INTERACTION OF CYTOTOXIC ANTIBIOTIC DACTYLARIN WITH GLYCOLYTIC THIOL ENZYMES IN EHRLICH ASCITES CARCINOMA CELLS

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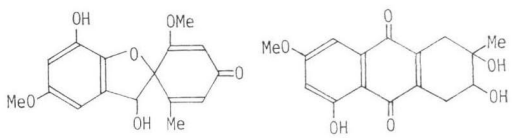
Cytotoxic effect of dactylarin on Ehrlich ascites carcinoma cells is caused by the inhibition of some SH-dependent glycolytic enzymes, especially of hexokinase (EC 2.7.1.1), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) and 6-phosphofructokinase (EC 2.7.1.11). Dactylarin interacts with thiols, which explains its inhibitory effectiveness on the above glycolytic enzymes.

Dactylarin was isolated from the culture medium[1] and from the mycelium[2] of *Dactylaria lutea* Routien. The antibiotic exhibits antiprotozoal activity *in vitro* against *Leishmania braziliensis* and *Entamoeba invadens*[1], and is highly cytotoxic to HeLa[3] and Ehrlich ascites carcinoma[4] cells. Its production from *D. lutea* was previously described[2]. The structure of dactylarin (A) proposed by KETTNER *et al.*[1] was revised by BECKER and co-workers[5] (B). These authors showed the identity of dactylarin with altersolanol B (2,3,5-trihydroxy-7-methoxy-2-methyl-1,2,3,4-tetrahydroanthraquinone).



A B

The results presented in this paper indicate that dactylarin is a thiol-combining agent and demonstrates its ability to inhibit glycolysis in Ehrlich ascites carcinoma cells.

Materials and Methods

Chemicals

Dactylarin was provided by Dr. KETTNER, Pharmacology Institute of Slovak Academy of Sciences, Bratislava, Czechoslovakia. In all experiments it was applied as a solution in dimethylsulfoxide, the most suitable solvent for this type of experiments[6]. All biochemicals used for enzyme assays and for glucose and lactate determinations were supplied by Boehringer, Mannheim, Germany. L-[U-^{14}C]-valine (0.1 mCi per 0.094 mg) and [8-^{14}C]adenine sulfate (0.1 mCi per 0.307 mg) were obtained from Institute for Research, Production and Application of Radioisotopes, Prague, Czechoslovakia. 5,5′-Dithiobis-2-nitrobenzoic acid was supplied by Fluka, Buchs, Switzerland. Dimethylsulfoxide and other chemicals of analytical grade were obtained from Lachema, Brno, Czechoslovakia.

Tumor Cells

Ehrlich ascites carcinoma (EAC) cells were obtained from the peritoneal cavity of mice on the 8th day after the transplantation. Cells were suspended in Krebs-Ringer phosphate medium, pH 7.4 without calcium, but with ascitic serum 2.5% v/v and glucose 3.0 mmole/liter. The cell concentration was adjusted to 3×10^6 per ml. Further details according to Reference 7.

Determination of Metabolic Effects

Solutions of dactylarin in dimethylsulfoxide in various concentrations, or ^{14}C-precursors were

added to the suspension of EAC cells. The incubation proceeded statically at 37°C. Aliquot parts of the suspension were taken out at appropriate time intervals and used for the determination of glucose and lactate, incorporation of ^{14}C-precursors, enzyme assay, SH groups determination, transplantability studies. The glucose and lactate concentrations were determined in the supernatant obtained after the precipitation of EAC cells with ice-cold perchloric acid[7]. Incorporation of ^{14}C-precursors (adenine, valine) was followed by measuring the radioactivity incorporated in the trichloroacetic acid precipitates of EAC cells as described MIKO and DROBNICA[7]. The cell free extracts, crude enzyme preparations of EAC cells for the enzyme assays, were obtained as follows. The stock suspension of EAC cells was centrifuged at 500 *g*, 4°C, for 10 minutes, the sediment was resuspended in ice-cold redistilled water, 1×10^7 cells per 0.2 ml and suspension obtained was frozen and thawed for 3 times at −20°C; +20°C. Homogenate recovered was centrifuged at 4,000 *g*, 4°C, for 20 minutes and the activities of enzymes were determined in the crude extracts using enzyme-optical method. The assay conditions were selected to ensure zero-order kinetics[8]. Concentrations of the total thiol groups (TSH) were estimated in the crude extracts with Ellman reagent as described by DROBNICA *et al.*[9].

Reaction of Dactylarin with Thiols

Kinetic measurements of the dactylarin reaction with thiols were carried out in Clark-Lubs buffered reaction mixtures (pH 5 ~ 8), containing at least 20-fold excess of thiols spectrophotometrically at 300 ~ 600 nm. The reactions were pseudomonomolecular under these conditions and obeyed the equation of the first-order reactions, time dependence of the log ΔA values is linear. The first order rate constants, $k_{obs}(s^{-1})$, were obtained from the slopes of the linear dependence of $\log(A_\infty - A_t)$ plotted against time after calculating according to equation 1:

$$k_{obs}=2.3[\log(A_\infty - A_{t2}) - \log(A_\infty - A_{t1})](t_2 - t_1) \quad (1)$$

The second order rate constants $k(\text{liter}\cdot\text{mole}^{-1}\cdot s^{-1})$ were calculated from equation 2:

$$k=k_{obs}\cdot C_o^{-1}\cdot K_a^{-1}(K_a + C_{H^+}) \quad (2)$$

where K_a is the dissociation constant of the thiol, C_o(mole/liter) its initial analytical concentration and C_{H^+}(mole/liter) is the concentration of the H^+ ions in the buffered system. Details are shown in previous papers[10,11].

Results

Effect of Dactylarin on EAC Cells

Dactylarin has been found to significantly inhibit glucose uptake and lactate production of EAC cells in Krebs-Ringer phosphate medium with glucose and ascitic serum, pH 7.4. Time and concentration dependences of this effect is seen in Table 1. This table also shows the inhibitory effect of dactylarin on several glycolytic enzymes in EAC cells. Activities of these enzymes were determined in the same experiment together with other parameters, glucose uptake, lactate formation, ^{14}C-precursors incorporation. From the decrease in activities of enzymes compared to controls *i.e.* without inhibitor being added, it comes out that mainly hexokinase, glyceraldehyde-3-phosphate dehydrogenase and 6-phosphofructokinase are sensitive on dactylarin. The enzymes mentioned above exhibit the same sensitivity on dactylarin after the application of the inhibitor into crude extracts. These findings indicate that dactylarin directly modifies the target enzymes. Furthermore, this assumption is also supported by the results obtained with isolated and purified enzymes. In the case of glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle, 66 μg protein per ml; carbonate buffer, pH 8.6, complete inhibition of enzyme activity is possible 60 minutes after application of dactylarin in a concentration 25.0 μg/ml.

Simultaneously with the inhibition of glycolysis by dactylarin the marked inhibition of ^{14}C-adenine and ^{14}C-valine incorporation into appropriate macromolecules occurred (Table 1). It is of interest to

Table 1. Effect of dactylarin on glucose uptake, lactate production, activities of some glycolytic enzymes, incorporation of ^{14}C-precursors and total SH groups content in EAC cells.

Consumption of glucose and formation of lactate are expressed in nmole/min/3×10^6 cells and calculated for 0~30 and 90~120 minutes intervals. Incorporation of ^{14}C-precursors was estimated for the same intervals as the radioactivity increase (CPM) in TCA-insoluble fractions of EAC cells. Enzyme activities are expressed in nmole/min/3×10^6 cells and thiol content in nmole/3×10^6 cells. Numbers in brackets indicate percentage of inhibition (%) against control.

Parameter	30 minutes			120 minutes	
	0	6.25 μg/ml	25 μg/ml	0	25 μg/ml
Glucose	13.3	8.7(35)	6.3(54)	13.3	<1.0(>93)
Lactate	23.4	18.3(22)	13.3(43)	23.4	<1.0(>93)
Hexokinase	17.9	12.4(31)	9.2(49)	15.9	4.9(69)
Phosphofructokinase	69.5	54.9(21)	51.8(26)	61.5	7.4(88)
Glyceraldehyde-3-phosphate dehydrogenase	640	425 (34)	199 (69)	587	35.2(94)
Lactate dehydrogenase	1,710	1,310 (23)	1,180 (31)	1,690	878.8(48)
Glucose-6-phosphate dehydrogenase	16.4	15.8(4)	15.4(6)	15.1	2.6(83)
^{14}C-Adenine	2,800	1,750 (37)	1,000 (64)	2,250	210 (91)
^{14}C-Valine	3,750	1,700 (55)	800 (79)	3,150	250 (92)
SH groups content	76.0	63.1(17)	44.1(42)	74.1	23.6(68)

Fig. 1. Spectrophotometric indication (A) and kinetics (B) of the dactylarin reaction with 2-mercaptoethanol in phosphate (0.1 mole/liter) buffer, pH 6.0 at 25°C.

Initial concentration of dactylarin 2.5×10^{-4} mole/liter and that of thiol 2.5×10^{-2} mole/liter. The absorption spectra of reaction mixture were measured for 10 (2), 20 (3), 40 (4), 80 (5), 120 (6) and 420 (7) minutes after the start of reaction. Curve 1 represents the spectrum of dactylarin in buffer without 2-mercaptoethanol.

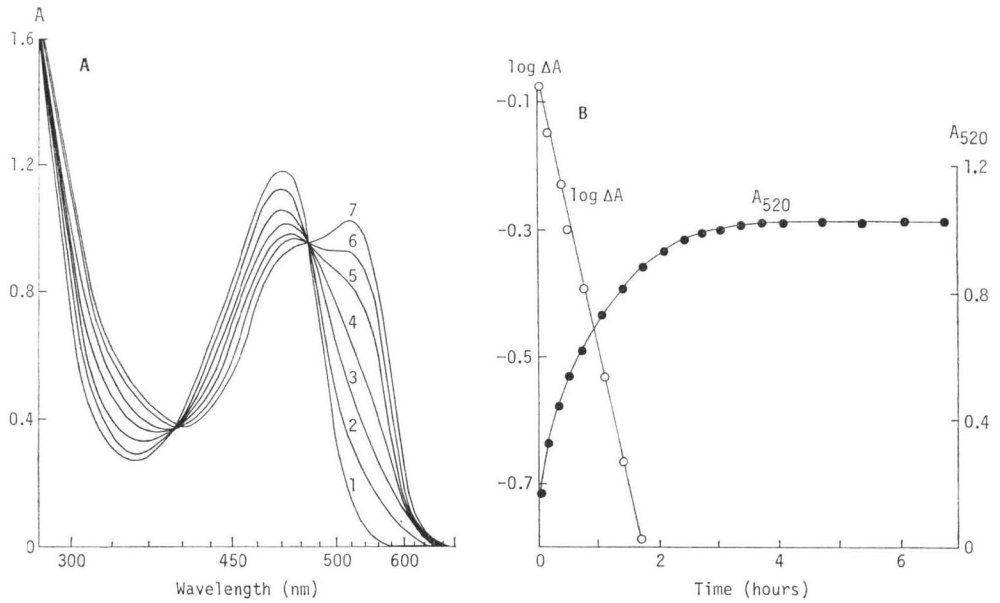


Table 2. Values of first-order (k_{obs}) and second-order rate constants (k) for reactions of dactylarin with 2-mercaptoethanol in phosphate (0.1 mole/liter) buffers, pH 5.0~7.0 at 25°C.

The initial concentration of ionized form (C_{RS^-}) of thiol is inserted for the comparison.

pH	C_{RS^-} (mole/liter)	k_{obs} (s^{-1})	k (liter·mole^{-1}·s^{-1})
5.0	1.0×10^{-6}	2.9×10^{-5}	2.9×10
5.5	3.2×10^{-6}	9.9×10^{-5}	3.1×10
6.0	1.0×10^{-5}	3.2×10^{-4}	3.2×10
6.5	3.2×10^{-5}	1.1×10^{-3}	3.5×10
7.0	1.0×10^{-4}	3.3×10^{-3}	3.3×10

Table 3. The second-order rate constants k (liter·mole^{-1}·s^{-1}) for reactions of dactylarin, benzyl isothiocyanate (BITK), iodoacetamide (IAA) and N-ethylmaleimide (NEM) with cysteine, glutathione and 2-mercaptoethanol at 25°C.

The values of rate constants for thiol-combining agents according to Ref. 12.

Thiol	Dactylarin	BITK	IAA	NEM
Cysteine	3.9×10^{0}	2.8×10	9.9×10^{-1}	3.1×10^{4}
Glutathione	7.2×10	2.5×10	—	3.8×10^{4}
2-Mercapto-ethanol	3.2×10	—	—	—

note that EAC cells which have been treated with dactylarin at concentrations 12.5 and 25.0 μg/ml did not evoked tumor formation in mice. Nevertheless, the lower concentrations, 3.1 and 6.2 μg/ml of dactylarin, have not prevented the tumor growths but significantly retarded them, thus elongating the survival of mice[4].

Reactions of Dactylarin with Thiols

The results summarized in Table 1 show that the inhibitory effect of dactylarin on EAC cells is connected with significant decrease of the total, protein and non-protein, content of SH groups. This finding could indicate that dactylarin interacts directly with thiols. Fig. 1 illustrates spectrophotometrically the course of the dactylarin reaction with 2-mercaptoethanol as a model thiol. This reaction and also reactions with glutathione and cysteine were carried out in buffers, pH 5.0~8.0. The observed rates of the dactylarin reactions with 2-mercaptoethanol characterized in Table 2 by the first-order rate constants $k_{obs}(s^{-1})$, are strongly dependent on the pH values. The reaction course is faster in more alkaline conditions, indicating that dissociated form of thiol, RS^-, takes part in the reactions. The corresponding second order anion rate constants k (liter·mole^{-1}·s^{-1}) have practically equal values (Table 2). For the calculation we took into consideration the concentration of the dissociated form of thiol instead of its analytical concentration. The second-order rate constants are practically independent on pH values. For this reason they are much more convenient for the characterisation of the dactylarin reactivity with thiols. Table 3 summarizes rate constants for reactions of dactylarin with cysteine, glutathione and 2-mercaptoethanol as model thiols. The table also contains, for comparison, the second order rate constants for some known thiol-combining agents estimated under the analogical conditions.

Discussion

The finding that dactylarin is a thiol-combining agent is not surprising when we realize that it is the naphthoquinone derivative. The fact that the naphthoquinones react with thiols has been known for a long time. The products of these reactions are addition products, or oxidative-reductive originated hydroquinones and disulfides[13]. From the biological point of view, however, the reactivity and lipophilicity of dactylarin determines its mode of action. The first parameter determines the time-course dependence, the second one the site of action[14]. The data presented in Table 3 show that the reactivities of dactylarin towards thiols are approximately 4-times higher than the reactivity of iodoacetamide, which is regarded to be the classical inhibitor of glycolysis. The mechanism of glycolysis inhibition by iodo-

acetamide is a result of the chemical modification, alkylation, of the catalytic active SH groups of glyceraldehyde-3-phosphate dehydrogenase[15]. However, the course of the glycolysis inhibition caused by dactylarin in EAC cells was, in relation to iodoacetamide slower (DROBNICA, unpublished results). It could be related to the slower retention of the antibiotic into the cells, since the compound possesses a few hydrophilic groups in its skeleton. The dissociated hydroxy-group in the site 5 could decrease the partition coefficient, lipophilicity as well as the ability of the compound to penetrate through the membrane into the cell. Relatively considerable decrease of the SH group content in EAC cells after the effect of dactylarin, as shown in Table 1, points at multitarget intervention of the antibiotic. Nevertheless, from the view point of mechanism of dactylarin action, its ability to inhibit the glycolysis is decisive, because this pathway is superimposed in the metabolism. It appears that glycolysis inhibition is initiated by the inactivation of hexokinase, rate limiting enzyme of glycolysis in non treated cells[16], phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase. All the enzymes mentioned above are SH dependent[13]. Inhibition of these enzymes with dactylarin could be considered the result of chemical modification of their essential thiol groups. The logical consequence of inhibited glycolysis is the slowing down of ^{14}C-precursors incorporation into the appropriate macromolecules of EAC cells. The biosynthetic processes followed are dependent on glycolysis not only because of appropriate intermediate requirements but also from aspect of energy demands. It is therefore conceivable to realize that the glycolysis inhibition is the very deep intervention of the dactylarin into the physiological steady-state of EAC cells. The consequence of this is a loss of transplantability of EAC cells, elimination of which is probably the result of viability disappearance of cells during the pretreatment with dactylarin[4].

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