Inhibition of Energy Metabolism in Ehrlich Ascites Cells Treated with Dactylarin in Vitro

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ABSTRACT

The effect of the new antibiotic dactylarin as a potential anticancer agent on energy-yielding and energy-requiring processes in Ehrlich ascites cells has been investigated. At concentrations higher than 10 µg/ml, the antibiotic causes a rapid and practically full inhibition of both aerobic glucose utilization and lactate formation. At lower concentrations (6.25 µg/ml), dactylarin causes a progressive inhibition of glycolysis; after 90 min, glycolysis is completely stopped. At concentrations as low as 3 µg/ml, however, glycolysis is stimulated. This stimulation of glycolysis, according to our previous results, suggests that the possible mode of action of this antibiotic is the uncoupling of oxidative phosphorylation. Dactylarin (Substance 66A1) and dactylarin-like antibiotics (Substances 66A2 or 66A3) stimulated endogenous respiration of these cells. The stimulation of respiration over 100% was attained by concentrations higher than 40 µg/ml. On the other hand, these antibiotics were able to release oligomycin-inhibited respiration of the cells studied. Dactylarin and its derivatives, however, have shown little effect on oxygen uptake by pigeon heart and rat liver mitochondria in State 4 or in State 3 respiration, respectively.

Proportionally to the increasing concentrations and in a time-dependent manner, dactylarin inhibits the synthesis of macromolecules in whole cells of Ehrlich ascites carcinoma measured by the incorporation of labeled amino acid, adenine, and thymidine into acid-insoluble material. The incorporation of uracil into the appropriate trichloroacetic acid-insoluble fraction was unaffected.

We conclude that dactylarin interferes primarily with energy-yielding processes. Secondary consequences of this interference were both the inhibition of some biosynthetic processes (mainly DNA and protein) and the loss of transplantability of Ehrlich ascites cells.

INTRODUCTION

Dactylarin (Substance 66A1) is a new antibiotic of the geodin group which was isolated from the culture medium (40) and from the mycelium of the predacious fungus Dactylaria lutea Routien by Kettner et al. (39) in 1973. Isolation, purification, properties, and structure (Chart 1A) as well as high activity against protozoa and nematodes have also been reported (40). Dactylarin was the most cytotoxic antibiotic of those studied in the geodin groups (59) and was strongly cytotoxic to HeLa cells (34). When exponentially proliferating HeLa cells were treated with varying concentrations of dactylarin, cell growth was retarded at low drug concentrations. At higher concentrations, there was an absolute decrease in the total cell count. Cells exposed to dactylarin were primarily inhibited in interphase, and it was demonstrated that cells were blocked in G2 just prior to mitosis (34). A relatively short in vitro exposure of L5178Y cells to dactylarin affected the transplantability of cells in C57BL x DBA/2 F1 mice (34). A significant cancerostatic effect of this antibiotic has also been observed (39, 54).

Recently, Becker et al. (5) investigated other metabolites of D. lutea Routien and isolated 3 hydroxylated derivatives of antraquinone as well. They revised the structure proposed by Kettner et al. (40) for dactylarin (Chart 1B) and proved that dactylarin was in fact altersolanol B (Chart 1B), which was previously isolated from Alternaria solani (70).

In the course of our screening tests to find antitumor-active natural or synthetic substances (22, 52, 57, 58), the antibiotic dactylarin has been found to significantly inhibit the incorporation of both [14C]adenine and [14C]valine into the acid-insoluble fraction of Ehrlich ascites carcinoma cells at very low concentrations (54). Since both nucleic acid and protein synthesis require energy, the possibility that the inhibitory effect of the antibiotic may be due to its interference with energy generation or utilization deserved study.

In view of the interesting biological activities shown by dactylarin in the present work, we have tried to elucidate why dactylarin is cytotoxic and cancerostatic (HeLa and Ehrlich ascites cells). Therefore, the effect of dactylarin (altersolanol B), as a new potential anticancer agent, on some bioenergetic processes in Ehrlich ascites cells in vitro and the loss of transplantability have been investigated. This site of action of dactylarin has not been considered previously in the literature and may be relevant to the mechanism by which it produces toxicity in tumor cells. Inhibition of energy-generating systems of the cell due to decreased activity of the glycolytic pathway and oxidative phosphorylation might result in loss of cell viability and impairment of other intracellular events (23). Drugs that interfere with ATP synthesis and are potent stimulators of glycolysis in intact cells are also potent inhibitors of cell proliferation (69). The possible significance of the data collected is therefore relevant to cancer therapy.

We have worked with ascites tumor cells, which under appropriate physiological conditions are suitable for such investigations (1, 7): (a) they can be grown i.p. in mice or in cell cultures under defined conditions; (b) they can be readily manipulated as uniform cell suspensions; and (c) their energy metabolism has been extensively studied (1, 7, 13, 30, 32, 58, 67). In our previous papers, Ehrlich ascites cells were used in primary screening for potential anticancer agents (22, 57, 58) and for the study of mechanism of action of some antibiotics (56), ethidium bromide (49), isothiocyanates (50), and other known cancerostatics (25, 48, 51). Some of the results have been presented in a preliminary report (54).

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MATERIALS AND METHODS

**Cells.** Ehrlich ascites carcinoma cells were maintained and propagated in white mice, strain H (Institute of Experimental Pharmacology, Slovak Academy of Sciences, Dobra Voda, Czechoslovakia), about 10 weeks old and 20 to 25 g body weight as described previously (16, 53, 55). ICR albino mice inoculated with Ehrlich-Leütre ascites cells were provided by Dr. E. Patterson of the Cancer Institute, Fox Chase, Pa. Ehrlich-Leütre hyperdiploid ascites tumor cells were grown in ICR albino mice after i.p. injection of 0.4 ml of ascitic fluid (approximately 10^8 cells) (21). These cells were used to measure endogenous respiration only. Ehrlich ascites carcinoma cells were transplanted at 7-day intervals by i.p. injection of 0.2 ml of ascitic fluid collected under sterile conditions. The tumor cells were obtained from the peritoneal cavity of mice and were packed by low-speed centrifugation (600 × g for 10 min at 4°C). Ascitic plasma was poured off, and an incidental layer of erythrocytes was removed (10). The cells were suspended in Krebs-Ringer phosphate buffer, pH 7.4, without calcium but with ascitic serum (2.5%, v/v) and glucose (final concentration, 3.0 mM). The number of the cells was adjusted to 3 × 10^6/ml of medium. Cell concentration was calibrated against the absorbance of the cell suspension (55). All operations were performed at 0–4°C.

**Materials.** Materials were purchased as follows. Dactylarin (Substance 66A,) and its analogs (Substances 66A2 and 66A3) were kindly supplied by Dr. M. Kettner, Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, Czechoslovakia. All the tested substances were chromatographically pure (40). Dactylarin is a weakly acidic compound which forms red plates, m.p. 201–205°C (with decomposition) and is quite soluble in acetone, ethyl acetate, and DMSO.2 It is also weakly acidic; acid solutions are yellow, basic dark red. It is soluble in DMSO and acetone, less so in ethyl acetate, and slightly so in butanol and water. It is not soluble in chloroform, benzene, and petrol ether, m.p., 192–205°C (with decomposition). Substance 66A3 crystallizes from methanol (or acetone) in the form of orange crystals. It is weakly acidic; acid solutions are yellow, basic dark red. The substance is soluble in DMSO, acetone, and methanol; less soluble in butanol and ethyl acetate; and not soluble in chloroform, benzene, or petrol ether, m.p. 168–178°C. [8-^{14}C]Adenine sulfate (specific activity, 44 mCi/mmol), [L-^{14}C]valine (specific activity, 175 mCi/mmol), [2-^{14}C]thymidine (specific activity, 53 mCi/mmol), and [2-^{14}C]-uracil (specific activity, 44 mCi/mmol) came from the Institute for Research, Production and Applications of Radioisotopes, Prague, Czechoslovakia. Radiochemical purity of all the radioactive precursors was checked by paper chromatography or thin-layer chromatography before use. Chemicals and enzymes for the determination of glucose and lactate were supplied by Boehringer/Mannheim, Federal Republic of Germany. All other reagents were obtained from Sigma Chemical Co., St. Louis, Mo. Oligomycin was added in an ethanolic solution.

**Glucose Uptake and Lactic Acid Production.** The kinetics of aerobic glucose uptake and lactic acid production by the methods described in previous papers was followed (16, 53, 55). Glucose and lactate concentrations were determined enzymatically in the supernatant obtained after 1 ml of the Ehrlich ascites cell suspension was precipitated with 1 ml of 0.6 M perchloric acid in an ice bath using commercial tests (Boehringer). The precision of these measurements is ±3%.

**Endogenous Respiration.** The effect of dactylarin on the endogenous respiration of Ehrlich-Leütre ascites cells was determined on the basis of oxygen consumption in 154 mM NaCl, 6.2 mM KCl-11 mM sodium phosphate buffer, pH 7.4 (10). Dactylarin dissolved in DMSO was mixed with 2.0 ml of this medium, and 200 μl of the cell suspension in the same buffer solution were added (49, 50). In control experiments, DMSO replaced the dactylarin solution. Rates of oxygen uptake by Ehrlich-Leütre hyperdiploid ascites cells as well as isolated mitochondria were measured polarographically with a Clark-type oxygen electrode in a thermostatically controlled reaction vessel equipped with a stirring device. The precision of these measurements is ±3%.

**Isolation of Mitochondria.** Intact pigeon heart and rat liver mitochondria were obtained in a medium containing 225 mM mannitol, 75 mM sucrose, 0.2 mM EDTA, and 2 mM morpholino-1-propanesulfonate, pH 7.2, according to the method of Chance and Hagiwara (9). If not indicated otherwise, the mitochondria were suspended in the same medium. Protein concentration of the final suspension was determined by the biuret method with bovine serum albumin as standard (24). All mitochondrial preparations were checked for structural integrity using the criterion of respiratory control (8). Oxidation rates and ADP:O ratio with ADP as P, acceptor were defined and calculated according to the method of Chance (8).

**Incorporation of Labeled Precursors.** The rate of synthesis of nucleic acids and proteins in whole Ehrlich ascites carcinoma was determined by the incorporation of corresponding radioactive precursors into the TCA-insoluble fraction of the cells. The procedure used in evaluating the inhibitory effect of dactylarin was similar to that used when testing other metabolic inhibitors (16, 17, 52, 58). Five Erlenmeyer flasks containing...
the suspension of Ehrlich ascites cells (60 ml, 3 × 10⁶ cells/ml) were placed in an ice bath and supplemented with 600 μl DMSO with various concentrations of the dactylarin. From each flask, 4 series were prepared each comprising 24 test tubes with 1 ml of the suspension. The incorporation of (Series 1) [¹⁴C]adenine, (Series 2) [¹⁴C]valine, (Series 3) [¹⁴C]thymidine, and (Series 4) [¹⁴C]uracil into acid-insoluble material was studied. The remaining suspensions of Ehrlich ascites carcinoma cells in the Erlenmeyer flasks were used to measure the rate of glycolysis and the transplantability of these cells. The cells were incubated in a water bath at 37° without shaking. At the indicated time intervals, samples of suspensions were analyzed for radioactivity in acid-insoluble material, glucose utilization, and lactic acid production. The rest of the suspension in the Erlenmeyer flasks was used for the determination of transplantability of Ehrlich ascites cells. In the control experiments, only DMSO was used. The final concentration of DMSO was 1% which does not affect the metabolic processes investigated (15). The pH of the incubation mixture was not altered by adding DMSO. Samples for determining [¹⁴C] precursor incorporation were transferred at appropriate time intervals into an ice bath, and 1 ml of 5% TCA was added to each of them. The samples were filtered through Synpor membrane filters, pore size 4 μm (Synthesia, Prague, Czechoslovakia); the precipitate was washed with 10 ml of cold 2.5% TCA and 10 ml of water and dried at 105°. Radioactivity was measured on a methane flow counter (Frieseker und Hoepfner GmbH, Erlangen, Federal Republic of Germany). In some cases, the nature of the labeled material was checked by alkaline-acid hydrolysis. In the case of adenine incorporation, 60.6% of the incorporated radioactivity corresponds to the RNA fraction and 39.4% corresponds to that of DNA. In the case of thymidine, 90% of its incorporation was found in DNA; in case of uracil, 87.5% of the radioactivity was found in the RNA fraction (in all cases fractionation of perchloric acid precipitates) (55). All experimental points are from duplicate determinations. The precision of these measurements is ±5%.

Transplantability of Ascites Tumor Cells. A 20-ml sample of Ehrlich ascites cell suspension that had been used for determining glucose utilization and lactic acid production was centrifuged after 2 hr incubation in the presence of different concentrations of dactylarin (600 × g for 5 min at 4°), and the pellet was resuspended in sterile 0.9% NaCl solution (6 ml) (17). From such a suspension, 0.5 ml (5 × 10⁶ cells) was immediately inoculated into 4 groups of mice (10 in each) for the bioassay. As a control, an additional group of mice was simultaneously inoculated with the same number of Ehrlich tumor cells incubated for 2 hr in the presence of DMSO by the same procedures mentioned above. All the procedures were performed under aseptic conditions. All the mice thus inoculated were inspected daily for 60 days, and the survival rate of each group was recorded. Autopsy was performed in the case of death. If no tumors were found after 60 days, the cells were considered to have lost the ability for transplantation. Untreated cells caused death in animals 15.7 ± 0.5 days (S.E.) after inoculation. The results were subjected to statistical evaluation by the t test.

RESULTS AND DISCUSSION

Effect of Dactylarin on Energy-yielding Processes. Chart 2 demonstrates the effect of dactylarin on aerobic glucose uptake and lactic acid formation by Ehrlich ascites cells. The antibiotic at concentrations higher than 10 μg/ml causes a

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Chart 2. The effect of dactylarin on the kinetics of aerobic glucose utilization and lactic acid formation by Ehrlich ascites tumor cells. The cells were incubated at 37° in the presence of different concentrations of antibiotic as described in "Materials and Methods." The initial glucose concentration was 3 mm. At various times, 1-ml samples of suspension were taken up and analyzed for the concentrations of both glucose and lactate. Dactylarin concentrations (μg/ml): O, none; A, 3.12; △, 6.25; O, 12.5; O, 25. Points, averages of the duplicate samples.
rapid and practically full inhibition of glycolysis, as judged from the point of stopping glucose consumption or lactate formation. At lower concentrations (6.25 μg/ml), it causes a progressive inhibition; after 90 min, glycolysis stops completely. At concentrations as low as 3 μg/ml, however, glycolysis is stimulated. The conversion of glucose to lactate in control cells is approximately 76% (calculated for the first 30 min of measuring glycolysis) which is in good agreement with our previous results, where the transformation of glucose into lactate was in the range of 75.0 to 83.5% (55). The inhibition of glycolysis by dactylarin was elucidated by direct investigation of enzyme activities of the glycolytic pathway after dactylarin action. It was shown that dactylarin inactivated thiol enzymes of this pathway, concretely, hexokinase, phosphofructokinase, and glyceraldehyde-3-phosphate dehydrogenase. The inhibition of these enzymes occurs as a result of chemical modification of catalytically active sulfhydryl groups (16). On the other hand, stimulation of glycolysis by low concentrations of dactylarin is similar to the effect of p-bromophenylisothiocyanate. The latter, a well-known thiol-combining agent, is a powerful uncoupler of oxidative phosphorylation (50).

Diamond et al. (14) have also shown that agents such as dinitrophenol and oligomycin that interfere with ATP synthesis, markedly stimulated lactic acid production by intact quiescent 3T3 cells, but the effect of oligomycin occurs at much lower concentrations than that of dinitrophenol. Such a stimulation of glycolysis by dactylarin pointed to its potential interference with respiratory processes in Ehrlich ascites cells. It was also postulated that anticancer chemotherapy based on specific inhibitors of NADH-linked respiration may be worth investigating (20, 25). Our results indicate that a substantial percentage of anticancer agents are active respiratory inhibitors (25, 26).

Effects of anticancer drugs on respiration can, in some cases, provide information relevant to the mechanism of action, mechanism of toxicity, and biochemical side effects of the compounds (26, 27).

The cytostatic action of some of the antileukemic agents can be correlated with their inhibitory effect on glycolysis and respiration. Chlorambucil and the nitrosourea compounds not only impair cellular respiration but also can produce sufficient respiratory inhibition and irreversible damage in individual cells to cause death (23). From this point of view, the effect of dactylarin and dactylarin-like antibiotics on the endogenous respiration of Ehrlich-Lettre ascites cells has been investigated. As shown in Chart 3, dactylarin and dactylarin-like antibiotics stimulated endogenous respiration of the cells according to the concentrations. Maximal stimulation of respiration (over 100%) was reached at concentrations higher than 40 μg/ml.

Stimulation of oxygen consumption by an optimal concentration of iodoacetate, another sulfhydryl reagent and a known inhibitor of glycolysis, to Ehrlich ascites carcinoma in the presence of glucose was shown by Coe (12) for the hypotetraploid cells and by McKee et al. (47) for the hyperdiploid Ehrlich-Lettre cells. Sauerman (68) has shown that iodoacetate and bromoacetate activated glucose oxidation in ascites tumor cells, but in his stimulated system there was an increase in carbon dioxide as well as oxygen consumption.

Such a stimulation is one of the typical properties of uncouplers of oxidative phosphorylation. To verify our assumption, we used the antibiotic oligomycin, a known inhibitor of oxidative phosphorylation. Addition of oligomycin caused inhibition of the endogenous respiration of tumor cells (49, 50). Some compounds are able to release the respiratory inhibition caused by oligomycin, which defines them as acting on the mitochondrial energy transfer pathway closer to the respiratory chain than the site of oligomycin inhibition.

The more detailed polarographic traces in Chart 4 indicate that the addition of 4.6 μg of oligomycin per ml to the respiring tumor cells caused 66 and 68% inhibition of respiration. On the other hand, oligomycin-inhibited respiration was partially reversed by subsequent addition of Substance 66A1 (dactylarin) or dactylarin-like substances 66A2 or 66A3 (77% and 45% release of respiration, in comparison with the initial rate of respiration). The observed reversal of oligomycin inhibition of respiration indicates further evidence for the possible uncoupling activity of dactylarin and its derivatives. Reversal of oligomycin-inhibited respiration by different concentrations of dactylarin (Substance 66A1) and dactylarin-like antibiotics 66A2 or 66A3 is shown in Chart 5. It is noteworthy that maximal release of oligomycin-inhibited respiration was obtained by the same concentrations of dactylarin or dactylarin-like antibiotics that were needed for maximal stimulation of endogenous respiration of tumor cells.

From the above results, it is evident that dactylarin affects the energy-yielding processes in Ehrlich ascites cells. On the basis of this, we also presume the interference of the drug with some mitochondrial functions in isolated mitochondria. When studying the effects of cancerostatics on the energy metabolism of Ehrlich ascites cells, we (25, 48, 51) and also other investigators (26, 27, 35) have used pigeon heart and rat liver mitochondria. A number of anticancer agents of high efficiency and yet considerable toxicity interact with bioenergetics and ion transport of not only the target tumor cells but also those of the host organs, particularly the heart and liver (25). It has been reported that mitochondria isolated from tumors differ in

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* L. Drobnica, manuscript in preparation.
it is not unreasonable to think that their respiration may be selectively inhibited and that such selective inhibition, combined with glycolytic inhibitors, might be useful in cancer chemotherapy (13).

The results, obtained when pigeon heart and rat liver mitochondria were treated in vitro with dactylin and dactylarin-like antibiotics, respectively, indicated that the oxidation rates of succinate measured in the absence of phosphate acceptor (State 4) were the same as those in the control experiments. However, when glutamate plus malate were used as substrates, a low stimulation of oxygen uptake was observed in rat liver mitochondria at concentrations as low as 2.5 μg/ml by all antibiotics investigated. Maximal increase of oxygen uptake from 22 to 35 nanomols of oxygen per mg protein per min was observed in the presence of Substance 66A3 (results not shown). A further increase of dactylarin concentration up to 40 μg/ml does not stimulate respiration any more.

The effects of these antibiotics on oxidative phosphorylation (ADP present, State 3) are summarized in Table 1. Both dactylin (Substance 66A1) and Substance 66A3 at concentrations of 25 μg/ml caused an inhibition of oxidative phosphorylation from 276 to 282 nanomols of oxygen per mg protein per min in the presence of glutamate plus malate as substrates. The inhibition by Substance 66A3 is weaker. A weak inhibition of oxidative phosphorylation occurred also in the presence of Substances 66A2 and 66A3 when succinate was used as substrate. The 490 quinone, a natural sulfhydryl-arylating reagent from the mushroom Agaricus bisporus inhibits also energy production by rat liver mitochondria (72).

Effect of Dactylin on Macromolecular Biosynthesis and Transplantability. Charts 6 and 7 demonstrate the effect of dactylin on 14C precursor incorporation into the TCA-insoluble fraction of Ehrlich ascites cells under the same experimental conditions as have been described for the study of glycolytic inhibition. These results show that dactylarin inhibits incorporation of adenine and thymidine as well as valine proportionally to its concentrations. However, the incorporation of [14C]thymidine is stimulated. We observed a similar effect in the presence of iodooacetate (Chart 8). The complete inhibition of [14C]thymidine incorporation was reached at the highest concentrations of dactylarin. These concentrations almost completely depressed the glycolysis of Ehrlich cells (Chart 2). Our data are in agree-

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<th>Antibiotic</th>
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<th>Control State 3</th>
<th>Antibiotic (25 μg/ml) State 4</th>
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<td>34.4</td>
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<td>89.5</td>
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<td>34.4</td>
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a Succinate, 150 μM ADP.

b Glutamate-malate, 200 μM ADP.
Chart 6. The effect of dactylarin on macromolecular synthesis of Ehrlich ascites cells. Incorporation of radioactive precursors into acid-insoluble fractions was determined by incubating Ehrlich ascites cells with [8-¹⁴C]adenine sulfate (final concentration, 0.312 μCi/ml) and L-[U-¹⁴C]valine (final concentration, 0.275 μCi/ml), in the presence of dactylarin at various concentrations. Dactylarin and precursors were added to the cells at the same time. The test tubes were incubated at 37°, and 1-ml samples of each suspension were analyzed for radioactivity in acid-insoluble material. The results are expressed as cpm/3 x 10⁶ cells. Dactylarin concentrations (μg/ml): ○, none; △, 3.12; Δ, 6.25; ●, 12.5; □, 25. Points, averages of the duplicate samples.

Chart 7. Different inhibitions of thymidine and uracil incorporations into Ehrlich ascites cells. Incorporation of radioactive precursors into acid-insoluble fractions was determined by incubating Ehrlich ascites cells with [2-¹⁴C]thymidine (final concentration, 1 μCi/ml) and [2-¹⁴C]uracil (final concentration, 1 μCi/ml), in the presence of dactylarin at various concentrations. Dactylarin and precursors were added to the cells at the same time. The results are expressed as cpm/3 x 10⁶ cells. Other experimental conditions and symbols are the same as for Chart 6. Points, averages of the duplicate samples.

ment with those of Ferrero et al. (19), who found a close correlation between inhibition of glycolysis and inhibition of incorporation of [¹⁴C]leucine into protein by whole cells of Yoshida ascites hepatoma cells. At lower concentrations, dactylarin does not affect and/or stimulate glycolysis; however, biosynthesis of macromolecules is at the same time significantly depressed. This effect of low concentrations of antibiotic may be attributed to the depression of mitochondrial functions, evidence for which is very difficult, since methods are not yet available to determine the degree to which respiration is cou-
The effect of iodoacetate on the incorporation of thymidine and uracil into acid-insoluble material of Ehrlich ascites cells. Iodoacetate and precursors were added at the same time. The results are expressed as cpm/3 \times 10^6 cells. Other experimental conditions are the same as for Chart 7. Iodoacetate concentrations (\mu g/ml): ○, none; △, 3.75; Δ, 7.5; ●, 15; ○, 30.

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Chart 8. The effect of iodoacetate on the incorporation of thymidine and uracil into acid-insoluble material of Ehrlich ascites cells. Iodoacetate and precursors were added at the same time. The results are expressed as cpm/3 \times 10^6 cells. Other experimental conditions are the same as for Chart 7. Iodoacetate concentrations (\mu g/ml): ○, none; △, 3.75; Δ, 7.5; ●, 15; ○, 30.

We must take into consideration that multitarget inhibitors, especially in the case of thiol reagents, besides affecting the bioenergetic processes, also directly inhibit nucleic acid precursors and reactions of polymerization themselves. A variety of sulfhydryl reagents have been evaluated for possible use as antitumor agents (41). These agents (including iodoacetate) and rifamycins depress incorporation of thymidine into DNA, affect accumulation of label in TTP, and depress incorporation of label to slightly lysine-rich histones in intact and animal cancer cells in vitro (42). In these experiments, the incorporation of uridine into RNA was less sensitive than that of thymidine into DNA. According to these authors, selected sulfhydryl inhibitors appear to show relatively preferential effects against DNA polymerase activity of intact cancer cells (41). It is presumed that such compounds are likely to act at many sites within the cell, but the consistent susceptibility of the \( \alpha \) class mammalian DNA polymerases to these agents (46) points to inhibition of these enzymes as a critical mode of action. There is considerable evidence that DNA polymerase \( \alpha \) plays a more major role in DNA replication than does DNA polymerase \( \beta \). Thus, inhibition of the former is presumed to have greater cytological significance (4). The 490 quinone, a natural sulfhydryl-arylating reagent, markedly inhibits DNA polymerase \( \alpha \) from the murine L1210 leukemia while minimally inhibiting DNA synthesis and obscure specific drug effects. The rate of DNA synthesis is rapidly affected by the lowering of the level of any of the 4 deoxyribonucleotide triphosphates (73, 75). Interference with the generation of high-energy phosphate bonds is one of the mechanisms available for induction of nucleotide deficiency (18, 63, 66). A depletion of nucleotide pools can serve as an efficient tool to inhibit cellular growth and to induce cell death under some circumstances (37).
polymerase $\beta$ from this source. The actions of the 490 quinone parallel other sulfhydryl reagents, p-chloromercuribenzoate and N-ethylmaleimide (29).

Although chemotherapeutic agents have generally been considered to exert their cytotoxic effects by directly interfering with DNA metabolism or by inhibiting enzymatic pathways in purine and pyrimidine nucleotide metabolism, Hill (33) emphasizes that this is an oversimplification. Most agents have multiple effective target sites within the cell, and the primary cytotoxic events responsible for their clinical effectiveness remain to be elucidated.

The question arises as to why dactylarin as well as other thiol-combining agents examined in our laboratory during the last 10 to 15 years do not inhibit the incorporation of labeled uracil into the TCA-insoluble fraction of Ehrlich ascites cells (Charts 7 and 8). This cannot be explained satisfactorily on the basis of the results presented. It is well known that in mammalian cells uracil is preferentially utilized by the reaction involving the uridine phosphorylase-uridine kinase salvage pathway (11). In Ehrlich ascites cells, for example, 95% of the nucleotide incorporated into RNA is formed via the salvage pathway (45). Incorporation characteristics of uracil, uridine, and orotic acid into neoplastic cells have been recently investigated in detail (31, 61, 71). Reports dealing with uridine uptake and phosphorylation (61–64) suggest that the pattern of uridine incorporation into RNA is dependent on the rate of uridine entry into the cell and is not simply due to changes in the rate of uridine phosphorylation. As has already been mentioned, Walters and Ratliff (73) suggest that reduction of any of the 4 deoxyribonucleotide triphosphate pools below a certain critical level will inhibit DNA synthesis more rapidly than RNA synthesis, probably because the cells become depleted of dATP more rapidly than of ATP. This can be expected, since the concentrations of deoxyribonucleotides are 2 to 3 orders of magnitude lower than those of ribonucleotides (28). Furthermore, ATP could, to some extent, be replaced by GTP, dGTP, dCTP, TTP, and dATP as phosphate donors (45). To obtain significant inhibition of [14C]uracil incorporation into Ehrlich ascites cells, concentrations of dactylarin and/or iodoacetate, as high as 50 and 100 $\mu$g/ml, respectively, had to be used. Dactylarin even at a concentration of 50 $\mu$g/ml was a more effective inhibitor than was iodoacetate at a concentration of 100 $\mu$g/ml (results not shown). Inhibition effects of both dactylarin and iodoacetate on 14C precursor incorporation could not be abolished by the washing of pretreated Ehrlich ascites cells in Krebs-Ringer phosphate medium. To fully explain the effects of sulfhydryl reagents on the incorporation of uracil into Ehrlich ascites cells, further experiments are planned in cooperation with Dr. J. F. Henderson, University of Alberta.

Finally, Table 2 summarizes the results concerning the effect of dactylarin on both biosynthetic processes and transplantability of Ehrlich ascites cells. The antibiotic at concentrations of 12.5 $\mu$g/ml and above causes serious metabolic disorders, manifested by the inhibition of glycolysis and even by the inhibition of biosynthetic processes. As a consequence, there is a loss of transplantability of these cells. The decrease in this parameter was probably due to a loss of viability of the cells during the pretreatment. This possibility is supported by the failure of cell washing to reverse the inhibition of macromolecular synthesis. The lower concentrations do not prevent tumor growth but significantly retarded it, thus prolonging the survival time of mice. The inhibition of tumor cell proliferation may have also resulted from an interference of dactylarin with the free SH-SS interchange, essential for malignant growth (2). It was found that the proliferation of murine tumor cells, for example, depends upon a free interchange between thiols and disulfides. Suspended in Hanks’ medium and incubated for 1 hr with 1 mM iodoacetate or N-ethylmaleimide, such cells are still viable but are no longer transplantable (2).

Ehrlich cells from 7-day-old ascites tumors are essentially starved since they have been growing under conditions of limited availability of oxygen, glucose, and other nutrients (6). It was noted previously that repeated washing of the cells by centrifugation, even at minimal force and time to sediment the cells, tends to damage some cells and thus causes the loss of their nucleotides (38, 61) as well as other nutrients (6). Furthermore, our results proved that the inhibitory effect of dactylarin was not abolished after the cells were washed in Krebs-Ringer medium. Therefore, unwashed cells were used for all transplantation experiments after dactylarin treatment.

A number of anticancer drugs of current clinical and research interest contain quinone or hydroquinone systems (for review, see Ref. 65). The recently characterized antibotic dactylarin can be included in this group. The biochemical elucidation of cytotoxic (34) and cancerostatic effects (39) of this compound evidently indicated its interference with the energy metabolism of the cell, probably due to the modification of the thiol group of cell components (16). Inhibitory effects of quinones on electron transport, oxidative phosphorylation, respiration, and glycolysis are well known (32, 44, 74). The data on the anthracycline quinones, particularly on Adriamycin, also indicate that their antitumor activities could be due, at least in part and in addition to the interaction with DNA, to the inhibition of ubiquinone enzymes in electron transfer processes of cell respiration.

Table 2

<table>
<thead>
<tr>
<th>Inhibition of incorporation (%)</th>
<th>Range of survival times (days)</th>
<th>Survival of mice (days)</th>
<th>$p$ (Student's t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>Valine</td>
<td>Thymidine</td>
<td>Uracil</td>
</tr>
<tr>
<td>------</td>
<td>------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.12</td>
<td>5.9</td>
<td>19.8</td>
<td>39.35</td>
</tr>
<tr>
<td>6.25</td>
<td>13.5</td>
<td>40.3</td>
<td>55.0</td>
</tr>
<tr>
<td>12.50</td>
<td>40.0</td>
<td>65.5</td>
<td>77.9</td>
</tr>
<tr>
<td>25.00</td>
<td>50.6</td>
<td>72.4</td>
<td>85.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of mice developing tumors/number inoculated.

<sup>b</sup> Mean ± S.D.

<sup>c</sup> +, stimulation over 100% against the control.
Dactylarin, as a quinone, has multiple sites of action similar to those of the other quinone-containing anticancer agents (29, 32, 35, 44). Recently, Bachur et al. (3) have investigated the mechanism of action of quinone-containing anticancer agents in more detail and propose that the intracellular activation of these drugs (interaction with mammalian microsomes) to a free radical state may be primary to their cytotoxic activity. As free radicals, these drugs have the potential to be "site-specific free radicals" following their activation intracellularly. This mechanism could be a possibility for dactylarin and in fact for any quinone.

Our finding concerning the inhibition of glycolysis by dactylarin is in contrast to the report of Horakova et al. (34). According to the above-mentioned authors, inhibition of glycolysis by dactylarin is not a prerequisite for growth inhibition of HeLa cells. At higher concentrations of dactylarin, however, full inhibition of glycolysis was observed. This is in agreement with our results illustrated in Chart 2. In the aforementioned paper, the effect of dactylarin was measured only in 2 concentrations of antibiotic, which were much lower than ours (1.56 and 0.78 μg/ml). We were able to show that the effect of dactylarin on glycolysis is concentration dependent.

In conclusion, it is very difficult to assign a definite causal relationship between observed biochemical aberrations caused by a drug and physiological responses of neoplastic tissue to the antibiotic. Although in the case of many antineoplastic agents attention has been focused upon their effects on DNA, RNA, and protein synthesis, the work by Farber (18) and others indicates that the inability to synthesize ATP in a cell leads to multiple secondary derangements in cellular metabolism. Our data suggest that the cytotoxic and cancerostatic action of dactylarin and its derivatives lies primarily in the exclusion of key processes in the energy metabolism of Ehrlich ascites cells.

Experiments on in vivo antitumor activity are now in progress, and a paper will be published elsewhere.

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REFERENCES


Inhibition of Energy Metabolism in Ehrlich Ascites Cells Treated with Dactylin in Vitro

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