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Comparison of the Response of Synchronized HeLa Cells to Talisomycin and Bleomycin

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Summary. *The cytotoxic activities of the antitumor antibiotics talisomycin (TLM) and bleomycin (BLM), were compared with asynchronous and synchronous populations of HeLa cells. The sensitivities of asynchronous and synchronous cells to TLM and BLM were expressed as biphasic dose-response survival curves. Of the cell cycle populations investigated, mitotic phase cells were the most sensitive and G_1 phase cells the most resistant to TLM and BLM cytotoxic activity. A significantly greater portion of mid-G_1 cells was more resistant to TLM than to BLM. Mid-S-phase cells showed greater resistance to TLM than to BLM in the initial portion (primary slope) of the dose-response curve.*

Introduction

The bleomycins are a group of glycopeptide antibiotics isolated from *Streptomyces verticillus* [29] that have been shown to be effective against a variety of neoplasms [7]. The primary target for bleomycin (BLM) cytotoxicity appears to be interaction with cellular DNA. The effects of BLM on isolated DNA have been shown to include liberation of free bases [10, 20], site-specific and non-specific single- and double-strand breakage [10, 15, 17, 18, 21, 23], non-covalent intermolecular crosslinks [16], and reduction of DNA melting temperature [21]. BLM produced breakage of DNA in cells grown in tissue culture, and the extent of degradation was correlated with cell cycle-specific cytotoxicity of the drug [6]. Mitosis has been reported to be the phase of the cell cycle most sensitive to BLM [1].

Talisomycin (TLM) is a new antitumor antibiotic related structurally to BLM. Structures of the two major components of the antibiotic, TLM A and TLM B, have been determined. They contain two new amino acids and a unique sugar, 4-amino-4,6-dideoxy-L-talose, that have not been previously found in the BLM complex [14]. The drug has exhibited significantly greater antibiotic activity against a variety of bacteria and fungi than did BLM [13]. Both TLM A and TLM B showed antitumor activity in experimental animal tumor systems [13]. Like BLM, TLM has been shown to cause DNA breakage in cells grown in tissue culture as well as single- and double-strand breaks in isolated DNA [26]. However, the relative single- and double-strand breakage activities [19] and the site-specificity of fragmentation of DNA moved by the two antitumor antibiotics appear to be different [18].

The purpose of this study was to compare the cytotoxic activities of TLM and BLM in asynchronous and synchronous cell cycle populations of HeLa cells.

Materials and Methods

Cells and Culture Techniques

HeLa S-3 cells were used and maintained as monolayer cultures in Minimal Essential Medium (Grand Island Biological Co., Grand Island, NY, USA) supplemented with 10% calf serum, 1% glutamine and antibiotics in a 5% CO_2 humidified incubator at 37° C. Under these conditions, the average cell cycle time was 24 h, with a 1-h mitotic period, a 9-h pre-DNA synthesis period (G_1), a 10-h DNA synthesis phase (S), and a 4-h post-DNA synthesis period (G_2) as determined by the method of Stubblefield [27]. Cultures were routinely checked for *Mycoplasma* contamination and found not to be contaminated, as evidenced by tests with Hoechst 33258 stain and fluorescent microscopy [5].

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The abbreviations used are: BLM, bleomycin; TLM, talisomycin; TdR, thymidine; L.I., labeling index; M.I., mitotic index; Do, mean lethal dose expressed as the inverse of the slope of the linear part of a survival curve
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Synchronization of Cells

To obtain large numbers of cells synchronized in S phase, a double treatment of excess TdR (2.5 m*M*) was used [25]. The degree of synchrony was monitored in replicate cultures by 30-min pulse labeling with 1 μCi TdR-^{3}H/ml, to determine the percentage of cells in S phase and by scoring the M.I.

In experiments requiring treatment of cells in M or G_1 phase, synchronized cells were obtained by the use of a single 24-h excess TdR block followed by treatment by the N_2O pressure method [24]. Cells growing at an exponential growth rate in 75-ml tissue culture flasks (Corning Glass Co., Corning, NY, USA) were blocked for 24 h with 2.5 m*M* TdR. Cells were then washed twice and incubated in fresh medium for 5 h, after which they were placed in a stainless steel container (Amicon Corp., Lexington, Mass) that was then flushed with a mixture of 5% CO_2, 95% air, and 90 N_2O psi applied for 8.5 h at 37° C. The cell-containing flasks were removed from the container and the rounded mitotic cells were shaken loose and collected. These cells were replated and the degree of synchrony monitored in replicate plates as described above.

In all radiographic procedures a 50% solution of Ilford K5 emulsion (Ilford, Ltd., England) in distilled water was used. Labeled cells were identified by the presence of five or more grains overlying the nucleus [8]. L.I. and M.I. were determined by scoring 500 cells for each sample point.

BLM and TLM Treatment of Cells

The survival response of HeLa cells to increasing concentrations of BLM A_2 and TLM A (Bristol Laboratories, Syracuse, NY) was determined at selected points of the cell cycle. Cells incubated for 3 h and 7.5 h in fresh medium after removal of the second excess TdR block (as described in the double TdR block) were selected as representative of mid-S phase and mid-G_2 phase cells, respectively. Cells incubated for 0.25 h and 4 h under normal conditions following release from the N_2O block were selected as representative of M phase and mid-G_1 phase cells. Non-synchronized cells growing at an exponental growth rate were selected as representative of an asynchronous population. At these selected times, replicated plates were incubated in the presence of 1, 3, 10, and 25 μ*M* BLM A_2 or TLM A for 1 h. After this incubation period, the cells were washed twice with fresh medium and harvested with 0.25 ml 0.1% trypsin solution. In the case of M phase cells treated with drug, both the cells remaining in the medium and those adhering to the petri dishes were collected and pooled. These cells were then washed twice in fresh medium by centrifugation and the cell pellet resuspended in medium. Following these harvesting procedures, which produced single cell suspensions, the cells were counted with an electronic particle counter (Coulter Electronics, Inc., Hialeah, Fl.) Appropriate cell concentrations were seeded in 60 × 15 mm petri dishes (Falcon, Oxnard, CA, USA), so that approximately 500 colonies would appear in control plates (containing clones from cells which underwent all experimental manipulations but were not incubated with drug) after a 1-week incubation period. The colonies were rinsed with 0.15 *M* NaCl, fixed, and stained with crystal violet. Cell survival was defined as the capacity of a single cell to generate colonies of 50 or more cells. Colonies were counted with a Biotran III Automatic Count Totalizer (New Brunswick Scientific Co., Edison, NJ, USA). Controls were run in parallel for each experiment. Percentage of survival was calculated in reference to controls. Each experiment was repeated at least twice with triplicate plates at each drug concentration and six control plates.

Results

Survival of HeLa Cells (Asynchronous)

The survival of cells following a 60-min treatment with increasing concentrations of TLM or BLM is shown in Fig. 1. Both drugs produced biphasic dose-response curves. However, TLM was less active than BLM at each concentration used in these experiments.

Degree of Synchrony of Cells Following Double Thymidine and N_2O Blocks

At 3 h after the removal of the second and final TdR block 94% of the cells were in S phase. Within 7 h after removal of the block 90% of the cells moved

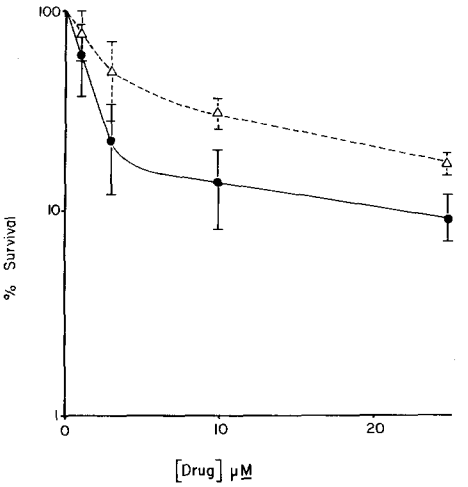

Fig. 1. Survival of HeLa cells (asynchronous) following 1 h treatment with BLM (●——●) or TLM (△ – – – △). *Bars* (I) indicate standard deviations obtained for three replicate plates. Curves were drawn by best-fit approximation

Table 1. L.I. and M.I. for synchronized cell populations treated with TLM and BLM

Cell cycle phase of drug-treated cells	Method of Synchronization	L.I.[a]	M.I.[b]
M	N_2O	0.00	0.93
Mid-G_1	N_2O	0.09	0.02
Mid-S	Double TdR	0.90	0.02
Mid-G_2	Double TdR	0.10	0.06
Asynchronous	None	0.38	0.02

[a] Labeling index measured by scoring the number of cells with five or more exposed silver grains per nucleus per 500 cells following incubations with TdR-^{3}H and autoradiography

[b] Mitotic index measured by scoring the number of cells containing mitotic figures per 500 cells

synchronously into G_2 as estimated by the L.I. and M.I. A M.I. peak of 20% was associated with a 5% L.I. at 11 h after removal of the TdR block. At the time of release from the N_2O block, a M.I. of 93% was observed and within 2 h 95% of the cells had moved synchronously into G_1 as determined by the L.I. and M.I. The L.I. remained at 0% until 8 h after release of the N_2O block and peaked at 75% at 11 h after release. These cells retained the normal population-doubling time (T_D) of 24.5 h as determined by monitoring the cell number in replicate plates.

Survival Response of Synchronized Cells

The L.I. and M.I. for cells at times selected for drug treatment corresponding to various cell cycle points following synchronization are shown in Table 1. Dose-response survival curves were obtained by exposing synchronized cells to increasing concentrations of TLM or BLM at selected points of the cell cycle (Fig. 2). At all four stages (M, mid-G_1, mid-S_1, and mid-G_2) biphasic survival curves were obtained for cells treated with TLM or BLM. The proportion of sensitive cells (percentage of cells responding to drug in the primary slope of the dose-response curve) and the corresponding Do values (inverse of primary slope), and the Do values of the relatively more resistant populations of cells (inverse of secondary slope) obtained for each of the cell cycle stages investigated in response to TLM and BLM are shown in Table 2. M phase cells showed similar levels of sensitivity at equal concentrations of either TLM or BLM, as did synchronized G_2 cells. However, synchronized mid-S and mid-G_1 cells differed in their survival response to TLM and BLM. Although similar Do values in response to TLM and BLM were obtained with respect to the sensitive population of

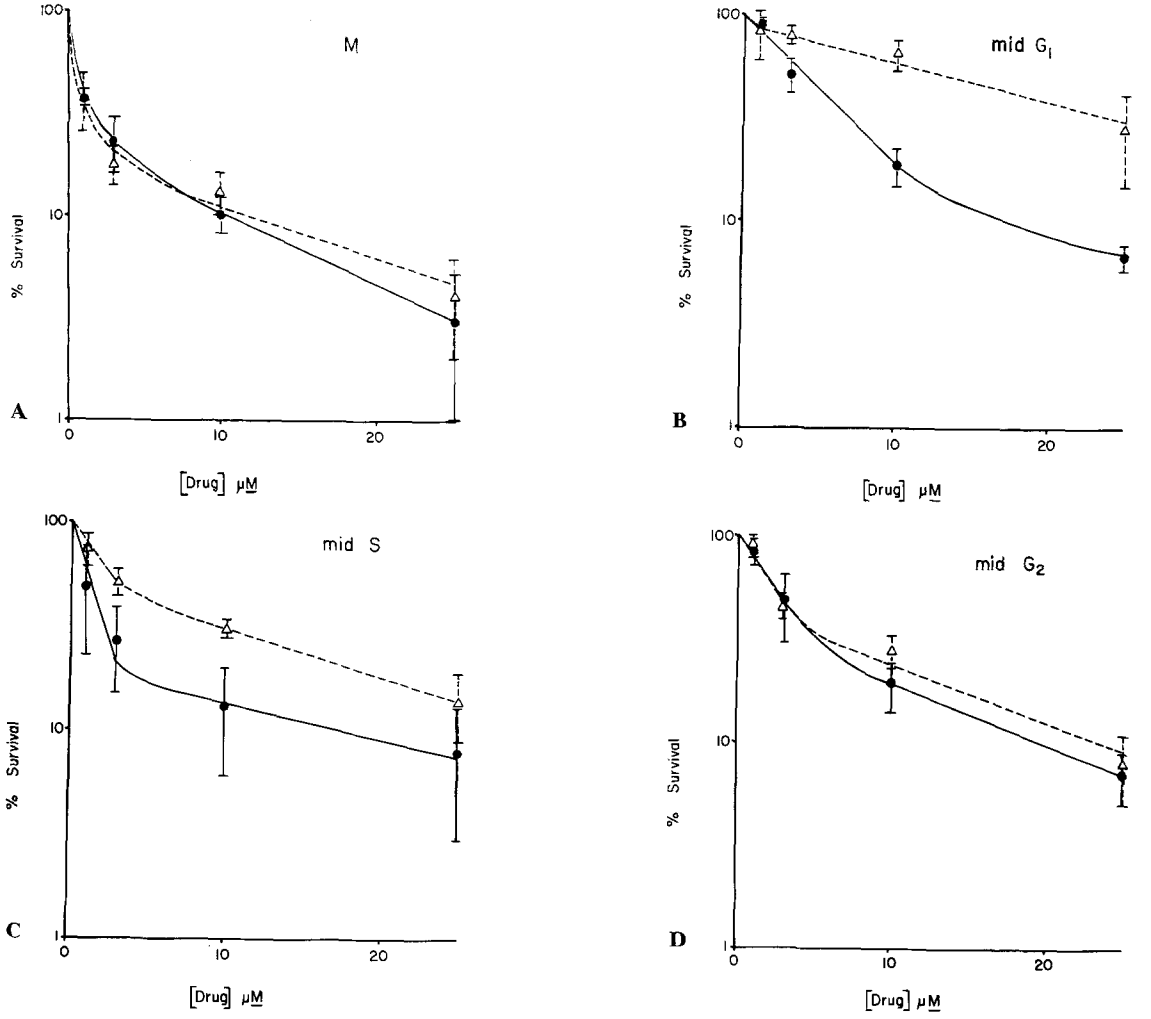

Fig. 2A–D. Survival of synchronized HeLa cells following 1-h treatment with BLM (●———●) or TLM (△– – –△). Cell cycle phase of treatment is indicated in the upper right corner of each graph

Table 2. Proportions of sensitive and less sensitive synchronized HeLa cells treated with BLM and TLM at different phases of the cell cycle

Phase	Drug	Do′ (μM)[a]	% Sensitive population[b]	Do″ (μM)[c]	% Survivors at 25 μM conc. of drug
Asynchronous cells	BLM	2.1	83	37	9
	TLM	3.3	63	27	17
M	BLM	0.7	82	18	4
	TLM	0.7	81	15	7
G_1	BLM	5.6	81	15	7
	TLM	5.6	14	24	30
S	BLM	2.0	82	26	8
	TLM	4.3	60	20	14
G_2	BLM	4.2	74	15	7
	TLM	4.5	63	16.0	8

[a] Do, primary mean lethal dose expressed as the inverse of the primary slope of the linear part of dose-response curve

[b] Percentage of cells responding to drug (inhibition of colony survival) in the primary slope of the dose-response curve

[c] Do″, secondary mean lethal dose expressed as the inverse of the secondary slope of the linear part of the dose-response curve

mid-G_1 phase cells, a significantly smaller proportion of mid-G_1 phase cells were sensitive to TLM (14%) than to BLM (81%). TLM was less active than BLM against synchronized mid-S phase throughout the concentration range of drugs employed.

Discussion

The biphasic intoxication of mammalian cell populations by increasing concentrations of BLM was first reported by Barranco and Humphrey [1]. Studies in which synchronized populations of cells have been used in different stages of the cell cycle suggest that the biphasic dose-response curve results from two distinct cell populations, in terms of sensitivity to drug, which co-exist within seemingly homogenous cell lines [1, 8]. Thus, the biphasic curve results from differential killing of these two populations, sensitive and resistant, regardless of their position in the cell cycle [9]. In this study we have demonstrated that like BLM, TLM produces biphasic dose-response curves in asynchronous and synchronous populations of cells.

The degree of cell synchrony obtained by the methods used in our study compares favorably with that reported by other investigators. Using the technique of a double block by excess thymidine to synchronize HeLa cells, Rao and Engelberg [25] reported that 98% of the cells were initially accumulated in early S phase. This value is closely comparable to that obtained in our studies, in which 94% of the cells were synchronized in early S phase following removal of the second excess thymidine block. The 93% synchrony in M phase following a N_2O block in our experiments is close to the value of 90% reported by Rao [24].

Controversy exists as to which phase of the cell cycle is most sensitive to BLM [7]. Much of this controversy may be due to comparisons of experiments in which different cell types and cell lines have been used as well as variations in experimental conditions. Our results with BLM A_2 indicate M phase cells to be the most sensitive, followed in order of decreasing sensitivity by mid-S, mid-G_2, and mid-G_1. These findings compare favorably with those of Terisima and Umezawa, who used HeLa cells and the mitotic shake off method as their mode of synchronization [28]. Therefore, this suggests that the experimental manipulations used in our studies did not result in selective changes in the response of cells to the drug. Thus the use of two different cell synchronization techniques to obtain a greater degree of synchrony in each of the cell cycle phases than could be obtained with only one synchronization method appears to be valid in attempts to study drug effects during the cell cycle. Clarkson and Humphrey [6], using *two methods* of synchronization, excess thymidine and mitotic shake off, demonstrated that BLM had greatest cytotoxic and DNA breakage activities in M phase when compared with its activities in the other cell cycle phases. Other investigators have also found mitosis to be the most

sensitive phase of the cell cycle to BLM [1] and with one exception [3] G_1 has been shown to be the least sensitive phase of the cell cycle [2, 8, 11, 22, 30, 31].

Our results indicate that TLM is also most effective at killing HeLa cells in mitosis and least effective against mid-G_1 cells (Table 2). However, the sensitivity of mid-S and mid-G_2 cells to TLM can not be differentiated in terms of the Do values of the sensitive populations between these two cell cycle phases. The TLM Do value of the resistant mid-G_2 cells was slightly lower than that of mid-S phase cells.

The most significant differences observed in these experiments between the response of synchronized populations of HeLa cells to TLM and BLM occurred in mid-G_1 and mid-S phase cells (Fig. 2). The decreased cytotoxic activity of TLM compared with BLM on these two synchronized cell cycle populations may account for the lower activity of TLM relative to BLM on asynchronous populations of HeLa cells (Fig. 1). Approximately 14% of the total synchronized population of mid-G_1 phase cells were among the sensitive population in response to TLM, as against approximately 80% in response to BLM (Table 2). TLM was also less active than BLM on cells comprising the sensitive populations of synchronized mid-S phase cells.

In conclusion, our data indicate that TLM, like BLM, kills cells more efficiently in mitosis than in the other three cell cycle phases investigated. Both drugs appear to be equally potent in M phase as well as in mid-G_2 phase. However, these two antitumor antibiotics differ in their respective abilities to kill cells in mid-S and mid-G_1 phase. These apparent differences in the cell cycle phase sensitivities to TLM and BLM may be related to a number of mechanisms involved in their cytotoxic activities, which include cellular transport of the drugs, differences in their DNA breakage activities found in tissue culture and in vitro [26, 19] and differences in the site-specific fragmentation of DNA by the two drugs [18]. Further investigation into the cytotoxic mechanisms of TLM and BLM may eventually lead to the design of new, more selective cytotoxic bleomycin analogs.

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