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Antitumor Agents XLI: Effects of Eupaformosanin on Nucleic Acid, Protein, and Anaerobic and Aerobic Glycolytic Metabolism of Ehrlich Ascites Cells

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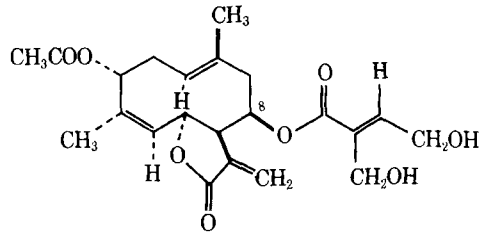
Abstract □ The major effect of eupaformosanin as an antineoplastic agent on Ehrlich ascites cell metabolism was to inhibit deoxyribonucleic acid synthesis, specifically at deoxyribonucleic acid polymerase and thymidylate synthetase enzymatic sites. Both pyrimidine and purine systems of Ehrlich ascites were marginally inhibited. Ribonucleic acid synthesis and messenger and ribosomal polymerase activities also were suppressed. Cyclic adenosine monophosphate levels were increased significantly, which correlated with the drastic reduction of histone phosphorylation. Eupaformosanin also suppressed a number of glycolytic and Krebs cycle enzymes as well as oxidative phosphorylation *in vitro*. All of the inhibited enzymes are known thiol-bearing enzymes that can undergo a Michael-type addition with the α-methylene-γ-lactone moiety of eupaformosanin, as shown with other sesquiterpene lactones.

Keyphrases □ Antitumor agents—eupaformosanin, effect on nucleic acid, protein, and anaerobic and aerobic glycolytic metabolism, Ehrlich ascites cells □ Eupaformosanin—antitumor activity, effect on nucleic acid, protein, and anaerobic and aerobic glycolytic metabolism, Ehrlich ascites cells □ Deoxyribonucleic acid synthesis, inhibitors—eupaformosanin, effect on nucleic acid, protein, and anaerobic and aerobic glycolytic metabolism, Ehrlich ascites cells

A new germacranolide, eupaformosanin (I), has been isolated from the whole plant of *Eupatorium formosanum*, and its stereochemistry and physical characteristics were reported previously (1). As an antineoplastic agent, eupaformosanin is a potent inhibitor of Walker 256 carcinosarcoma cell proliferation at 2.5 mg/kg/day (T/C = 471) and of P-388 lymphocytic leukemia cell proliferation at 25 mg/kg/day (T/C = 147) (2). The effects of eupaformosanin on nucleic acid, chromatin protein, purine, pyrimidine, and protein metabolism, anaerobic and aerobic glycolysis, and oxidative phosphorylation of Ehrlich ascites tumor cells are reported here.

EXPERIMENTAL

Male CF_1 mice, ~30 g, were implanted intraperitoneally on Day 0 with 10^6 Ehrlich ascites tumor cells from donor mice housed in these laboratories. Eupaformosanin suspended by homogenization in 0.05% polysorbate 80–water was administered intraperitoneally at 25 mg/kg/day for 8 days to determine the inhibition of tumor growth (3). For the metabolic studies, mice were treated on Days 7–9 with a subacute dose of 0.25 mg ip. Animals were sacrificed on Day 10, and the ascites fluid was collected from the peritoneal cavity. *In vitro* metabolic studies were determined on untreated, harvested, Day 10 Ehrlich ascites cells. The number of tumor cells per milliliter and the 0.4% trypan blue uptake were determined with a hemocytometer (4).



I

Incorporation of thymidine into deoxyribonucleic acid was determined by the method of Chae *et al.* (5). One hour prior to the animal sacrifice, 10 μCi of [^{14}C-*methyl*]-thymidine[1] (53.2 mCi/mmole) was injected intraperitoneally. The deoxyribonucleic acid was isolated, and the carbon-14 content was determined in two parts of toluene, one part of octoxynol, 0.4% 2,5-diphenyloxazole, and 0.01% 1,4-bis[2-(5-oxazolyl)]-benzene scintillation fluid and corrected for quenching. The deoxyribonucleic acid concentration was determined by UV spectrophotometry at 260 nm with calf thymus deoxyribonucleic acid as a standard.

Uridine incorporation into ribonucleic acid was determined in an analogous manner utilizing 10 μCi of 5-^{3}H-uridine (20.0 Ci/mmole). Ribonucleic acid was extracted by the method of Wilson *et al.* (6). Leucine incorporation into protein was determined by the method of Sartorelli (7) using 8 μCi of 1-^{14}C-leucine (54.4 mCi/mmole). The effect of eupaformosanin on 1-^{14}C-acetic acid (57.8 mCi/mmole) incorporation into cholesterol of Ehrlich ascites cells also was measured (8, 9).

Nuclear deoxyribonucleic acid polymerase activity was determined on isolated nuclei (10). The incubation medium was that of Sawada *et al.* (11), except that [^{3}H-*methyl*]-deoxyribothymidine-5′-triphosphate (53.1 mCi/mmole) was used and the insoluble nucleic acids were collected on glass fiber paper (GF/F) by vacuum suction. Deoxythymidine and deoxythymidylate monophosphate and diphosphate kinase activities were determined by the method of Maley and Ochoa (12), which is based on the disappearance of 0.1 μmole of reduced nicotinamide adenine dinucleotide at 340 nm.

Thymidine synthetase activity was assayed by the method of Kampf *et al.* (13) utilizing a postmitochondrial supernate (9000×*g* for 10 min) and 5 μCi of 5-^{3}H-deoxyuridine monophosphate (11 Ci/mmole). Mes-

[1] New England Nuclear was the source of all radioisotopes used in this study. Biochemical reagents were purchased from Sigma Chemical Co. and Calbiochem Co.

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senger, ribosomal, and hormone-sensitive ribonucleic acid polymerase activity were determined by the methods of Anderson *et al.* (14) and Hall *et al.* (15). Variation of ammonium sulfate concentrations was used to measure the respective type of ribonucleic acid polymerase. Insoluble ribonucleic acid was collected on nitrocellulose fiber paper by vacuum suction.

Phosphorylation of histones was determined by injecting intraperitoneally 10 μCi of γ-^{32}P-adenosine triphosphate (30.0 Ci/mmole) 1 hr prior to sacrifice. The nuclei were isolated (10), and the histone chromatin protein was extracted by the method of Raineri *et al.* (16). Nonhistone chromatin phosphorylation by nuclear protein kinase (17) was assayed utilizing 2 nmoles of γ-^{32}P-adenosine triphosphate (30.0 Ci/mmole). Chromatin protein was collected on nitrocellulose filter membranes. The effect of eupaformosanin on hydrolytic enzymes also was determined. Deoxyribonuclease activity was measured at pH 5.0 by a modification of the de Duve method (18). Ribonuclease activity and acid cathepsin activity were determined at pH 5.0 by the method of Cho-Chung and Gullino (19).

^{14}C-Formate incorporation into purine was measured by the method of Spassova *et al.* (20) using 0.5 μCi of ^{14}C-formic acid (4.95 mCi/mmole). Separation of purines was carried out on silica gel TLC plates eluted with *n*-butanol–acetic acid–water (4:1:5). The plates were scraped, and the radioactive content was determined. Phosphoribosyl–pyrophosphate amino transferase activity was determined by the method of Wyngaarden and Ashton (21), which is based on the reduction of 0.6 μmole of nicotinamide adenine dinucleotide at 340 nm. Carbamyl phosphate synthetase acitivity was determined with ornithine and ornithine transcarbamylase present by the method of Kalman *et al.* (22).

The colorimetric determination of citrulline was performed according to the method of Archibald (23). Aspartate carbamyl transferase activity was assessed by the method of Kalman *et al.* (22) in the presence of aspartate transcarbamylase. The colorimetric determination of carbamyl aspartate was carried out according to the method of Koritz and Cohen (24). Dihydrofolate reductase activity was determined by the procedure of Ho *et al.* (25), which is based on the disappearance of 0.1 μmole of reduced nicotinamide adenine dinucleotide phosphate at 340 nm. *S*-Adenosyl methionine transferase activity was determined by the method of Borchardt *et al.* (26) utilizing 0.05 μCi of [^{14}C-*methyl*]-*S*-adenosyl-L-methionine (53 mCi/mmole). Ribonucleotide reductase activity was determined by the method of Moore and Hurlbert (27) using 5-^{3}H-cytidine-5-diphosphate (26.1 Ci/mmole).

Cyclic-3′,5′-adenosine monophosphate levels were determined by the method of Gilman (28) using a ^{3}H(G)-adenosine-3′,5′-cyclic phosphate (39.8 Ci/mmole) radioimmunoassay.

UV binding studies (4) were conducted *in vitro* with eupaformosanin (85 μg/ml) with deoxyribonucleic acid or deoxyguanosine monophosphate (38 μg/ml) in 0.1 *M* phosphate buffer, pH 7.2, over a range of 200–340 nm for 24 hr at 22°.

The following *in vitro* glycolytic and Krebs cycle enzymatic activities were determined both for controls and in the presence of 1 μmole of eupaformosanin: hexokinase (29), phosphofructokinase (30), lactic dehydrogenase (31), glucose-6-phosphate dehydrogenase (32), glyceraldehyde-3-phosphate dehydrogenase (33), glucose-6-phosphatase (34), fructose-1,6-diphosphatase (35), malic dehydrogenase (36), succinic dehydrogenase (37), and adenosine phosphatase (38). *In vitro* effects of eupaformosanin on Ehrlich ascites tumor cell oxidative phosphorylation were measured in the presence and absence of adenosine diphosphate utilizing succinate, a flavin adenine dinucleotide-linked dehydrogenase, and α-ketoglutarate, a nicotinamide adenine dinucleotide-linked dehydrogenase (39). The inorganic phosphate was determined by the method of Chen *et al.* (40). The protein content for all assays was determined by the method of Lowry *et al.* (41).

Probable (*p*) significant differences were determined by the Student *t* test. Data are expressed in Tables I and II as percent of the control with standard deviations; *n* equals the number of animals per group.

RESULTS

Daily administration of eupaformosanin resulted in 97% inhibition of Ehrlich ascites tumor growth. The normal number of Ehrlich ascites cells per milliliter on Day 10 was 223 × 10^{6}. Eupaformosanin treatment at a subacute dose of 0.25 mg (12.5 mg/kg) on Days 7–9 reduced the cell count by 82%. Drug treatment resulted in no significant difference in the percentage of nonviable cells or in the size or general morphology of the cell as observed by light microscopy.

For the control animals, the thymidine incorporation into deoxyribonucleic acid was 241,675 dpm/mg of deoxyribonucleic acid. Drug treatment inhibited deoxyribonucleic acid synthesis by 76% (Table I). Uridine incorporation into ribonucleic acid for Day 10 Ehrlich ascites cells was 92,489 dpm/mg of ribonucleic acid. Eupaformosanin treatment reduced ribonucleic acid synthesis by an average of 23%. Leucine incorporation into protein for the control was 2484 dpm/mg of protein, which was not affected by drug treatment. Acetic acid incorporation into cholesterol for membrane synthesis for the control was 273,271 dpm/mg of protein. Drug treatment reduced cholesterol synthesis by 59%.

Table I—*In Vivo* Effects of Eupaformosanin on Nucleic Acid and Protein Metabolism of Ehrlich Ascites Tumor Cells (n = 6)

Parameter	Percent Control: Control (0.05% Polysorbate 80), $\bar{x} \pm SD$	Percent Control: Treated on Days 7–9 with 0.25 mg of Eupaformosanin, $\bar{x} \pm SD$
^{14}C-Thymidine incorporation into deoxyribonucleic acid	100 ± 22	24 ± 8[a]
^{3}H-Uridine incorporation into ribonucleic acid	100 ± 15	77 ± 17[b]
^{14}C-Leucine incorporation into protein	100 ± 19	102 ± 24
^{14}C-Acetic acid incorporation into cholesterol	100 ± 23	41 ± 13[a]
Number of tumor cells per milliliter of fluid	100 ± 14	18 ± 3[a]
Thymidine kinase	100 ± 2	102 ± 5
Thymidylate monophosphate kinase	100 ± 3	88 ± 4[a]
Thymidylate diphosphate kinase	100 ± 9	103 ± 7
Deoxyribonucleic acid polymerase	100 ± 8	58 ± 10[a]
Messenger ribonucleic acid polymerase	100 ± 15	69 ± 6[c]
Ribosomal ribonucleic acid polymerase	100 ± 10	73 ± 10[c]
Hormone-sensitive ribonucleic acid polymerase	100 ± 6	283 ± 7[a]
Thymidylate synthetase	100 ± 7	69 ± 4[a]
^{14}C-Formate incorporation into purines	100 ± 9	66 ± 8[a]
Phosphoribosyl–pyrophosphate amino transferase	100 ± 3	87 ± 2[a]
Carbamyl phosphate synthetase	100 ± 10	73 ± 9[c]
Aspartate carbamyl transferase	100 ± 11	77 ± 13[b]
Dihydrofolate reductase	100 ± 13	106 ± 7
S-Adenosyl-L-methionine transferase	100 ± 4	91 ± 3[c]
Ribonucleotide reductase	100 ± 5	89 ± 3[c]
Phosphorylation of histone proteins	100 ± 14	40 ± 6[a]
Phosphorylation of nonhistone proteins	100 ± 10	90 ± 9
Cyclic-3′,5′-adenosine monophosphate levels	100 ± 11	252 ± 49[a]
Deoxyribonuclease	100 ± 7	73 ± 7[a]
Ribonuclease activity	100 ± 9	97 ± 8
Cathepsin activity	100 ± 9	95 ± 10

[a] p = 0.001. [b] p = 0.025. [c] p = 0.005.

In vitro studies of the binding of eupaformosanin with deoxyribonucleic acid and deoxyguanosine monophosphate demonstrated that eupaformosanin possessed a maximum absorption peak at 230 nm. Combination with nucleic acids increased the absorption at 230 nm, indicating nonspecific binding. Untreated Day 10 Ehrlich ascites cells demonstrated a change in absorbance for thymidine kinase activity of 1.328 optical density units/hr/mg of protein. Thymidylate monophosphate kinase activity was 1.361 optical density units/hr/mg of protein, and thymidylate diphosphate kinase activity was 2.100 optical density units/hr/mg of protein. Eupaformosanin treatment had little effect on the kinase activities, with only a 12% reduction of thymidylate monophosphate kinase activity. Nuclear deoxyribonucleic acid polymerase activity, *i.e.*, alpha, beta, and gamma, for the control was 45,734 dpm/mg of nucleoprotein, which was reduced 42% by drug treatment.

^{14}C-Formate incorporation into purines for Day 10 Ehrlich ascites cells was 17,088 dpm/mg of protein, which was reduced 34% by drug therapy. Phosphoribosyl–pyrophosphate amino transferase activity for the control resulted in an absorption increase at 340 nm of 0.376 optical density unit/min/mg of protein based on 0.75 μmole of nicotinamide adenine dinucleotide, which was significantly reduced 13% by eupaformosanin. Dihydrofolate reductase activity for the control was 0.514 optical density unit/hr/mg of protein, which was unaffected by eupaformosanin treatment. *S*-Adenosyl-L-methionine transferase activity for the Day 10 Ehrlich ascites cells was 3268 dpm/mg of protein, which was suppressed 9% by drug treatment. Carbamyl phosphate synthetase activity for the control was 0.128 mg of carbamyl phosphate formed/hr/mg of protein,

Table II—*In Vitro* Effects of Eupaformosanin on Anaerobic and Aerobic Metabolism of Ehrlich Ascites Cells ($n = 6$)

Parameter	Percent Control: Control (0.05% Polysorbate 80), $\bar{x} \pm SD$	Percent Control: Treated on Days 7–9 with Eupaformosanin, $\bar{x} \pm SD$
Hexokinase activity	100 ± 4	85 ± 4[a]
Phosphofructokinase activity	100 ± 5	25 ± 9[a]
Lactic dehydrogenase activity	100 ± 9	43 ± 4[a]
Glucose-6-phosphate dehydrogenase activity	100 ± 16	3 ± 2[a]
Glyceraldehyde-3-phosphate dehydrogenase activity	100 ± 19	35 ± 4[a]
Glucose-6-phosphatase activity	100 ± 5	72 ± 12[a]
Fructose-1,6-diphosphatase activity	100 ± 9	59 ± 3[a]
Malic dehydrogenase activity	100 ± 4	26 ± 10[a]
Succinic dehydrogenase activity	100 ± 7	11 ± 6[a]
Adenosine triphosphatase activity	100 ± 16	96 ± 8
Oxidative phosphorylation processes		
Succinate: State 4	100 ± 3	69 ± 12[a]
Succinate: State 3	100 ± 4	69 ± 9[a]
α-Ketoglutarate: State 4	100 ± 5	52 ± 14[a]
α-Ketoglutarate: State 3	100 ± 11	53 ± 8[a]

[a] $p = 0.001$. [b] $p = 0.005$. [c] $p = 0.025$.

which was inhibited 27% by eupaformosanin treatment. Aspartate carbamyl transferase activity for the Day 10 Ehrlich ascites cells was 6.245 mg of carbamyl aspartate formed/hr/mg of protein, which was suppressed 23% by eupaformosanin.

Thymidylate synthetase activity for the control was 103,328 dpm/mg of protein, which was reduced 31% by drug treatment. Ribonucleotide reductase activity for the control was 155,065 dpm/mg of protein, which eupaformosanin reduced by 11%. Messenger ribonucleic acid polymerase activity for the control was 6434 dpm/mg of protein, ribosomal ribonucleic acid polymerase activity was 7340 dpm/mg of protein, and hormone-sensitive polymerase activity was 8254 dpm/mg of protein. Eupaformosanin reduced messenger activity by 31% and ribosomal activity by 27% and elevated hormone-sensitive polymerase activity by 183%. The cyclic adenosine monophosphate level for the control was 3.65 pmoles/10^6 cells, whereas the level rose by 152% after drug treatment. Histone phosphorylation of chromatin protein was at a rate of 2954 dpm/mg of chromatin protein, which was reduced 60% by eupaformosanin treatment. Nonhistone phosphorylation of chromatin protein was at a rate of 9803 dpm/mg of chromatin protein, which was inhibited 10% by drug treatment.

The catabolic rate of nucleic acids and proteins also was determined to eliminate the possibility that the drug accelerated hydrolytic enzymatic activity and thus indirectly lowered the incorporation of precursors into nucleic acids and proteins. Deoxyribonuclease activity for Day 10 Ehrlich ascites cells was 247 μg of deoxyribonucleic acid hydrolyzed/hr/mg of protein, which was inhibited 27% by eupaformosanin treatment. Ribonuclease activity for the control tumor cells was 43 μg of ribonucleic acid hydrolyzed/hr/mg of protein, which was unaffected by eupaformosanin. Cathepsin activity for the control was 869 μg of protein hydrolyzed/hr/mg of protein, which was unchanged by drug therapy.

Hexokinase activity for the control Ehrlich ascites tumor cells was calculated as a change in absorbance equal to 0.195 optical density unit/min/mg of protein based on 500 μmoles of nicotinamide adenine dinucleotide phosphate, which was suppressed 15% by 1 μmole of eupaformosanin *in vitro* (Table II). Phosphofructokinase activity for the control was a change of 0.176 optical density unit/min/mg of protein based on 1.125 μmoles of reduced nicotinamide adenine dinucleotide, which was reduced 75% by eupaformosanin presence. Lactic dehydrogenase activity resulted in an optical density unit change of 0.623/min/mg of protein based on 0.12 μmole of reduced nicotinamide adenine dinucleotide, which eupaformosanin reduced by 57%. Glyceraldehyde dehydrogenase activity for the control was 2.623 optical density units/min/mg of protein based on 20.3 μmoles of nicotinamide adenine dinucleotide, which was suppressed 65% by the drug.

Glucose-6-phosphate dehydrogenase activity for the control was 0.284 optical density unit/min/mg of protein based on the presence of 0.0056 μmole of nicotinamide adenine dinucleotide phosphate. Eupaformosanin inhibited this dehydrogenase activity by 97%. Glucose-6-phosphatase activity for the control was 20.15 μg of phosphorus released/hr/mg of protein, which was suppressed 28% by the drug treatment. Fructose-1,6-diphosphatase activity for the control was 24.87 μg of phosphorus released/hr/mg of protein, which was reduced 41% by eupaformosanin. Malic dehydrogenase activity for Day 10 Ehrlich ascites cells was 0.80 optical density unit/min/mg of protein, which was reduced 74% by the drug. Succinic dehydrogenase activity for the control was 0.069 optical density unit/min/mg of protein based on 15 μmoles of potassium ferricyanide, which eupaformosanin suppressed by 89%. Adenosine triphosphatase activity measured as inorganic phosphate released for the control was 17.02 μg of phosphorus hydrolyzed/hr/mg of protein. The presence of eupaformosanin resulted in no change in enzymatic activity.

The basal respiration (state 4) of Day 10 Ehrlich ascites tumor cells with succinate as the substrate was 5.273 μl of oxygen consumed/hr/mg of protein. In the presence of adenosine diphosphate (state 3), stimulated respiration on coupled oxidative phosphorylation was 8.725 μl of oxygen consumed/hr/mg of protein. Eupaformosanin inhibited state 4 and state 3 respiration by 31%. Use of α-ketoglutarate as a substrate resulted in a state 4 respiration of 3.569 μl of oxygen consumed/hr/mg of protein and in a state 3 respiration of 5.156 μl of oxygen consumed/hr/mg of protein. Eupaformosanin inhibited state 4 respiration by 48% and state 3 respiration by 47%.

DISCUSSION

Eupaformosanin administration significantly lowered deoxyribonucleic acid and cholesterol synthesis, whereas ribonucleic acid synthesis was only moderately inhibited in Day 10 Ehrlich ascites cells. One possible explanation of the reduction of radiolabeled precursor into these macromolecules is that drug treatment accelerated catabolism of macromolecules. However, treatment with eupaformosanin did not accelerate hydrolytic enzymatic activity responsible for the catabolism of these macromolecules; rather, the drug moderately reduced deoxyribonuclease activity and had no effect on ribonucleic and cathepsin activities at pH 5.0.

The major site of eupaformosanin inhibition of deoxyribonucleic acid synthesis was on the deoxyribonucleic acid polymerase nuclei enzymes, whereas thymidylate synthetase activity appeared to be reduced moderately. Purine synthesis also was inhibited, but the major site did not appear to be at the regulatory enzyme of the cycle, *i.e.*, phosphoribosylpyrophosphate amino transferase, which was only slightly inhibited. Pyrimidine synthesis was moderately inhibited at the carbamyl phosphate synthetase and aspartate carbamyl transfer steps. *S*-Adenosyl-L-methionine transferase and ribonucleotide reductase activity also were slightly inhibited.

It was demonstrated previously that compounds containing the O=CC=CH_2 system can undergo a rapid Michael-type addition with biological nucleophiles, *e.g.*, sulfhydryl groups (4). Both α- and γ-deoxyribonucleic acid polymerase of the nucleus (42), thymidylate synthetase (43), aspartate carbamyl transferase (44), and ribonucleotide reductase (44) possess exposed sulfhydryl groups which can be alkylated. Contained in the structure of eupaformosanin is the O=CC=CH_2 system both as an α-methylene-γ-lactone and within the C-8 ester side chain. The α-methylene-γ-lactone moiety undergoes a rapid Michael-type addition with L-cysteine and reduced glutathione (4, 45). A similar phenomenon occurs with the sesquiterpene lactones helenalin and tenulin (4, 45) and the germacranolide eupahyssopin (46).

Cellular proliferation is regulated by phosphorylation of chromatin histones, *e.g.*, the F-1 fraction by protein kinase (47). This effect occurs predominantly during G_1 and early S of the cell cycle, when cyclic adenosine monophosphate levels are decreasing and enzymes are synthesized for nucleic acid replication. Eupaformosanin treatment significantly inhibited phosphorylation of histone chromatin protein and increased the intracellular level of cyclic adenosine monophosphate levels in Day 10 Ehrlich ascites cells. The latter effect is associated with reversal of tumor morphology (48), reduced cellular proliferation, and enhanced cellular differentiation (49, 50).

The UV spectral studies suggest that eupaformosanin interacts with deoxyribonucleic acid. The fact that messenger ribonucleotide polymerase activity also was suppressed by drug treatment may indicate that the transcription processes utilizing deoxyribonucleic acid as a template were impaired. The inhibition of purine and pyrimidine nucleotide synthesis is not the reason why messenger and ribosomal ribonucleic acid polymerase activities are inhibited since triphosphate ribonucleotides were added to the medium. Hormone-sensitive ribonucleotide polymerase may be elevated due to the elevated level of cyclic adenosine monophosphate, a modulator of this polymerase (15).

Compounds containing the α-methylene-γ-lactone moiety inhibit

enzymes of the Embden–Meyerhof and Krebs cycles (35, 51–53). Eupaformosanin inhibited phosphofructokinase and lactic acid dehydrogenase significantly and hexokinase marginally *in vitro*. These enzymes contain sulfhydryl groups (35, 51–53) that can be alkylated by the O=CC=CH_2 moiety. Enzymes involved in reversed glycolysis, *e.g.*, glucose-6-phosphatase and fructose-1,6-diphosphatase, were inhibited significantly by eupaformosanin, as was observed previously with other germacranolides (46) and pseudoguaianolides (35). The regulatory enzyme of the hexose monophosphate shunt, glucose-6-phosphate dehydrogenase, was reduced drastically by eupaformosanin. Succinic and malic dehydrogenase activities were suppressed significantly *in vitro*. Dehydrogenases of the Krebs cycle contain sulfhydryl groups in the enzymes and iron inorganic sulfide flavin proteins as integral components of the dehydrogenase enzymes (54). Theoretically, this group also can be alkylated.

Eupaformosanin suppressed basal respiration and coupled oxidative phosphorylation or mitochondrial adenosine triphosphate synthesis *in vitro*, and this suppression was not due to an accelerated adenosine triphosphatase activity brought about by the presence of the drug. The drug inhibited respiration with α-ketoglutarate, a nicotinamide adenine dinucleotide-linked dehydrogenase that contains an iron–sulfur center (55) more than with succinate, which is a flavin adenine dinucleotide-linked enzyme. Numerous antitumor agents inhibit respiration and oxidative phosphorylation of Ehrlich ascites cells (56) including other sesquiterpene lactones (2).

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