EFFECT OF DACTYLARIN ON HeLa CELLS

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Dactylarin is a new antiprotozoal antibiotic which also exhibits cytotoxic activity. The decrease of mitotic index in treated HeLa cells indicates that the antibiotic inhibition is expressed prior to mitosis. Dactylarin inhibited the progression of G_2 cells into mitosis. One of the earliest changes of dactylarin-treated cells was an increase in the cell size. Dactylarin induced unbalanced growth of HeLa cells in which DNA and RNA content per unit of cell volume decreased, while protein content remained directly proportional to the increased cell volume. Under the same experimental conditions only a slight effect on glucose utilization was observed. In the light of these findings, the possible lethal action of dactylarin was discussed.

Dactylarin is a new antiprotozoal antibiotic of the geodin group derived from a culture of *Dactylaria lutea* Routien[1]. It has strong inhibitory activity *in vitro* against *Leishmania braziliensis* and *Entamoeba invadens* and is slightly active against Gram-positive bacteria. No activity was observed against *Trypanosoma cruzi*, *Tetrahymena pyriformis*, Gram-negative bacteria, yeasts or molds. Dactylarin was the most cytotoxic antibiotic from the studied geodin group[2]. A relatively short *in vitro* exposure of L5178Y cells to dactylarin affected the transplantability of cells in BDF_1 mice (unpublished observation). Miko and Drobnica[3] observed that dactylarin primarily interferred with the energy metabolism. The secondary consequence of this interference was the inhibition of other metabolic key processes, such as protein synshesis of nucleic acids in Ehrlich ascites carcinoma (EAC) cells.

The present studies were undertaken to determine those biochemical processes in HeLa cells which result in lethality when inhibited by dactylarin.

Materials and Methods

Cell Culture System

Monolayer cultures of HeLa cells were grown at 37°C in Eagle's basal medium (USOL, Prague) supplemented with 6% calf serum and adjusted to the pH 7.0 with sodium bicarbonate. Exponentially growing cells in Müller flasks were treated for 48 hours with dactylarin which was dissolved in DMSO. Control cultures received an identical concentration (1%) of DMSO. After particular time intervals, flasks were harvested in triplicate by treatment with 0.25% trypsin and cell numbers and cell sizes were determined, as previously described[4].

Entry into Mitosis

The effect of treatment on the entry of G_2 cells into mitosis was studied using the "collection function" parameter of Puck and Steffen[5]. Colcemid (0.04 μg/ml, final concentration) was added to replicate 5-ml cultures simultaneously with dactylarin. At 2-hour intervals following the addition of colcemid, duplicate flasks were treated with trypsin and the cells thus

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released were fixed in ethanol—acetic acid (3:1, v/v). Smears of the fixed cells were stained with GIEMSA and the fraction of the cell population in mitosis was determined by microscopic examination. The accumulation of mitotic figures in cultures exposed to colcemid was expressed in terms of the mitotic collection function[5].

In determinations of mitotic index, at least 1,000 cells were examined.

Biochemical Procedures

After removal of medium, cells were harvested from culture flasks by trypsinization, washed once with 0.9 % NaCl and suspended in 0.9 % NaCl. Portions of this suspension were used for determinations of cell number and protein content[6]; another portion was alternately frozen and thawed three times to disrupt cells and then extracted with 5 % trichloroacetic acid (90°C, 20 min.) to recover the nucleic acid contents. The DNA content of such extracts was estimated by the diphenylamine method[7] and that of RNA by the orcinol method[8].

Glucose concentration in the culture medium was determined by using the Bio-La-test (Lachema, Brno).

Results

Inhibition of Cell Multiplication

Dactylarin inhibited the proliferation of HeLa cells at doses from 0.78 to 6.25 μg/ml (Fig. 1). With the highest concentration of dactylarin there was a marked decrease in the number

Fig. 1. Proliferation of HeLa cells during exposure to various concentrations of dactylarin.

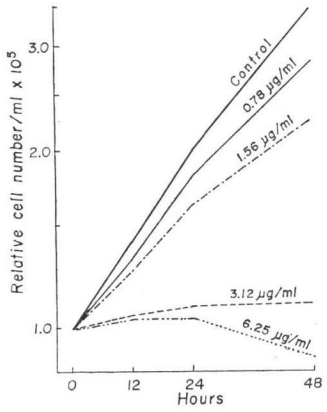
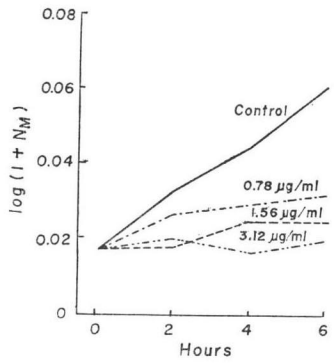


Fig. 2. The effect of dactylarin on the entry G_2 cells into mitosis.
The antibiotic was added to the medium simultaneously with colcemid at 0 hour at the concentrations of 0.78, 1.56 and 3.12 μg/ml.



of living cells compared to the control cultures. After 24-hour treatment there were detached dead cells floating in the medium. With 3.12 μg/ml of dactylarin 100 % inhibition of the growth was observed from the first day after the treatment. At the doses of 1.56 and 0.78 μg/ml retardation of cell replication was observed which was related to the dactylarin concentration.

Effect of Dactylarin on the Entry of Cells into Mitosis

At various times after antibiotic exposure, experiments were carried out to determine the entry rate of cells into the mitotic stage. The frequency of mitosis was inhibited at low doses (Table 1) and practically disappeared within 12 hours following the exposure to higher doses of dactylarin. On the basis of the above observations it can be concluded that the stage prior to initiation of mitosis is particularly sensitive to the action of dactylarin. In order to test

this hypothesis the method of PUCK and STEFFEN[5] was used which employs mitotic indices of culture samples taken serially after the addition of colcemid. Two to six hours after addition of dactylarin at 0.78 μg/ml the accumulation of mitosis was markedly retarded (Fig. 2). With the higher doses no actual

Table 1.

Incubation time in hours	Mitotic index		
	Control	Dactylarin (μg/ml)	
		0.78	1.56
12	49	37	31
24	56	45	30
48	53	43	35

Fig. 3. Enlargement of HeLa cells produced by dactylarin.

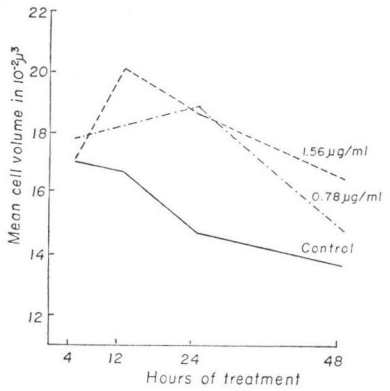
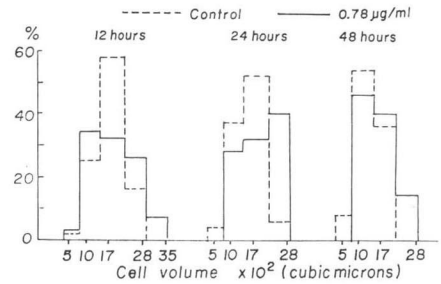


Fig. 4. The changes in size distribution of HeLa cells produced by dactylarin.



mitotic figures accumulated immediately after the cells were exposed to the antibiotic. These results suggest that the cells are blocked in G_2 phase, just prior to M phase.

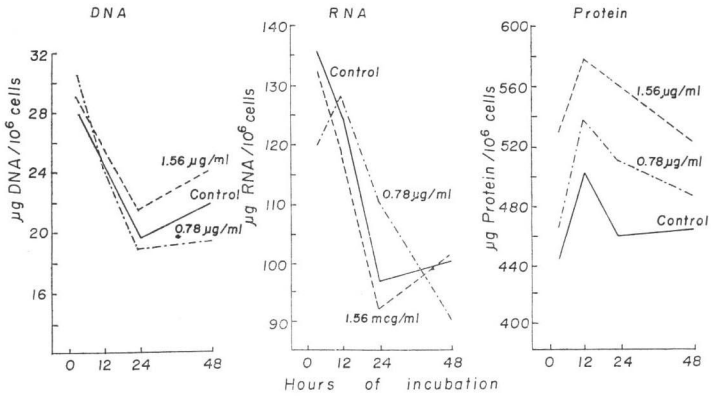
Effect of Dactylarin on Cell Size

Since direct microscopic observation indicated an enlargement of treated HeLa cells at all tested concentrations compared to control cells, the following experiments were aimed at the qualitative and quantitative evaluation of this effect. By 4 hours after drug addition, cells increased in size, reaching maximum values at 12 and 24 hours for cells exposed to 1.56 and 0.78 μg/ml dactylarin (Fig. 3). Analysis of the cell volume distribution profiles presented in Fig. 4 shows that although the population of treated cells had overlapped the distribution of untreated cells, the percentile representation of each cell size group changed in favor of larger cells.

Table 2. The effect of exposure to dactylarin on DNA, RNA and protein content per unit volume of HeLa cells

Hours after addition of dactylarin	μg DNA × $10^3/\mu^3$			μg RNA × $10^3/\mu^3$			μg Protein × $10^3/\mu^3$		
	0 μg/ml	0.78 μg/ml	1.56 μg/ml	0 μg/ml	0.78 μg/ml	1.56 μg/ml	0 μg/ml	0.78 μg/ml	1.56 μg/ml
12	1.49	1.36	1.28	7.4	7.0	5.9	30	29	28
24	1.29	0.98	1.16	6.4	5.9	4.9	31	27	30
48	1.60	1.30	1.45	7.3	6.0	6.1	33	32	31

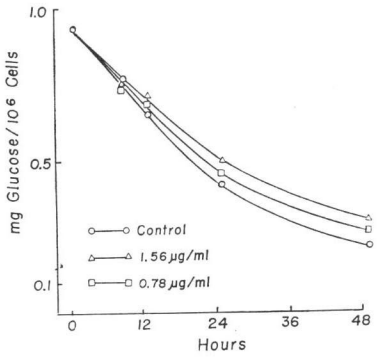
Fig. 5. Cell content of DNA, RNA and protein during the cytotoxic reaction of dactylarin on HeLa cells.



Effect of Dactylarin on Cell Content of Macromolecules

Changes in the amount of DNA, RNA and protein per 10^6 cells resulting from exposure of the cells to dactylarin are illustrated in Fig. 5. The differences in the RNA and DNA content of treated and untreated cultures were relatively minor. On the other hand, a significant increase in protein content per cell was observed already in the first hours after the drug addition.

Fig. 6. Glucose consumption by HeLa cells in the presence and absence of dactylarin.



When DNA, RNA and protein contents were expressed in terms of a unit cell volume (Table 2), it was found that in dactylarin-treated cells the relationship between these parameters were disturbed. While the content of protein remained directly proportional to the increased cell volume, the content of DNA and RNA per unit cell volume decreased. The RNA decrease was more apparent at 1.56 μg/ml of dactylarin, the concentration which caused 50% inhibition of HeLa cell proliferation.

The Utilization of Glucose

Glucose consumption from the culture medium by HeLa cells in the presence and absence of dactylarin was determined. As seen in Fig. 6, glucose consumption was only slightly inhibited by dactylarin at the tested concentrations.

Discussion

Dactylarin is a new antiprotozoal antibiotic produced by *Dactylaria lutea* ROUTIEN[1]. In the present investigation, the effect of dactylarin upon HeLa cells was studied. When exponentially proliferating HeLa cells were treated with varying concentrations of this antibiotic, the growth of cells was retarded at low drug concentration. At higher concentrations, there was an absolute decrease in the total cell count.

Judging from the effect on the frequency of mitosis, the dactylarin-exposed cells were primarily inhibited in interphase. Using PUCK and STEFFEN's method[5] it was demonstrated that

cells are blocked in G_2 phase just prior to mitosis (Fig. 2).

The decrease in cell proliferation was associated with the increase in cell size which was demonstrated by sequential measurements of cell diameter (Figs. 3,4) and by cinemicrography[2]. This increase in cell size is an aspect of the unbalanced growth[9]. At the time intervals selected for study, *i.e.* 12, 24 and 48 hours, the content of DNA and RNA per unit cell volume decreased, but the content of protein remained directly proportional to the increased cell volume (Table 2). Glucose consumption was only slightly inhibited by dactylarin. Investigation of the *mechanism* of action of dactylarin in EAC cells has shown that the loss of transplantability of these cells is the consequence of primary interference of the antibiotic with the energy metabolism[3]. The investigation with HeLa cells suggests that although the inhibition of glycolysis by dactylarin might contribute to the toxic effect of the antibiotic, the primary interference with the energy metabolism is not a prerequisite for its lethal effect on HeLa cells.

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