phases

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ABSTRACT

The drugs studied included nogalamycin and its derivative 7-con-O-methylnogalarol, dis-nogamycin and its derivative 7-con-O-methylnogalarol, and Adriamycin. All of these drugs, especially at high doses, were lethal to cells in every phase of the cell cycle, indicating that they were not phase specific. However, there were significant differences in drug sensitivity of cells in different parts of the cell cycle.

Nogalamycin and Adriamycin were most lethal to the cells in S phase, whereas cells in M, G1, and G2 were much less sensitive. In contrast, the nogalamycin derivative 7-con-O-methylnogalarol was almost equally lethal to cells in all phases of the cell cycle.

Dis-nogamycin was most lethal to cells in postmetaphase and in early S phase. Cells in mid- and late G1, late S, and G2 were much less sensitive.

The pattern of sensitivity to 7-con-O-methylnogalarol was different from that of dis-nogamycin. 7-con-O-Methylnoagarol was most lethal to cells in early G1, S, and G2. Only cells in mid- and late G1 were much less sensitive to this drug.

INTRODUCTION

Nogalamycin is an anthracycline antibiotic which was markedly cytotoxic in vitro and was active against several tumor systems in vivo (9). However, its toxicity in animals precluded its clinical trial (9). Recently, Wiley et al. (20) prepared several nogalamycin analogs with the hope of increasing the therapeutic efficacy of the drug. Among these analogs, 7-con-OMEN, 7-con-O-methylnoagarol, dis-nogamycin, and nogalamycin were the most active against P388 leukemia and B16 melanoma in mice (17). Li et al. (15, 16) showed with L1210 cells that nogalamycin, 7-con-O-methylnoagarol, and dis-nogamycin strongly interacted with DNA, which resulted in significant inhibition of RNA synthesis. In contrast, 7-con-OMEN bound to DNA to a much lesser extent and inhibited DNA and RNA synthesis minimally at doses that were significantly lethal (16). Therefore, we decided to study in detail the sensitivity of different phases of the cell cycle to these 4 drugs and to compare the responses to those with Adriamycin. The phase-specific lethality of drugs can be useful in determining the schedule of drug administration (7) and their effect in combination with other drugs (4). Parts of these results were presented previously (5).

MATERIALS AND METHODS

Cell Cultures. CHO cells were maintained in monolayer culture in F-10 medium supplemented with 10% calf serum, 5% fetal calf serum, and CaCl2 (0.05 mg/ml).

Synchronized cultures were prepared by planting mitotic (mainly metaphase) cells harvested after a 2-hr Colcemid (0.033 μg/ml) block. We used a modification of the method described by Tobey et al. (19). Exponentially growing CHO cells were planted (10⁷/roller) in 490-sq cm plastic roller bottles (Corning 25130 disposable polystyrene tissue culture roller bottles; Corning Glass Works, Corning, N. Y.) in 150 ml medium. The bottles were rolled at 1 rpm for 48 hr. Then, the old medium containing dislodged cells was discarded, and 100 ml fresh medium containing Colcemid (0.033 μg/ml) were added. The bottles were placed on the roller apparatus for 2 hr. Then, the medium was discarded and replaced with 25 ml cold medium. The bottles were rolled back and forth rapidly and tapped to dislodge mitotic cells. The mitotic cells in the supernatant medium were collected by centrifugation. The mitotic cell pellet (between 90 and 95% mitotic) was dispersed in fresh medium, and 10⁵ cells were plated in 5 ml medium in T-30 plastic bottles. The cell survival was determined as described below.

To obtain mitotic cells without Colcemid pretreatment, the cells were grown in roller bottles as described above. The cells were then harvested according to the protocol described by Tobey et al. (19). In brief, the first 5 harvests were discarded, after which cells entering mitosis every 10 min were collected and stored on ice. The mitotic cells were selectively dislodged as described above.

Drug Exposure and Cell Survival. The compounds were dissolved in 0.1 M glucuronic acid in H2O at 1 mg/ml and further diluted in medium prior to addition of 1 ml of drug or appropriate vehicle to the cells. Adriamycin (NSC 123127) was obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. HSA [³H]dThd (20 Ci/mmol) was obtained from New England Nuclear, (Boston, Mass.). Nogalamycin and its analogs were prepared by Wiley et al. (20, 21) at The Upjohn Company and have the structures shown in Chart 1.

At different times after planting mitotic cells, the culture was exposed to drug for 2 hr. After drug exposure, the cell monolayer was harvested with trypsin, and the cells were centrifuged and washed once with 5 ml medium to remove drug. The cells were then diluted in medium, and 2 ml of cells were planted to give 20 to 100 colonies in plastic Linbro plates. The cells were incubated in a humid 5% CO2 atmosphere for 8 days, after which the colonies were stained and counted. In all cases, the experiments were repeated. The cloning efficiency of the untreated (control) cells was normalized to 100%, and the cloning efficiency of the treated cells was expressed as a percentage.
of control survival. The coefficient of variation (the standard deviation expressed as percentage of the mean) in determining cell survival was about 15%. The cloning efficiency of asynchronous cells and synchronized cells was about 70 and 40%, respectively. Mitotic cells harvested without Colcemid pretreatment had 60% cloning efficiency.

To determine the position of the cells in the cell cycle during drug exposure, the HSA [3H]dThd suicide method was used. Cells were exposed to HSA [3H]dThd (20 Ci/mmol; 10 μCi/ml) for 2 hr and then washed with 5 ml of dThd (2 μg/ml). Cell survival was determined as described above.

**Cell DNA Distribution Analysis.** Cells were analyzed for DNA content in a fluorescence-activated cell sorter (FACS II; Becton-Dickinson Co., Mountain View, Calif.). For this purpose, the cells were stained with mithramycin according to the protocol described by Crissman and Tobey (10). The results were expressed as a histogram representing the relative number of cells with a given DNA content. The DNA histograms were analyzed by the method of Krishan and Frei (14). In this method, the histogram was arbitrarily divided into G1, S, and G2 + M regions based on the position of G1 and G2 + M peaks. Due to the overlap of late G1-phase and early S-phase cells at the G1-S boundary, this method tends to give low values for the percentage of S-phase cells. Similarly, low values for the percentage of S-phase cells are obtained at the G2-S border. This can be seen for the 8- and 14-hr samples in Chart 2.

**Pulse-labeling Index.** Cells were exposed at different times after planting mitotic cells to [3H]dThd (2 Ci/mmol; 10 μCi/ml) for 30 min. The cells were then prepared for autoradiography by standard procedures. After the proper emulsion exposure period, 1000 cells were counted, and cells with more than 5 grains were considered to be labeled.

**RESULTS**

**Position of Cells in the Cell Cycle Based on Sensitivity to HSA [3H]dThd.** Synchronized cultures used in these experiments were prepared by planting mitotic cells harvested after a 2-hr Colcemid block. In every experiment, the survival response of cells exposed to HSA [3H]dThd (20 Ci/mmoll) was used to determine the position of the cells in the cell cycle at the time of drug exposure. Since HSA [3H]dThd is incorporated only into DNA, the percentage of cells killed will equal the percentage of S-phase cells. The position of cells in the cell cycle at different times after planting mitotic cells was also determined either by pulse labeling the cells with [3H]dThd or by DNA distribution analysis. The results are shown in Chart 2. In general, the percentage of S-phase cells determined either by pulse labeling or HSA [3H]dThd suicide correlated quite well. However, it must be realized that for the suicide index the cells were exposed to HSA [3H]dThd for 2 hr (the same as for drug exposure), whereas for the pulse-labeling index they were exposed to [3H]dThd for 0.5 hr. The DNA histograms showed that from 1 to 5 hr after planting mitotic cells the cell population consisted of about 90% G1 cells. By 8 hr, the cells consisted of G1 and early S cells, whereas by 11 hr they were almost all (80 to 90%) S-phase cells, with the majority being late S-phase cells. By 14.5 hr, the cell population consisted of G2 + M and G1 cells, with contamination by late S-phase cells. Therefore, the results obtained at 15 hr after planting mitotic cells should not be interpreted as the response of only G2 + M cells.
Different phases exposed to several levels of the drugs are shown in Charts 3 to 7. The results show the following.

At high doses, the drugs killed cells in all phases of the cell cycle. Therefore, unlike the S-phase-specific drugs 1-β-D-arabinofuranosylcytosine or hydroxyurea, none of these drugs can be considered to be absolutely phase specific. However, there were marked differences in the sensitivity of different phases of the cell cycle to these drugs.

Nogalamycin and Adriamycin were most toxic to cells in S phase and least toxic to cells in G1 and G2 or M (Chart 3). The increase in lethality with increased dose was much greater for cells exposed in S phase (9-hr sample) than for cells exposed in postmetaphase plus early G1 (1-hr sample). Thus, the percentage of survival for the 1-hr cells decreased from 100% at 2.5 µg/ml to 32% at 7 µg/ml. In contrast, the percentage of survival of 9-hr cells decreased from 60% at 2.5 µg/ml to 0.45% at 7 µg/ml.

dis-Nogamycin was most lethal to cells exposed during postmetaphase plus early G1 and early S (Chart 4). Cell sensitivity decreased as the cells progressed through G1, so that the mid-G1 (4-hr sample) and late G1 (6-hr sample) cells were the least sensitive. Mid- and late G1 cells were almost equally sensitive to the drug. Cell sensitivity increased as the cells entered early S phase (9 hr). At 10 µg of dis-nogamycin per ml, the cells in late S (12 hr) and G2 (15 hr) were significantly less sensitive than those in early S (9 hr). However, at higher doses (20 and 40 µg/ml), the sensitivity of early S- and late S-, and G2-phase cells was approximately the same.

7-con-OMEN was most lethal to postmetaphase plus early G1 (1 hr) cells. Cell sensitivity decreased as the cells progressed through mid- to late G1. Chart 5 shows that, unlike dis-nogamycin, 7-con-OMEN was significantly more lethal to mid-G1 than to late G1 cells. Cell sensitivity increased as the cells progressed into S phase. Cells in G2 + M were about as sensitive as cells in late S.

7-con-O-Methylnogalarol was about equally lethal to cells in all parts of the cycle (Chart 6).

In the above experiments, all the drugs were not tested simultaneously in one experiment. Since it is possible for subtle variations to occur in the progression of the synchronized cells through the cell cycle, we felt it was necessary to compare all the drugs in the same experiment. The results of such an experiment showed that the response pattern of the drugs was similar to that described above.

When the lethality of dis-nogamycin and 7-con-OMEN to cells in the first 2 hr after planting metaphase cells was investigated, marked differences in their behavior were observed (Chart 7). During the first 0.5 hr postmetaphase, the cells were about equally sensitive to dis-nogamycin and 7-con-OMEN. However, after the first 0.5 hr, sensitivity to dis-nogamycin decreased rapidly, whereas sensitivity to 7-con-OMEN kept increasing for 2 hr. This indicates that, for dis-nogamycin, postmetaphase cells are the most sensitive, whereas 7-con-OMEN was most toxic to early G1 (2 hr) cells.
In all of the above experiments, mitotic cells were harvested after 2 hr exposure to Colcemid. The results in Table 1 show the response of a synchronous culture established from mitotic cells harvested without exposure to Colcemid. 7-con-OMEN was more lethal to the mitotic cells harvested with or without exposure to Colcemid than to cells in late G1.

Chart 8 shows the survival response of cells in different phases to varied levels of nogalamycin and 7-con-OMEN. It was quite obvious that the difference in sensitivity of different phases was mainly due to differences in the slopes of the exponential region of the cell survival curve. The dose-response curves were used to calculate the LD50 of the drugs for different phases of the cell cycle. These values are in the same range as the LD50 values for asynchronous cells (Table 2).

DISCUSSION

The selective detachment of mitotic cells as outlined by Terasima and Tolmach (18) does not subject the cells to any perturbation and would therefore be the preferred method for synchronizing cells. However, the low yields of mitotic cells necessitated the present method of harvesting mitotic cells after Colcemid pretreatment. Colcemid is toxic to cells and has been reported to cause chromosomal nondisjunction (12). Therefore, we wondered whether our results on phase specificity of different drugs would be affected by the Colcemid treatment prior to mitotic harvest. We have compared the cell cycle times and drug sensitivity of synchronous cultures of mitotic cells with and without Colcemid pretreatment (4). The latter (mitotic cells harvested without Colcemid) took less time to traverse G1 and S phases and were uniformly less sensitive to 1-β-D-arabinofuranosylcytosine, ellipticine, and actinomycin D than the former. However, the pattern of sensitivity of different phases to these 3 drugs was essentially similar, irrespective of whether Colcemid pretreatment was used.

Synchronous populations can also be obtained through centrifugal elutriation. This method generates a large number of...
cells in a short period of time without exposure to any synchrony-inducing agent. However, the synchronous CHO populations generated by this method consisted of 88% for G1, 71% for S phase, and 75% G2 (11). Therefore, although the G1 and S populations were not superior to those in this paper, the G2 population was clearly more synchronous. This would probably be the method of choice for future studies with cell lines that can be synchronized by this method.

The lethality of these drugs for asynchronous cells was in the same concentration range as for the synchronized cells. For example, the \( L_{D_{50}} \) of nogalamycin for asynchronous cells was 3 \( \mu \)g/ml compared to a range of 2.7 to 5.4 \( \mu \)g/ml for the synchronous cells in different phases (Table 2). Similarly, for 7-con-OMEN, the \( L_{D_{50}} \) for asynchronous cells was 0.28 \( \mu \)g/ml as compared to 0.18 to 0.3 \( \mu \)g/ml for synchronous cells in different phases. These results indicate that exposure to Colcemid to prepare synchronous cultures did not cause any unusual changes in drug sensitivity.

In the present study, the position of cells in the cell cycle at different times after planting mitotic cells was based on the HSA \([3H]dThd\) suicide method, the pulse-labeling index with \([3H]dThd\), and DNA distribution analysis by flow cytometry. The pooled results (Chart 2) indicated that from 1 to 5 hr the cell population consisted of about 90% G1 cells. By 8 hr, the cells were a mixture of G1 and early S cells (80%), and by 11 hr they were mainly S-phase cells. Based on the DNA histogram, the survival response of 11-hr samples would be that of cells distributed throughout early, mid- and late S phase. By 14.5 hr, the population had lost its synchrony such that there was a significant percentage of cells in G2, S, and G2 + M. Therefore, the percentage of survival obtained at 14.5 hr should not be interpreted as the response of G2 + M cells only.

Our results showed that Adriamycin was most lethal to cells in S phase, whereas cells in G1, G2, and M were much less sensitive. Kim et al. (13) and Bhuyan et al. (6) obtained similar results with Adriamycin using synchronized HeLa and DON cells, respectively. Barranco ef al. (3) used CHO cells synchronized by 2 treatments with excess thymidine and reported that early S-phase cells were most sensitive to Adriamycin. Krishan and Frei (14) also found that Adriamycin was most cytotoxic to human lymphoblasts in the S phase.

The present results showed that nogalamycin was most lethal to S-phase cells. For example, the \( L_{D_{50}} \) of nogalamycin for S-phase cells (9-hr sample) was 1.5 \( \mu \)g/ml compared to 5 \( \mu \)g/ml for postmetaphase plus early G1 (1-hr sample) cells. However, our previous results using DON cells do not agree with the present results with CHO cells. With DON cells, nogalamycin and actinomycin D were most lethal to cells in G1-S or early S

### Table 1

Lethality of 7-con-OMEN to synchronous cultures established with or without prior exposure to Colcemid

<table>
<thead>
<tr>
<th>7-con-OMEN (( \mu )g/ml)</th>
<th>0 to 2 hr</th>
<th>5 to 7 hr</th>
<th>8 to 10 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>100 (32.2)</td>
<td>100 (36.5)</td>
<td>100 (30.6)</td>
</tr>
<tr>
<td>0.4</td>
<td>11 ± 0.3</td>
<td>13 ± 0.3</td>
<td>11.2 ± 0.9</td>
</tr>
<tr>
<td>0.6</td>
<td>1.1 ± 0.05</td>
<td>1.15 ± 0.05</td>
<td>22.2 ± 4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.5 ± 0.2</td>
</tr>
</tbody>
</table>

### Table 2

LD\(_{50}\) for asynchronous and synchronous CHO cells in different parts of the cell cycle

<table>
<thead>
<tr>
<th></th>
<th>Postmetaphase + G1 (1 hr)</th>
<th>Late G1 (6 hr)</th>
<th>Early S (9 hr)</th>
<th>Asynchronous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nogalamycin</td>
<td>5.4</td>
<td>3.8</td>
<td>2.7</td>
<td>3</td>
</tr>
<tr>
<td>dis-Nogalamycin</td>
<td>&lt;10</td>
<td>&gt;40</td>
<td>&lt;10</td>
<td>6</td>
</tr>
<tr>
<td>7-con-OMEN</td>
<td>0.18</td>
<td>0.3</td>
<td>0.22</td>
<td>0.28</td>
</tr>
<tr>
<td>7-con-O-Methyl-nogalarol</td>
<td>0.76</td>
<td>0.84</td>
<td>0.63</td>
<td>0.6</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>&lt;0.2</td>
<td>&gt;0.2</td>
<td>&lt;0.2</td>
<td>0.47</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, actual plating efficiency values for the control samples. Cells harvested without Colcemid had a higher plating efficiency than did cells exposed to Colcemid. These values were normalized to 100%.

* Mean ± S.D.
phase (8). Sensitivity decreased as the DON cells progressed into S phase. In contrast, with synchronized CHO cells, both actinomycin D (4) and nogalamycin (this paper) were most lethal in the S phase. DON cells have a very short G1 phase (<2 hr) compared to 6 hr for CHO cells. We do not know whether this would account for the difference in sensitivity of DON and CHO cells.

The sensitivity patterns of dis-nogamycin and 7-con-OMEN were markedly different from those of nogalamycin and Adriamycin. Both of the former agents were markedly cytotoxic to cells in postmetaphase plus early G1 and least cytotoxic to cells in late G1. For example, for 7-con-OMEN, the 90% lethal doses for cells in postmetaphase plus early G1 (1-hr sample) and in late G1 (6-hr sample) were 0.31 and 0.66 μg/ml, respectively. Sensitivity increased as the cells entered S phase. For dis-nogamycin at 10 μg/ml, sensitivity decreased sharply as the cells progressed through late S phase into G2. In contrast, for 7-con-OMEN, early and late S and G2 + M (15-hr) cells were all almost equally sensitive.

In contrast to the marked cyclic pattern seen with all of the above agents, 7-con-O-methylnogalarol was almost equally lethal to all parts of the cell cycle.

At present, it is not possible to explain the sensitivity of different phases on the basis of the known biochemical action of these drugs. Li et al. (15, 16) reported that nogalamycin, dis-nogamycin, 7-con-O-methylnogalarol, and Adriamycin bind extensively to DNA, resulting in marked changes in circular dichroism and the melting temperature of DNA. In contrast, 7-con-OMEN bound much less to DNA. The phase sensitivity pattern of 7-con-O-methylnogalarol was different from the other drugs, since it was almost equally lethal to all parts of the cell cycle. The other drugs (nogalamycin, dis-nogamycin, 7-con-OMEN, and Adriamycin) had one property in common, namely, that they were all more lethal to cells in early S than to cells in late G1. This common toxicity pattern cannot be explained in view of the difference in the DNA-binding property of 7-con-OMEN from that of the other drugs. Bachur et al. (1) have suggested that anthraquinones, such as Adriamycin and nogalamycin, are activated by mammalian microsomes to a free radical state that may account for their cytotoxic effect. Such a mechanism of action, which is common to these anthraquinones, may account for their common toxicity to S-phase cells. The lethality of an agent is determined by its transport and binding to various macromolecules and by the repair of the damage caused by the agent. We do not know at present to what extent these factors can account for the observed differences between the nogalamycin analogs.

One factor that has not been taken into consideration in studying the phase-specific cytotoxicity of these agents is the possibility of recovery from potentially lethal damage. Barranco and Bolton (2) showed that cells in different phases of the cell cycle differed in their ability to recover from potentially lethal damage caused by bleomycin. Further studies are needed to determine if this occurs with these agents and if it is a particular effect seen with cells dislodged by trypsin.

We hope that knowledge of the phase specificity of these agents can be translated into new combinations of drugs. For example, we can visualize a sequential combination of nontoxic dose of vincristine followed by 7-con-OMEN or dis-nogamycin. Vincristine would block cells in metaphase. The cells would then be exposed to 7-con-OMEN or dis-nogamycin after the vincristine block is removed. Similarly, hydroxyurea can be used to block cells at the G1-S border. When the cells are released from the block, they can be exposed to nogalamycin or 7-con-OMEN. Such combinations are being investigated.

REFERENCES

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