

Cell Cycle Phase-specific Cytotoxicity of the Antitumor Agent Maytansine¹

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ABSTRACT

The objective of this investigation was to study the effects of maytansine on the cell cycle kinetics of HeLa cells. The results of this study indicate that maytansine is a very potent mitotic inhibitor and that it has no effect on macromolecular synthesis. Maytansine-induced cytotoxicity was dependent upon the position of the cell in the cell cycle. Mitotic and G₂ cells are most sensitive to this agent, while G₁ phase cells are the most resistant, with S-phase cells being intermediate. Small (0.82×10^{-8} M) fractionated doses given at an interval of 8 hr have been found to be more cytotoxic than was a large (1.64×10^{-8} M) single dose. In evaluating the drug combinations, we observed that the schedule in which 1- β -D-arabinofuranosylcytosine treatment was followed by maytansine treatment exhibited greater cell kill than the reverse sequence. No schedule-dependent effects were observed when maytansine was tried in combination with Adriamycin.

INTRODUCTION

Maytansine, a naturally occurring ansa macrolide, isolated from the East African shrub *Maytenus serrata* (4, 5), has been reported to have significant antitumor activity against several experimental animal tumors, including P388 lymphocytic leukemia, B16 melanoma, and Walker 256 carcinoma (11). The antitumor activity of maytansine appears primarily to be due to its stathmokinetic effects, as in the case of *Vinca* alkaloids (12). Phase 1 clinical trials with maytansine in our department at M. D. Anderson Hospital and Tumor Institute appear to be promising because the antitumor activity of maytansine in patients with melanoma, breast carcinoma, and head and neck clear cell carcinoma is associated with little or no myelosuppression (2). Responses were also observed by other investigators in patients with acute lymphocytic leukemia, non-Hodgkin's lymphoma, ovarian cancer (3), and carcinoma of the breast (1). Maytansine is now in Phase 2 clinical trials.

Since maytansine is associated with some dose-dependent gastrointestinal toxicity (2, 3), we decided to study the effects of scheduling, dose, and dose fractionation of maytansine alone and in combination with ara-C³ or Adriamycin on HeLa cells *in vitro*.

¹ This research is supported in part by Grants CA-11520, CA-14528, and CA-19856 and Contract CM-53773 from the National Cancer Institute, and Grant GM-23252 from the National Institute for General Medical Sciences, NIH, Department of Health, Education, and Welfare.

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³ The abbreviations used are: ara-C, 1- β -D-arabinofuranosylcytosine; dThd, thymidine.

Received July 24, 1978; accepted May 15, 1979.

MATERIALS AND METHODS

Cells. HeLa cells used in this study were grown in Lux plastic dishes as monolayer cultures in McCoy's Medium 5A supplemented with 16% heat-inactivated fetal calf serum and 1% penicillin (10,000 units/ml):streptomycin (10,000 μ g/ml). These cells have a cell cycle time of approximately 22 hr and a plating efficiency of about 90%.

Cell Synchrony. HeLa cells were synchronized in S phase by the excess dThd (2.5 mM) double-block method (8). Cells in S and G₂ phases were obtained by trypsinizing monolayer cultures at 1 and 7 hr, respectively, after reversal of the second dThd block. A pulse label of 30 min with [³H]dThd gave a labeling index of 95% for S-phase population and 15% for the G₂ population. The mitotic index in these populations was less than 1%. Mitotic HeLa cells of 98% purity or better were obtained by selective detachment after the exposure to nitrous oxide (at 80 psi and 37°) of a monolayer culture that was partially synchronized into S phase by a single dThd block (7). G₁ phase cells were obtained by incubating the N₂O-arrested mitotic cells under regular culture conditions for 3 hr. During this 3-hr incubation, the mitotic index decreased from 98% to below 5%, indicating the successful completion of mitosis following reversal of the N₂O block.

Drugs. Maytansine (NSC 153858), ara-C (NSC 63878), and Adriamycin (NSC 123127) were supplied by the Drug Development Branch, National Cancer Institute, NIH. Stock solutions of these drugs were freshly prepared just before use and then serially diluted in complete culture medium to obtain the desired concentrations.

Cell Cycle Kinetics. A culture in exponential growth was trypsinized and plated in a number of Lux 35-mm plastic dishes at 2×10^5 cells/dish about 20 hr before the experiment. The experiment was begun by replacing the medium in the dishes with fresh medium containing maytansine. The drug concentrations studied were 0, 0.5, 1.0, 2.0, 4.1, 8.2, 16.4, and 32.8 nM. For each concentration, there were 2 dishes, one for an 18-hr continuous treatment and the other for a pulse treatment of 60 min (followed by a wash to remove the drug) and a posttreatment incubation of 17 hr in regular medium. At the end of this period, the cells were collected by trypsinization, deposited directly on clean slides by the use of a cytocentrifuge, fixed in absolute methanol:glacial acetic acid (3:1, v/v), stained with aceto-orcein, and scored for the percentage of cells in mitosis. Five hundred cells were scored for each point. The mitotic accumulation was plotted as a function of dose. The data presented represent an average of 3 experiments.

Dose-Survival Studies. The procedures for drug treatment and the determination of plating efficiency have been described previously (9). HeLa cells in exponential growth, which were trypsinized and plated in a number of dishes the day before

the experiment, were exposed to various concentrations of the drug for 1 hr or more, depending upon the purpose of the experiment. At the end of the treatment, medium containing the drug was removed, and cells were washed with drug-free medium, trypsinized, plated for colonies, and incubated for 10 days. The number of colonies observed in the treatments was expressed as a percentage of the value for the untreated control. The plating efficiency of the controls was $85 \pm 7\%$ (S.D.).

Evaluation of Drug Combinations. For the *in vitro* evaluation of combined drug effects, a random population of HeLa cells was exposed first to one drug that was then removed by washing before the second drug was added to the medium. Soon after the drug treatments, cells were washed with regular medium, trypsinized, and plated for colonies. In this study, 2-drug combinations, maytansine:ara-C and maytansine:Adriamycin, were examined. The various schedules included maytansine followed by either ara-C or Adriamycin and the reverse sequence. The duration of exposure of cells to maytansine and Adriamycin was 60 min. However, with ara-C, the treatment was 16 hr because it has been shown that a 16-hr incubation of a random population of HeLa cells with a sublethal dose ($0.8 \mu\text{g/ml}$) of ara-C reversibly blocked about 90% of the cells in S phase (9).

RESULTS

Effect on Cell Cycle Traverse and Macromolecular Synthesis. The purpose of this set of experiments was to determine the optimum dose and duration of treatment of HeLa cells with maytansine to produce the maximum cytotoxic effects. The primary effect of maytansine on HeLa cells was the arrest of cells in metaphase. The degree of mitotic accumulation in a random population of HeLa cells after 18 hr of continuous exposure or after 17 hr of incubation following a 1-hr treatment with maytansine is presented in Chart 1. In general, the effects of maytansine on mitotic accumulation were similar to those of Colcemid. The effects of a pulse (60 min) exposure of cells to the drug were reversible at lower (0.05 to 0.2×10^{-8} M) but

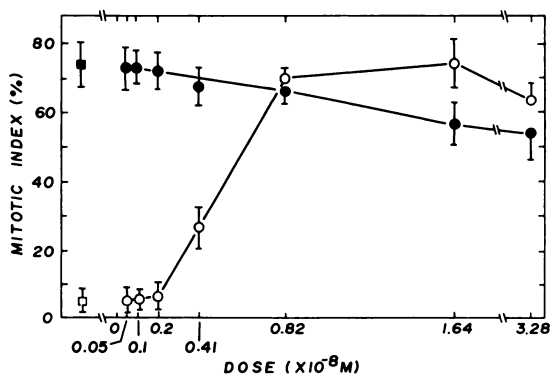


Chart 1. Effect of various concentrations of maytansine on the mitotic accumulation of HeLa cells in exponential growth. ○, a pulse treatment; cells were exposed to maytansine for 60 min; the drug was removed by washing and then incubated in regular medium for 17 hr. ●, cells were incubated with maytansine for 18 hr. Cells exposed to Colcemid (1.37×10^{-7} M or $0.05 \mu\text{g/ml}$) served as a control to monitor the antimetabolic effects of maytansine. ■, 18 hr continuous Colcemid treatment; □, 1 hr Colcemid treatment followed by 17 hr incubation in regular medium. Bars, S.D.

not at higher concentrations.

Maytansine has no inhibitory effect on the rate of incorporation of tritium-labeled precursors into DNA, RNA, and protein during a 3-hr period (Table 1). However, there was some increase in the incorporation of [^3H]leucine in the presence of Colcemid or maytansine in the medium.

Effect on Cell Survival. The effect of a 1-hr treatment with various concentrations of maytansine on the plating (cloning) efficiency of HeLa cells was studied. Initially, the plating efficiency decreased with an increase in dose, but it soon reached a plateau (Chart 2). Further increase in dose had little or no effect on survival until the concentration reached 6.56×10^{-8} M. However, an increase in the duration of treatment resulted in a decrease in the plating efficiency (Chart 3).

Effect of Dose Fractionation on Survival. Based on the dose-survival curve in Chart 2, a maytansine concentration of 1.64×10^{-8} M was selected. This dose was fractionated into 2 doses (0.82×10^{-8} M each), and each was applied for 1 hr, with or without an interval between them. These results indicate that the longer the interval between the split doses (up to a maximum of 8 hr studied) the greater was the decrease in cell survival (Chart 4).

Cell Cycle Phase-Specific Effects of Maytansine on Plating Efficiency. When HeLa cells synchronized in various phases of the cell cycle were exposed to maytansine (1.64×10^{-8} M) for 1 hr, the percentage of survival varied depending upon the phase of the synchronized population (Chart 5). The greatest drug sensitivity was observed in mitotic populations followed by G_2 , S, and G_1 , in order of decreasing sensitivity.

In Vitro Evaluation of Drug Combinations. In view of the cell

Table 1
Effect of maytansine on the incorporation of tritium-labeled precursors in DNA, RNA, and protein
The data represent the average of 4 experiments.

Treatment	Relative cpm/ 10^6 cells		
	[^3H]dThd	[^3H]Uridine	[^3H]Leucine
Control	100	100	100
Colcemid (0.05 $\mu\text{g/ml}$)	106.4 ± 4.39^a	94.5 ± 7.20	121.2 ± 11.10
Maytansine (0.656 $\times 10^{-9}$ M; 0.5 ng/ml)	102.7 ± 8.10	111.3 ± 10.60	152.0 ± 13.8

^a Mean \pm S.D.

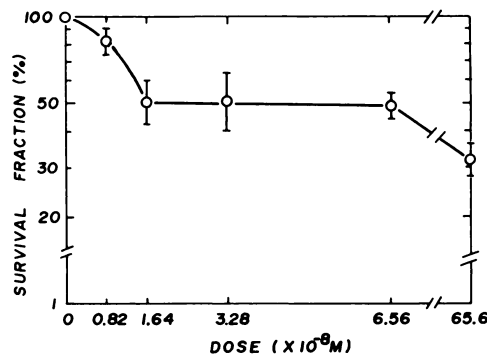


Chart 2. Effect of a 1-hr treatment with various concentrations of maytansine (○) on the plating efficiency of HeLa cells in exponential growth. Bars, S.D.

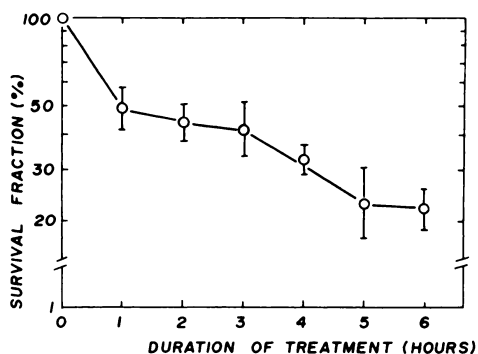


Chart 3. Effect of the duration of maytansine (1.64×10^{-8} M) (O) treatment on the plating efficiency of HeLa cells in exponential growth. Bars, S.D.

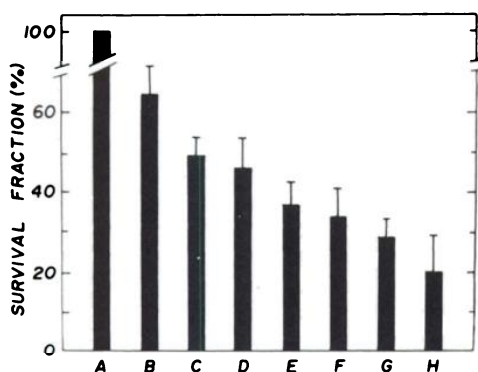


Chart 4. Effect of dose fractionation and the duration of interval between the treatments on the survival of HeLa cells in exponential growth. A, untreated control; B, maytansine treatment (0.82×10^{-8} M) for 1 hr; C, maytansine treatment (1.64×10^{-8} M) for 1 hr. For dose fractionation studies, 2 treatments of maytansine at 0.82×10^{-8} M for 60 min each were applied at zero (D), 2- (E), 4- (F), 6- (G), and 8- (H) hr intervals. Bars, S.D.

cycle phase-specific cytotoxicity of maytansine, we decided to study whether there would be any increase in cell kill by synchronizing with a low dose of ara-C and then exposing the cells to maytansine. Schedule-dependent cytotoxic effects were observed in the combination of maytansine with ara-C but not with Adriamycin (Chart 6). Exposure of cells to ara-C for 16 hr followed by a 1-hr treatment with maytansine reduced the plating efficiency to about 25%, as compared with 41% survival when the sequence was reversed. The data presented are the averages of 3 experiments.

DISCUSSION

The results of this study indicate that maytansine is primarily a mitotic inhibitor. As a mitotic inhibitor, it is effective over a wide range of concentrations (Chart 1). The lowest effective concentration (0.5 nM) for maytansine in HeLa cells is about 200 times smaller than that for Colcemid (1.37×10^{-7} M). Similarly, maytansine has been shown to be at least 100 times more potent as an antimetabolic agent than vincristine in sea urchin eggs (10).

Continuous treatment of HeLa cells with higher doses of maytansine may slow down the progression of cells through the cell cycle to some extent, as indicated by a slight reduction in the degree of mitotic accumulation (Chart 1). Such retarding

effects are not observed if the duration of treatment is limited to 1 hr. A 60-min treatment with concentrations of maytansine above 0.82×10^{-8} M produces a mitotic block in HeLa cells that remained irreversible up to 18 hr. At concentrations of 0.2×10^{-8} M or lower, the antimetabolic effects are quickly reversible by washing and resuspending the cells in drug-free medium. Similar results were obtained with murine leukemia cells by Wolpert-Defilippes et al. (12).

A 3-hr incubation of a random culture of HeLa cells with maytansine at concentrations of 6.6×10^{-8} M had no effect on the incorporation of [3 H]dThd and [3 H]uridine into DNA and RNA, respectively. A measurable increase in the uptake of [3 H]leucine into both Colcemid- and maytansine-treated cells is unexpected. Probably, these agents, due to their disorganizing effects on the cytoskeleton, may increase the permeability of

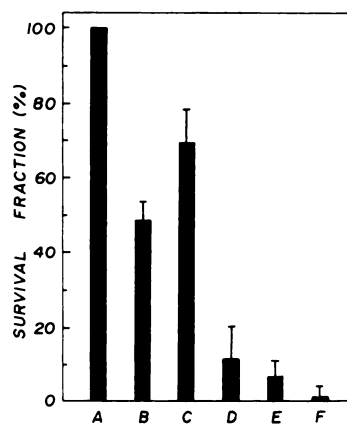


Chart 5. Effect of maytansine (1.64×10^{-8} M for 60 min) on the plating efficiency of HeLa cells synchronized in various phases of the cell cycle. The number of colonies observed in the treatment is expressed as a percentage of the untreated control for each phase of the cell cycle. A, control, a random culture. Maytansine treatments: B, random culture; C, G₂ phase; D, S phase; E, G₁ phase; F, mitotic populations. Bars, S.D.

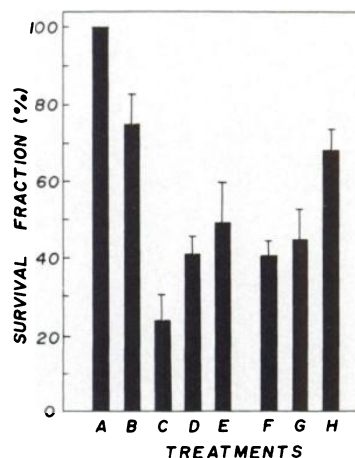


Chart 6. Effect of maytansine in combination with ara-C or Adriamycin on the survival of HeLa cells in exponential growth. The treatments included ara-C (0.8 μM) for 16 hr, Adriamycin (0.2 μg/ml) for 1 hr, and maytansine (1.64×10^{-8} M) for 1 hr. A, untreated control; B, ara-C treatment; C, ara-C followed by maytansine; D, maytansine followed by ara-C; E, maytansine alone; F, maytansine followed by Adriamycin; G, Adriamycin followed by maytansine; H, Adriamycin alone. Bars, S.D.

the cell membranes to [³H]leucine, thus facilitating greater uptake and consequently greater incorporation of label into the protein than occurs in the control. These results are at variance with those of Wolpert-Defilippes *et al.* (12), who reported that, in murine leukemic cells, DNA synthesis was inhibited to the greatest extent. However, they measured the uptake of labeled precursors into DNA, RNA, and protein by pulse-labeling of cells that were incubated with maytansine over a period of 12 hr. A 12-hr exposure to maytansine at 10⁻⁷ M would certainly block more than 50% of these cells in mitosis. The arrest of a significant fraction of the cell population in mitosis would result in a considerable reduction in the incorporation of label into DNA, RNA, and protein when compared with untreated control cultures.

The dose-survival curve for maytansine (Chart 2) quickly reaches a plateau with an increase in the concentration, indicating that there are 2 cell types in the population, one sensitive and the other relatively resistant. However, with a fixed dose, there is a direct correlation between the duration of treatment and the percentage increase in cell kill (Chart 3). Dose fractionation studies reveal that small fractionated doses are more cytotoxic than is a large single dose (Chart 4). The longer the interval between the fractionated doses, the lower is the plating efficiency up to 8 hr. These results suggest that most of the sensitive fraction in the cell population is killed by the first treatment, whereas the second treatment kills those that move from a resistant to a more sensitive phase of the cell cycle.

Studies with synchronized populations reveal that cells in mitosis are the most sensitive and those in G₁ are the most resistant to this agent (Chart 5). The closer the cell is to mitosis, the more sensitive it is to the cytotoxic effects of maytansine. Depolymerization and inhibition of polymerization of tubulin has been shown to account for the antimitotic effects of maytansine (10). Since oxidation of the sulfhydryl groups in tubulin inhibits its polymerization, the effect of maytansine may be due to its binding to these groups (10). It is also conceivable that tubulin, which is the most important constituent of the mitotic apparatus, accumulates gradually during the cell cycle reaching a peak at the beginning of mitosis (6). Thus, cells in G₂ and mitosis would have a full complement of these proteins, whereas those in G₁ would have the least proteins, with S-phase cells being intermediate. As soon as the cells were exposed to cytotoxic doses of maytansine, the spindle protein (tubulin) would be inactivated (depolymerized) by the irreversible binding of the drug. If the drug were removed after a brief exposure of 60 min, cells in G₁ and to some extent those in S could synthesize new tubulin and thus overcome the antimitotic effects of maytansine. Hence, G₁ cells would be more resistant to maytansine than those in other phases of the cell cycle, as we have observed. In the light of these observations, we can

explain the pattern of the dose-survival curve (Chart 1) as follows. The initial sharp decrease in the plating efficiency represents the killing of cells that were in the sensitive phases of the cell cycle by low concentrations of maytansine. On the other hand, the plateau is represented by the more resistant G₁ fraction, which constitutes about 50% of the population and remains unaffected over a relatively wide range of drug concentrations.

In vitro evaluation of the cytotoxic effects of maytansine in combination with ara-C revealed schedule-dependent effects (Chart 6). Administration of maytansine following a 16-hr ara-C treatment is more cytotoxic than the reverse sequence. This could be due to the synchronization of cells in S phase by the ara-C treatment. Our results (Chart 5) indicate that S-phase cells are more sensitive than those in G₁. Since Adriamycin does not induce cell synchrony, the sequence of administration of Adriamycin and maytansine made no difference in cell survival.

ACKNOWLEDGMENTS

We thank Dr. Howard Thames for his assistance in statistical analysis.

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Cancer Res 1979;39:3152-3155.

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