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ACTION OF A NEW SARCOMYCIN DERIVATIVE ON MITOCHONDRIAL ENZYMES OF EHRLICH MOUSE ASCITES CELLS

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Abstract—The inhibitory action of SB 21 Sarcomycin derivative (4-Ethyl-2,5-bis-piperonyliden-cyclopentanon-3-carboxylic acid) on the citric acid cycle oxidations and on some of the respiratory enzymes (succinate oxidase, succinate dehydrogenase, malate dehydrogenase, $NaDH_2$ cytochrome c reductase, cytochrome oxidase) has been studied, using Ehrlich mouse ascites tumor cells.

The SB 21 derivative inhibits oxygen uptake by tumor cells homogenates, using fumarate and pyruvate as substrates. In the same experimental conditions the 2,4-dinitrophenol-stimulated respiration is suppressed by the drug.

The succinate oxidase, succinate dehydrogenase and malate dehydrogenase activities are remarkably lowered by the action of the Sarcomycin derivative, while it does not at all affect the $NADH_2$–oxidase system $NADH_2$ cytochrome c reductase and cytochrome oxidase.

IN PREVIOUS papers[1, 5] the relation between chemical structure and biological activity of new Sarcomycin derivatives has been reported. Evidence was presented that the most fruitful modification of the Sarcomycin molecule was obtained by introducing into its fundamental ring (cyclopentanon-3-carboxylic acid) an ethyl radical and two substituted methylene groups. This modification led to the synthesis of a new compound (code no. SB 21, Fig. 1), whose antiblastic action appeared to be strongly increased in respect to that of the original substance (Dihydrosarcomycin). The effectiveness of the new derivative was tested as inhibitory action on the growth of Ehrlich ascites tumor, and evaluated from morphological and biochemical alterations induced on tumor cells.

In view of defining the mechanism of action of the Sarcomycin derivatives, the effects of SB 21 on the metabolism of Ehrlich mouse ascites tumor have been investigated. The finding that SB 21 strongly inhibited the oxygen uptake of Ehrlich ascites cells at a concentration (250 μg/ml) at which the glycolytic rate was practically unaffected,[3] suggested an interference of the drug with the last steps of the oxidative metabolism.

Therefore it seemed interesting to investigate more deeply if the cytotoxic effect of the drug was to be ascribed or not to the inhibition of a definite enzymic system. Detailed data on the effects of SB 21 on the Krebs cycle and on some oxidative enzymes of Ehrlich ascites cells are reported in this paper.

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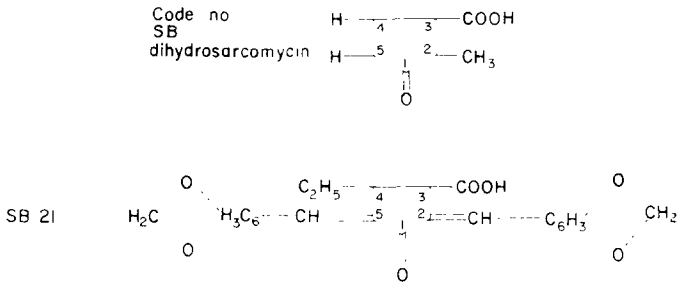

FIG. 1.

MATERIALS AND METHODS

The Ehrlich ascites tumor (Tetraploid Lipschutz IV strain) was chosen for this study. The ascitic cells, withdrawn from a donor mouse bearing the tumor since 6–9 days, were washed twice by centrifugation (600 *g*) with iso-osmotic Ringer phosphate solution.

The preparation of homogenates and the isolation of mitochondria from tumor cells were carried out according to Hawtrey and Silk.[11]

The mitochondrial protein content was determined by the biuret method according to Gornall *et al.*[10] using bovine serum albumin as standard.

The oxygen uptake has been measured for all the experiments by the conventional manometric Warburg apparatus. The following standard conditions have been used: final volume of the reaction mixture, 2·9 ml; in the center well, 0·10 ml NaOH 40%; gas phase, air; temperature, 38°; 10–15 min equilibration. The oxygen uptake by total homogenates using fumarate and pyruvate as substrates (Krebs cycle) has been evaluated according to Potter.[14]

The Succinate oxidase (SOX), Succinate: (phenazine) oxidoreductase (SDH) and L-Malate: NAD oxidoreductase (MDH) assays have been carried out on mitochondria from Ehrlich tumor cells.

The SOX has been assayed by the manometric technique[14] in the presence of Cyt c; the SDH assay has been carried out using the manometric method with phenazine methosulphate according to Bernath and Singer.[1] The MDH has been tested by a spectrophotometric procedure in presence of oxalacetate.[13]

The estimation of the $NADH_2$–oxidase system ($NADH_2$: cytochrome c oxidoreductase-$NADH_2$ Cyt-c-RED- and Cytochrome C : C_2 oxidoreductase-Cyt OX-) of isolated mitochondria has been carried out spectrophotometrically, according to Mahler[12] for the $NADH_2$ Cyt-c-RED and Cooperstein and Lazarow[6] for Cyt OX activities. In all the experiments SB 21 Sarcomycin derivative (4-Ethyl-2,5-bis-piperonyliden-cyclopentanon-3-carboxylic acid, mol. wt. = 420) synthetized by Giuliano *et al.* following their own original procedure,[7–9] was dissolved as sodium salt and the pH of the final solution adjusted at 7·4 with Ringer phosphate solution.

Cytochrome c (type III), $NADH_2$, Na-ATP and phenazine methosulphate were obtained from SIGMA Chemical Co.

RESULTS

In Table 1 are reported the main results collected studying the action of three Sarcomycins (Sarcomycin, Dihydrosarcomycin and SB 21 Sarcomycin derivative) on the metabolic properties of Ehrlich tumor cells. For each activity which has been investigated the Table shows the concentration of the drug which is able to give a half inhibition of the tested activity.

TABLE 1. ACTION OF SARCOMYCIN, DIHYDROSARCOMYCIN AND SB 21 SARCOMYCIN DERIVATIVE ON THE RESPIRATION AND OXIDATIVE ENZYMES OF THE EHRLICH ASCITES TUMOR CELLS

For each tested activity is given the concentration necessary to have 50 per cent inhibition. The inhibition are calculated on the basis of: Qo_2 for the respiration and Krebs cycle oxidations; and 'specific activities' for the SOX, SDH, MDH, $NADH_2$ Cyt-c RED and Cyt OX. All the experiments have been carried out in the presence of the drug.

	Compound	Concentration,* μg/ml	M 10^{-4}	Cellular fraction	Reference
Respiration	Sarcomycin	~670	47·8	whole cells	(2)
Respiration	Dihydrosarcom.	1000	70·5	,,	(3)
Respiration	SB 21	50	1·2	,,	(3)
Krebs cycle	,,	160	3·8	homogenate	this work
SOX	,,	90	2·1	mitochondria	,,
SDH	,,	190	4·5	,,	,,
MDH	,,	100	2·4	,,	,,
$NADH_2$Cyt-c RED	,,	1000	23·8	,,	,,
Cyt OX	,,	1000	23·8	,,	,,

* The reported values of the concentration giving 50 per cent inhibition are obtained for dihydrosarcomycin and SB 21 from the smooth curves in the plots of the percent inhibition against the concentration of the drug.

It is clear that SB 21 determines a remarkable depression of the oxidation rate of fumarate and pyruvate (Krebs cycle) by homogenates of Ehrlich ascites cells. The concentration of the drug giving 50 per cent inhibition is about three times higher than that found for the oxidation of the glucose by the whole cells (respectively 160 and 50 μg/ml). In the same experimental conditions the drug is able to suppress completely the 2,4-dinitrophenol (DNP) stimulated respiration (Fig. 2). The ratio between oxygen uptake in the presence and absence of DNP (Qo_2DNP/Qo_2), which is ranging from 1·4 to 1·7 for controls, assumes the value of $1 \pm 0{\cdot}02$ in the presence of SB 21 Sarcomycin derivative (conc. 167 μg/ml).

The consideration of these experimental evidence seems to exclude an interference of the compound with the oxidative phosphorylations, and suggests a disturbance of some reactions of the citric acid cycle or of the electron carriers.

The evaluation of SOX and SDH activities of mitochondria of Ehrlich tumor cells in presence of SB 21 Sarcomycin derivative reveals a marked sensitivity of these enzymic systems to the action of the drug. As it appears from Fig. 3, the inhibitory action of SB 21 is more marked on the SOX system for every concentration tested. This result could be explained by admitting that for the SOX system there is an additional effect, which is absent in the SDH determinations. The drug's concentration

necessary to halve the enzymic activity is equal to 90 μg/ml for SOX and 170–200 μg/ml for SDH.

The MDH seems to be the most sensible between the tested enzymic systems (Fig. 3). The concentration giving 50 per cent inhibition is, for this enzyme, equal to 100 μg/ml

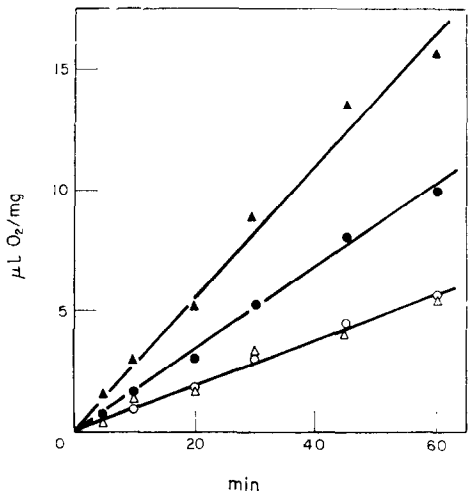

FIG. 2. Effect of SB 21 sarcomycin derivative on the 2,4-dinitrophenol stimulated respiration. The vassels contained the following reaction mixture: 30 μM K-K_2 phosphate buffer pH 7·3, 6 μM Na-fumarate, 6 μM Na-pyruvate, 12 μM $MgCl_2$, 3 μM Na-ATP, 485 μM sucrose, 6–10 mg dry weight Ehrlich tumor cells homogenate. Addition: ● nil; ▲ 0·15 μM 2,4-DNP; ○ SB 21 500 μg (167 μg/ml); △ SB 21 500 μg (167 μg/ml) + 0·15 μM 2,4-DNP.

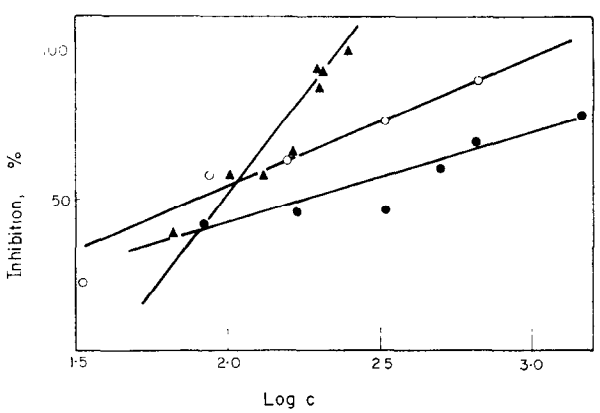

FIG. 3. Inhibitory action of SB 21 sarcomycin derivative on SOX, SDH, and MDH of mitochondria of Ehrlich ascites cells.

The percent inhibition is plotted against log c, where c is the drug concentration in μg/ml. The following reaction mixtures have been used: for SOX: 100 μM K-K_2 phosphate buffer pH 7·4, 150 μM Na-succinate; 0·04 μM Cytochrome c, 1·2 μM $CaCl_2$, 0·6 μM $Al_2(SO_4)_3$, about 0·75 mg of protein Ehrlich cells mitochondria. For SDH: 150 μM TRIS buffer pH 7·4, 5 μM $CaCl_2$, 3 μM KCN, 60 μM Na-succinate, 1% phenazine methosulphate 0·20 ml, 0·4–0·7 mg of protein Ehrlich ascites mitochondria. For MDH: 75 μM K-K_2 phosphate buffer pH 7·4, 0·15 μM $NADH_2$, 0·76 μM Na-oxalacetate, about 0·5 mg of protein Ehrlich cells mitochondria, water to 3 ml. The notations are as follows: ○ SOX; ● SDH; ▲ MDH.

and the inhibition reaches the 100 per cent at a concentration of 250 μg/ml (6×10^{-4} M).

Two of the components of the $NADH_2$ oxidase system ($NADH_2$-Cyt-c-RED and Cyt OX) are practically unaffected by the action of the drug (Table 2), confirming the results obtained when the Cyt OX activity was tested in different experimental conditions.[3] For both enzymic activities the concentration of SB 21 giving 50 per cent inhibition is higher than 1000 μg/ml.

DISCUSSION

The inhibitory action of SB 21 Sarcomycin derivative on the oxidative metabolism of Ehrlich mouse tumor cells is several times increased in comparison with that of the original compounds, Sarcomycin and Dihydrosarcomycin. Taking as term of a comparison the inhibition of the oxidation of the glucose by the whole cells (see Table 1), we may say that SB 21 is from 30–50 times more active than the two original drugs. The chemical basis of the increased effectiveness is linked with the introduction into the fundamental ring of Sarcomycins (cyclopentanon-3-carboxylic acid) of an ethyl radical and two substituted methylene groups[4] (Fig. 1).

As previously shown[3] the inhibitory action of SB 21 fundamentally concerns the oxidative metabolism of the tumor cells, without a noteworthy compromise of the glycolytic rate. Furthermore our results show that some electron carriers are practically unaffected by the action of the Sarcomycin derivative, also at very high concentrations (Table 2).

TABLE 2. ACTION OF SB 21 SARCOMYCIN DERIVATIVE ON $NADH_2$-CYT-C RED AND CYT OX OF MITOCHONDRIA OF EHRLICH ASCITES CELLS

The following reaction mixtures have been used:

For the $NADH_2$ Cyt-c RED: 150 μM K-K_2 phosphate buffer pH 7·4, 0·60 μM $NADH_2$, 0·071 μM Cyt-c, 3 μM KCN, about 0·10 mg of protein Ehrlich cells mitochondria, water to 3 ml.

For the Cyt OX: 90 μM K-K_2 phosphate buffer pH 7·4, 0·051 μM Cyt-c, about 0·5 mg protein Ehrlich cells mitochondria, water to 3 ml.

All the experiments have been carried out in the presence of the drug.

	$NADH_2$ Cyt-c RED		Cyt OX	
	Δ O.D. at 550λ, min^{-1} mg $prot^{-1}$	per cent inhibition	Δ O.D. at 550λ, min^{-1} mg $prot^{-1}$	per cent inhibition
Control	0·490	—	2·47	—
SB 21 167 μg/ml	0·410	16	—	—
,, 333 ,,	0·455	7	2·39	3
,, 500 ,,	0·363	26	—	—
,, 666 ,,	—	—	2·28	8
,, 1000 ,,	0·318	35	2·17	12

On the other hand the oxidative phosphorylations, indirectly assayed studying the action of SB 21 on the DNP-stimulated respiration, do not seem particularly affected. This result, although is in agreement with the data reported by Quastel *et al.*[2] on the action of Sarcomycin on the metabolism of the Ehrlich ascites carcinoma, does not give any further clarification to the intimate mechanism of action of these drugs on the adenylic acid reactions.

The findings reported in the present paper suggest that the citric acid cycle is the chiefly inhibited metabolic pathway. In fact SB 21 is able to decrease the MDH and SDH activities to a remarkable extent, since the concentration giving 50 per cent inhibition ranges, for these enzymic systems, from 2–5 $\times$ 10^{-4} M.

The mechanism by which SB 21 effects its antiblastic action is to be found in the antimetabolic activity of the compound. Although the drug is particularly active on some of the enzymic components of the Krebs cycle, its inhibitory action cannot be defined specific for one of them. In our opinion the biochemical basis of the antitumoral effect of this new Sarcomycin derivative is to be found in a complex action which develops along all the respiratory chains, although greater in some metabolic steps than others. However we do not wish to exclude the possiblity of other mechanisms not investigated in this study.

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