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# Effect of Actinomycin D on cells *in vitro*

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**ABSTRACT** The effect of Actinomycin D was studied on synchronized V 79B Chinese hamster lung cell line *in vitro* during the whole cell cycle by the colony forming assays and by the proliferative cell activity. The cells most susceptible were in the $G_1$ phase, at the beginning of the S phase and in the mitotic phase of the cell cycle. The minimal effective dose of Actinomycin D was 0.75µg per litre which inhibited colony forming ability and proliferative cell activity during the whole cell cycle. Lower concentrations (0.5 and 0.1 µg Actinomycin D per litre) caused inhibition of colony forming ability only when applied in $G_1$ phase, at the beginning of the S phase, and in the middle of M phase.

## INTRODUCTION

Studies of drug effects and their mechanism*in vitro* provide valuable information for more effective cancer chemotherapy (Bowman et al., 1991; Hill et al., 1991). For optimal cancer chemotherapy it is important to know what amount of drugs to apply and to determine the time interval (Decamaryo andFranco, 1991; Hudakova, 1992).

Bhuyan et al. (1977) investigated the effect of Actinomycin D on the mouse leukemic L1210 cell cycle by biochemical analysis, and found that it inhibited the biochemical processes most significantly in the $G_1$ phase. Muller et al. (1971) reported the effective influence of Actinomycin D in the M phase of the cell cycle.

In this study the effect of Actinomycin D is investigated on the V 79B cell line in vitro in regard to the cell ability to form colonies and to proliferate. Simultaneously the phase of the life cycle in which the cells are most susceptible to the drug was investigated.

## MATERIAL AND METHODS

The stabilized V 79B cell line used in the present experiments was derived from lung fibroblasts of a male Chinese hamster (Ford and Yerganian, 1958). The cells were maintained in Eagle's minimal essential medium (USOL Prague, Czech Republic) supplemented with 10% foetal bovine serum (University of Veterinary Medicine, Brno, Czech Republic) and antibiotics. The cell cultures were incubated at 37°C in a humidified atmosphere of 5% $CO_2$ in air.

The inhibitory effect of Actinomycin D (Serva, Heidelberg, Germany) was tested in synchronized V 79B cells. Synchronization of cells was performed by mitotic selection (Terasima and Tolmach, 1963) and the success was checked by measuring the incorporation of $^3$H-thymidine into DNA (1 µl per litre $^3$HTdr). $^3$H-thymidine was purchased from Ústav pro výzkum, výrobu a využiti radioaktivnich izotopu, Radiova 1, 10227 Praha 10, Czech Republic.

Cells at a density of $5 \times 10^2$ in 4 ml of culture medium were plated on Petri dishes with diameter of 40 mm. Actinomycin D dissolved in 1 ml of culture medium in respective concentrations was applied onto the synchronized V 79B cells after two hours, *i.e.* just after their adhesion to the dish. At the same time 1 ml of only culture media was added into dishes with control cells. After 7 days of cultivation the cell clones formed into colonies. The developed colonies were stained in medium for 20 - 30 minutes with 1.0% solution of methylene blue. The untreated V 79B cell ability to form colonies (*i.e.* plating efficiency) was 76%.

In preliminary tests the effect of the drug was studied at the concentrations 10.0µg, 1.0µg, 0.75 µg, 0.5 µg and 0.1 µg per litre.

To verify the most sensitive phase of the V 79 B cell cycle and to find out the minimal dose inhibiting the studied parameters, Actinomycin D was applied at concentrations 0.75 µg, 0.5 and 0.1 µg per litre to synchronized cells in two-hour intervals during the 14-hour cycle of the cells (Fig.1). The survival was determined by the in vitro cloning assay which measured the V 79 B cell ability to form colonies, as well as by the cell proliferative activity. The inhibition of the colony forming assay of the treated cells was compared with the colony forming ability of the untreated

cells which was concerned to be 100%. The kinetics of the cell proliferation in the individual colonies was found by direct counting of the cells after they had been trypsinized and resuspended. The number of cells in each colony was calculated in such a way that the total number of the cells in all the colonies growing in one Petri dish was divided by the total number of colonies per Petri dish.

The cell proliferative activity in the individual colonies resulted in their different size. The diameter of the colony size was measured and expressed in mm. The inhibition of the mentioned parameters in the affected cells was determined by comparison with the parameters of the untreated control. The colony size and the kinetics of the cell proliferation per colony in the V 79 B control cell during 7 days of cultivation is reported in table 1. The values are given as the mean value from three separate experiments.

## RESULTS AND DISCUSSION

The effect of Actinomycin D on the cell ability of synchronized V 79 B cell line in vitro to form colonies and on the kinetics of the cell proliferation in each colony were following. The untreated control cells formed visible colonies at 3rd day of cultivation and the average diameter of the colony was 0.1 mm. The cell number per colony was about 8. After another 4 days of cultivation the cell number per colony increased from 31 to 2008 and the diameter of colonies was 0.5 – 2.0 mm as shown in table 1.

**Table 1 : Proliferative activity in synchronized V 79B cell line colonies during 7 days of cultivation**

| *Day* | *Average number of cells per colony (X ± SD)* | *Diameter of colonies (mm)* |
|---|---|---|
| 1 | 1 ± 0 | – |
| 2 | 2 ± 0 | – |
| 3 | 8 ± 0.3 | 0.1 |
| 4 | 31 ± 3.9 | 0.5 |
| 5 | 114 ± 8.9 | 1.0 |
| 6 | 482 ± 20 | 1.5 |
| 7 | 2003 ± 40 | 2.0 |

The numberals represent the mean of nine samples (mean ± SD; cpm x $10^6$)

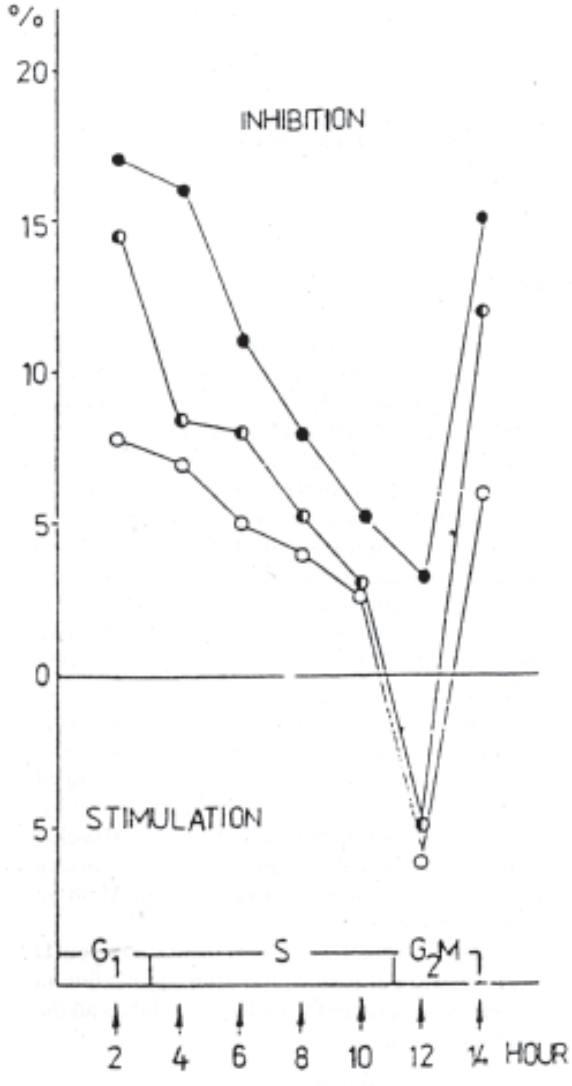
All studiend parameters, the ability of the individual cells to proliferate as well as the colony forming ability of the treated cells were significantly inhibited by Actinomycin D. As can be seen from table 2, Antinomycin D in concentration 10.0 µg per litre fully inhibits both the mentioned parameters. Concentration 1.0 µg per litre inhibits the colony forming ability in the cells to 72.0% and nearly absolutely blocks their proliferation. The results of their inhibition are colonies of minute size after 7 days of cultivation. There were about 7 - 8 cells per colony. The range of other three lower doses of Actinomycin D (0.75µg, 0.5 µg, and 0.1 µg per litre) caused comparatively low inhibition. However, concentration of 0.75 µg and 0.5µg per litre markedly inhibited the cell proliferation and, therefore, also the size of the grown colonies. In this case the inhibition is larger by 75% in comparison to the untreated colonies. Actinomycin D in the concentration of 0.1µg per litre did not block the cell proliferation so significantly, which resulted to the smaller size of the colonies by 50% compared with the controls.

**Table 2 : Colony forming ability of V 79 B synchronized cells treated by Actinomycin D after 7 days of cultivation**

| *Actinomycin D (µg per litre)* | *Inhibition of plating efficiency (% of control)* | *Diameter of colonies (mm)* |
|---|---|---|
| 10.0 | 100.0 | – |
| 1.5 | 72.0 | 0.1 |
| 0.75 | 15.3 | 0.5 |
| 0.5 | 13.4 | 0.5 |
| 0.1 | 7.2 | 1.0 |

Diameter of colonies in control = 2 mm
Average number of colonies in control = 360 = 100%

The next experiments submitted considered the three lower concentrations of Actinomycin D (0.75 µg, 0.5 µg, and 0.1 µg per litre) and their influence on the most susceptible phase of the cell cycle in V 79B cells. The above mentioned concentrations of the cytostatic have been applied to a synchronized cell line V 79 B during a 14 hr cell cycle in 2 hr intervals to evaluate inhibition of their cell colony forming ability and their proliferative activity (Table 3, Fig. 1). Only the concentration of 0.75 µg per litre inhibited the colony forming ability of the cells during the whole cell cycle. Both the lower concentrations of the drug (0.5µg and 0.1 µg per litre) given in the $G_2$ phase even stimulate the colony forming ability of the cells (Fig. 1).




**●- Actinomycin D - 0.75 µg per litre**
**◐- Actinomycin D - 0.5 µg per litre**
**○- Actinomycin D - 0.1 µg per litre**
**Average number of colonies in control = 380 = 100%**

**Fig. 1. Effect of Actinomycin D on colony forming assay of synchronized V 79B cells influenced in different time intervals of cell cycle after 7 days of cultivation**

The experiments showed that the most significant inhibition of the colony forming assay occurred after Actinomycine D in the concentration 0.75 µg per litre was applied to the cells in the $G_1$ phase in the 2nd hr (17.0%) or possibly at the beginning of the S phase in the 4$^{th}$hr (16.0%). In the following intervals - in the 6$^{th}$, 8$^{th}$, and in the 10$^{th}$ hr of the cell cycle the inhibition of the cells ability to form colonies decreased gradually to the level of the $G_2$ phase in the 12$^{th}$hr. During the mitotic phase in the 14$^{th}$hr the inhibition suddenly rose to 15.0%. The concentration of 0.5 µg per litre blocked the V 79B cell colony forming ability in the $G_1$ phase to 14.5% and at the beginning of the S phase to 8.5%. After the sudden rise of inhibition in the $G_2$ phase, the ability was again significantly blocked in the M phase to 11.9%. The lowest studied concentration 0.1 µg per litre of Actinomycin D inhibited the cell ability to form colonies in the $G_1$ phase to the value 7.9%, at beginning of S phase to the value 7.0% and in the M phase to the value 6.5% of the cell cycle (Fig.1).

Contrasted with the colony forming assay, the effect of the cytostatic studied is more clearly manifested by the inhibition of cell proliferation which results in the different size of the colonies (Table 3). Both the proliferative activity of V 79B cells and the size of the colonies are largly blocked by the concentration 0.75 µg per litre applied in the $G_1$ phase, at the beginning of S phase, and in the M phase, *i.e.* in the 2nd, the 4th and the 14$^{th}$hr of the cell cycle. After 7 days of cultivation the colonies were only 0.1 mm in diameter and the cell proliferation blocking was almost completed. The number of cells per colony did not exceed 9. It means, the inhibition of cell proliferation was large by 90% than the untreated colonies. When this concentration (0.75 µg per litre) was applied in the 6$^{th}$, 8$^{th}$ and the 10$^{th}$hr of the cell cycle, the diameter of the colonies increased to 0.5 mm which represented about 75% inhibition. The cells less sensitive to the mentioned concentration of Actinomycin D were in the $G_2$ phase in the 12$^{th}$hr of the cell cycle. The inhibition then was 50%.

The two lower concentrations (0.5 µg and 0.1 µg per litre) studied caused significant inhibition of cell proliferation in colonies too. The size of the cell colonies was blocked by 50% - 75% compared with the untreated control in the case the Actinomycin D application occured at any time

**Table 3: Colony forming ability and proliferative activity in V 79B synchronized cells influenced in different time intervals of cell cycle after 7 days of cultivation**

| *Actinimycin D (μg per litre)* | *Time of drug application (in hours)* | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *2* | | *4* | | *6* | | *8* | | *10* | | | *12* | | | *14* | | | |
| | *A* | *B* | *A* | *B* | *A* | *B* | *A* | *B* | *A* | *B* | | *A* | *B* | | *A* | *B* | | |
| 0.75 | 0.1 | 7 ± 0.9 | 0.1 | 8 ± 0.5 | 0.5 | 30 ± 2.0 | 0.5 | 31 ± 4.0 | 0.5 | 40 ± 4.5 | 1.0 | 100 ± 14.8 | | 0.1 | 9 ± 1.0 | | | |
| 0.5 | 0.5 | 29 ± 2.0 | 0.5 | 30 ± 1.9 | 0.5 | 3.8 ± 4.8 | 1.0 | 132 ± 10.0 | 1.5 | 509 ± 17.0 | 1.5 | 530 ± 17.5 | | 0.5 | 32 ± 2.4 | | | |
| 0.1 | 1.0 | 127 ± 12.1 | 1.0 | 129 ± 12.1 | 1.0 | 130 ± 10.0 | 1.0 | 140 ± 13.0 | 1.5 | 508 ± 21.0 | 1.5 | 520 ± 12.7 | | 1.0 | 127 ± 11.0 | | | |

Size of colonies in control = 2mm
Average number of cells per colony in control = 2.041 ± 39.0
A – Size of colonies in mm
B – Average number of cells per colony (x ± SD)
The numerals represent the mean of nine samples (mean ± SD ; cpm x $10^{-6}$)

except in the 10th and the 12thhr (the end of the S phase and the $G_2$ phase) of the cell cycle (Table 3). The concentration of 0.5 μg per litre of Actinomycin D was most effective on the cells in the $G_1$ in the 2ndhr, and et the beginning of the S phase in the 4th and 6thhr. The cell proliferation increased after the drug was applied after the 8th hr of the cell cycle. The rise culminated between the 10th and ther 12th hr. The inhibition of the cell proliferation and the colony growth in the M phase which appeared in 14th hr were similar to the state in the $G_1$ and at the beginning of the S phase. Actinomycin D in the concentration 0.1 μg per litre affected the cell proliferation and the formation of colonies less significantly than the two firstly mentioned concentrations (0.75μg and 0.5 μg per litre). The V 79 cells were sensitive to these concentrations of the drug in the $G_1$ (the 2ndhr), in the S phase (the 4th, 6th, 8thhr), and in the M phase (the 14thhr). The studied parameters were least affected in the 10th and 12th hr, *i.e.* at the end of the S phase and in the $G_2$ phase.

It can be concluded that a cell influenced by the lower concentrations (0.5 μg and 0.1 μg per litre) of Actinomycin D keeps its ability to adhere but loses its ability to divide. This information has also been stated by other authors. They reported, using other cell lines, that even very low doses of Actinomycin D inhibit cell proliferation (Muller et al., 1971; Bridget, 1976; Bhuyan et al., 1977). According to Fimiani and Minniti (1992), the application of very low concentrations of phaselly non-specific substances causes primary cell damage which results in blocking the cell proliferative activity.

The effect of Actinomycin D on cell cycle was studied in detail by other authors whose results are in agreement with our findings. Bhuyan et al. (1977) using L1210 cells concluded that inhibition effect in the $G_1$ phase was the result of the inhibited synthesis of RNA and of partial inhibition of DNA replication as a follow-up after intercalation complexes. Similar result have been obtained by selective inhibition of RNA synthesis in the $G_1$ phase of leucocytes (Cairo et al., 1986). Muller et al. (1971) reported effective influence of Actinomycin D in the M phase of the cell cycle.

It can be summarised, the results of this investigation provide the evidence that Actinomycin D, when tested in the V 79 B cell line isolated system *in vitro*, has significant influence on the cell colony forming ability and on the cell proliferative activity in the $G_1$ phase, at the beginning of the S phase and in the middle of the M phase of the cell cycle.

The minimal effective dose of Actinomycin D was 0.75 μg per litre which after application in whatever interval of the cell cycle inhibits all the studied parameters.

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