carried out. As shown in Table II, calcium salts of (+), (±)-isocitric acid and (±)-alloisocitric acid caused a positive inotropic effect on isolated guinea pig atria to the same degree as compound I at a concentration of 10^{-4} g/ml, while no toxic effect such as arrhythmia was observed.

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ACKNOWLEDGMENTS

The experiment was supported in part by a research grant from Banyu Pharmaceutical Co., Ltd.

The authors thank Mr. Y. Tanaka, Mr. I. Maetani, and Miss Y. Soeda for measurements of PMR, CMR, and mass spectra.

Antitumor Agents LIII: The Effects of Daphnoretin on Nucleic Acid and Protein Synthesis of Ehrlich Ascites Tumor Cells

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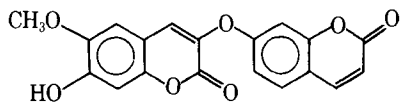
Received July 2, 1981, from the *Division of Medicinal Chemistry, School of Pharmacy, The University of North Carolina, Chapel Hill, NC 27514.* Accepted for publication October 7, 1981.

Abstract □ Daphnoretin, a dicoumaryl ether, has been shown to inhibit growth of Ehrlich ascites carcinoma cells. Dosing for three days at 6 mg/kg/day reduced the cell number per milliliter by 60%. *In vitro* DNA and protein synthesis studies demonstrated an $ID_{50} \cong 0.194$ m*M* and $ID_{50} \cong 0.340$ m*M*, respectively, for daphnoretin. Subsequently it has been shown that *in vivo* nucleic acid and protein synthesis were inhibited. Major sites in the DNA synthetic pathway inhibited significantly by daphnoretin were dihydrofolate reductase, orotidine monophosphate decarboxylase, thymidylate monophosphate kinase, and ribonucleotide reductase. Reduction of *in vitro* oxidative phosphorylation processes and acid hydrolytic enzymes were also inhibited in the presence of daphnoretin.

Keyphrases □ Daphnoretin—effects on nucleic acid and protein synthesis of Ehrlich ascites tumor cells, antitumor agent □ DNA—effects of daphnoretin, protein synthesis, Ehrlich ascites tumor cells □ Protein synthesis—effects of daphnoretin, DNA, Ehrlich ascites tumor cells □ Antitumor agents—the effects of daphnoretin on nucleic acid and protein synthesis of Ehrlich ascites tumor cells

Daphnoretin has been isolated from the whole plant of *Wikstroemia indica* C. A. Mey (Thymelaeaceae) (1), which is known as "Nan-Ling-Jao-Hua" or "Po-Lun" in Chinese folklore as a herbal remedy for the treatment of human syphilis, arthritis, whooping cough (2), and cancer (3). Daphnoretin was shown to have significant inhibitory activity *in vivo* against the Ehrlich ascites carcinoma growth in mice but did not demonstrate any activity against P-388 lymphocytic leukemia growth (4). A detailed examination of the effects of daphnoretin on nucleic acid and protein synthesis of Ehrlich ascites carcinoma cells is presented.



Daphnoretin

EXPERIMENTAL

CF_1 male mice (~30 g) were implanted with 2×10^6 Ehrlich ascites tumor cells intraperitoneally on day 0. Daphnoretin was suspended by homogenization in 0.05% polysorbate 80–water and 3–12 mg/kg ip was administered for 9 days to determine the inhibition of tumor growth. Mice were sacrificed on day 10, and the ascites fluid was collected from the peritoneal cavity. The volume and ascrit were determined for each animal and the inhibition of tumor growth was calculated (5). For the metabolic studies, mice were treated on days 7–9 with 6 mg/kg ip of daphnoretin. The animal was sacrificed on day 10 and the ascites fluid was harvested. The *in vitro* metabolic studies were performed at 0.340 m*M*.

In vitro incorporation of [^{3}H]thymidine, [^{3}H]uridine, or [^{3}H]leucine was determined using 10^6 Ehrlich ascites cells, 1 μCi labeled precursor, minimum essential medium, and varying final concentrations of drug from 0.035 to 0.35 m*M*. The tubes were incubated at 37° for 60 min and inactivated by trichloroacetic acid. The acid insoluble-labeled DNA was collected on glass filter discs[1], and RNA and protein were precipitated on nitrocellulose filters by vacuum suction (6). Results are expressed as disintegrations per minute of incorporated precursor per hour per 10^6 ascites cells.

For *in vivo* studies, incorporation of thymidine into DNA was determined by the method of Chae *et al.* (7). One hour prior to the animal sacrifice on day 10, 10 μCi of [6-^{3}H]thymidine (21.5 Ci/mmole, ip) were injected. The DNA was isolated and the tritium content was determined in a toluene based scintillation fluid[2]. The DNA concentration was determined by the diphenylamine reaction using calf thymus DNA as a standard. Uridine incorporation into RNA was determined using 10 μCi of [5,6-^{3}H]uridine (22.4 Ci/mmole). RNA was extracted by the method of Wilson *et al.* (8). Using yeast RNA as a standard, the RNA content was assayed by the orcinol reaction. Leucine incorporation into protein was determined by the method of Sartorelli (9) using 10 μCi of [4,5-^{3}H]leucine (52.2 Ci/mmole). Protein content was determined by the Lowry procedure using bovine serum albumin as a standard.

In vitro and *in vivo* nuclear DNA polymerase activity was determined on isolated Ehrlich ascites cell nuclei (10). The incubation was that described previously (11), except that [methyl-^{3}H]deoxythymidine triphosphate (82.4 Ci/mmole) was used. The acid insoluble nucleic acid was collected on filters and counted. Nuclear RNA polymerase activities were determined on enzymes isolated from nuclei. Messenger, ribosomal, and transfer RNA polymerase enzymes were isolated using 0.3, 0.04, and 0.0 *M* concentrations, respectively, of ammonium sulfate in magnesium chloride. The incubation medium was [^{3}H]uridine triphosphate (23.2 Ci/mmole) (12). The acid insoluble RNA was collected on nitrocellulose filters and counted.

Deoxythymidine, deoxythymidylate monophosphate, and diphosphate kinase activities were measured spectrophotometrically at 340 nm at 20

[1] GF/F glass filter discs.

[2] Fisher Scintiverse.

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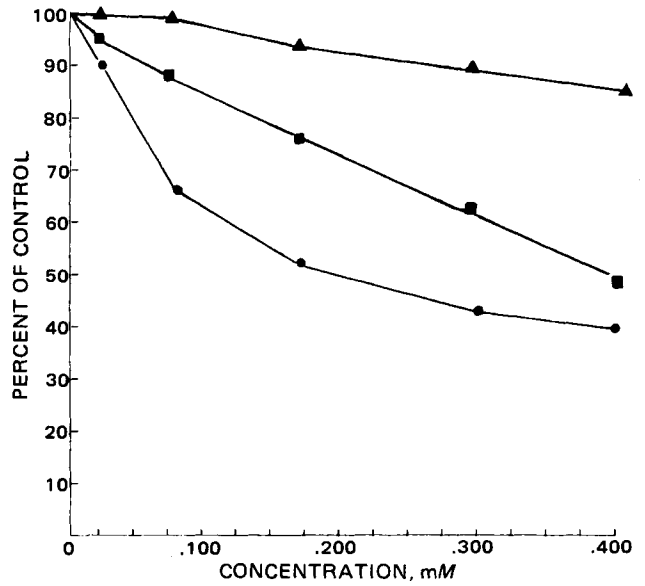

Figure 1—*The in vitro effects of daphnoretin on Ehrlich ascites nucleic acid and protein synthesis. Key: (●), [^{3}H]thymidine → DNA; (▲), [^{3}H]uridine → RNA; (■), [^{3}H]leucine → Protein.*

min using reduced nadide (0.1 μmole) (13). [6-^{3}H]Thymidine incorporation (21.5 Ci/mmole) into nucleotides was measured using the medium of Maley and Ochoa (13). The reaction medium was extracted with ether and the aqueous layer plated on polyethyleneiminecellulose plates and eluted with 0.5 *N* formic acid–0.6 *M* LiCl (1:1). Areas that correlated with the R_f values of thymidylate monophosphate, diphosphate, and triphosphate standards were scraped and counted.

Carbamyl phosphate synthetase activity was determined using the reaction medium of Kalman *et al.* (14) in the presence of ornithine and ornithine transcarbamylase. Citrulline formed from ornithine was measured at 490 nm by the method of Archibald (15). Aspartate transcarbamylase activity was assayed using the incubation medium of Kalman *et al.* (14). Colorimetric determination of carbamyl aspartate was conducted by the procedure of Koritz and Cohen (16). Orotidine monophosphate decarboxylase activity was assayed by the method of Appel (17) using 0.1 μCi of [^{14}C]orotidine monophosphate (34.9 mCi/mmole). The [^{14}C]carbon dioxide generated in 15 min was trapped in 1*M* KOH[3] and counted. Thymidylate synthetase activity was determined using a postmitochondrial supernate (9000×*g* for 10 min) and 5 μCi of [5-^{3}H]-deoxyuridine monophosphate (14 Ci/mmole) according to the method of Kampf *et al.* (18). [^{14}C]Formate incorporation into purines was determined by the method of Spassova *et al.* (19) using 0.5 μCi of [^{14}C]-formic acid (52.0 mCi/mmole). Purines were separated on silica gel TLC plates eluted with *n*-butanol–acetic acid–water (4:1:5).

After identifying R_f values consistent with the standards, adenine and guanine, the plates were scraped and the radioactive content determined. Phosphoribosyl-1-pyrophosphate amidotransferase activity was determined on a supernatant fraction (600×*g* for 10 min) measuring the reduction of 0.6 μmole of nadide at 340 nm for 30 min (20). Inosinic acid dehydrogenase activity was determined spectrophotometrically at 340 nm for 30 min using a supernatant fraction (600×*g* for 10 min). The assay medium was that of Magasanik, (21), which contained nadide. Dihydrofolate reductase activity was determined at 340 nm for 30 min as the oxidation of reduced nadide phosphate (22). Ribonucleotide reductase activity was determined by the method of Moore and Hurlbert (23) using [5-^{3}H]cytidine-5′-diphosphate (25 Ci/mmole). Ribose and deoxyribose nucleotide were separated on polyethyleneiminecellulose plastic precoated TLC plates eluted with 4% boric acid–4*M* LiCl (4:3) and scraped at the R_f values consistent with the standard deoxycytidine diphosphate.

In vivo phosphorylation of histones was determined by injecting 10 μCi of [γ-^{32}P]ATP (30.0 Ci/mmole) into mice 1 hr prior to sacrifice. The nuclei were isolated (10) and the histone chromatin protein was extracted by the method of Raineri *et al.* (24). *In vitro* nonhistone protein phosphorylation dependent on nuclear protein kinase was determined using 2.0 nmoles of [γ-^{32}P]ATP (30.0 Ci/mmole) and isolated nuclei chromatin protein was collected on nitrocellulose filters (25). Cyclic 3′,5′-adenosine monophosphate levels were determined by the radioimmunoassay method of Gilman (26) using a commercial kit.

In vitro oxidative phosphorylation studies (27) were conducted on Ehrlich ascites cells using the substrates, α-ketoglutarate or succinate. Basal oxygen consumption (State 4) was determined with an oxygen electrode connected to an oxygraph. The adenosine diphosphate was added to obtain State 3, or stimulated respiration. The number of microliters of oxygen consumed per hour per milligram of protein for States 3 and 4 were calculated. Deoxyribonuclease activity was measured at pH 5.0 by a modification of the de Duve method (28). Ribonuclease activity and acid cathepsin activity were determined at pH 5.0 by the method of Cho-Chung and Gullino (29). Protein content for the enzymatic assays was determined by the Lowry technique (30). UV studies between 210 and 340 nm for the binding of daphnoretin (20 μg/ml) to DNA (38 μg/ml) were performed in 0.1 *M* phosphate buffer, pH 7.2 (31) for 24 hr at room temperature.

[3] Hyamine hydroxide.

Table I—Antineoplastic Activity of Daphnoretin Against Ehrlich Ascites Tumor Growth

	N	Volume	Ascrit	Inhibition, %
Control	6	4.25	37.5	—
Daphnoretin				
3 mg/kg/day	6	0.08	59.5	97
6 mg/kg/day	6	0.16	35.0	96
12 mg/kg/day	6	0.45	36.2	90
6-Mercaptopurine	6	0.10	2.5	99

RESULTS

Daphnoretin successfully inhibited Ehrlich ascites growth at 3 mg/kg/day by 97%, at 6 mg/kg/day by 96%, and at 12 mg/kg/day by 90%. The normal number of Ehrlich ascites cells per milliliter on day 10 was 226 × 10^6. Daphnoretin treatment on days 7, 8, and 9 reduced the cell count by 60% (Table I).

Preliminary whole cell *in vitro* incorporation studies demonstrated that for DNA synthesis, the $ID_{50} \cong 0.194$ m*M* and for protein synthesis the $ID_{50} \cong 0.340$ m*M*. RNA synthesis was suppressed only marginally by daphnoretin *in vitro* demonstrating only 15% inhibition at 340 μmole (Fig. 1). Results from *in vitro* studies can be found in Table II, and results from *in vivo* studies can be found in Table III.

For the *in vivo* incorporation studies, the control values for 10-day Ehrlich ascites cells for thymidine incorporation into DNA were 107,533 dpm/mg of isolated DNA, 51,193 dpm/mg of isolated RNA for uridine incorporation, and 19,181 dpm/mg of isolated protein for leucine incorporation. After 3 days of dosing with daphnoretin at 6 mg/kg, DNA synthesis was reduced by 46%, RNA synthesis was suppressed by 42%, and protein synthesis was inhibited by 77%.

Nuclear DNA polymerase activity for the control was 76,528 dpm/hr/mg of nucleoprotein which was essentially unaffected by daphnoretin *in vitro* and was reduced by only 16% by *in vivo* administration of drug. Nuclear mRNA polymerase activity for the control was 4867 dpm/hr/mg of nucleoprotein, rRNA polymerase activity was 8751 dpm/hr/mg of protein, and tRNA polymerase activity was 10,792 dpm/mg of protein. Messenger RNA polymerase activity was suppressed by 10% *in vitro* and 17% *in vivo*, rRNA polymerase activity was elevated by 81% *in vitro* and 94% *in vivo*, and tRNA polymerase activity was reduced by 14% *in vitro* and 10% *in vivo*.

Ribonucleotide reductase activity for the control was 153,791 dpm/mg of protein which was suppressed by 31% *in vitro* and 39% *in vivo*. [^{14}C]-Formate incorporation into purines for the 10-day control was 28,786 dpm/mg of protein. Purine synthesis *in vitro* was inhibited by 32% and after drug administration for 3 days was suppressed by 30%. Phosphoribosyl pyrophosphate amidotransferase activity for the control was 0.544 optical density unit/hr/mg of protein. *In vitro* presence of drug resulted in a 39% reduction of enzymatic activity and *in vivo* administration of drug caused 31% suppression. Inosinic acid dehydrogenase activity for the control was 0.358 optical density unit/hr/mg of protein, reduced 19% by *in vivo* administration of drug. Dihydrofolate reductase activity for 10-day Ehrlich ascites cells was 0.514 optical density unit/hr/mg of protein which was inhibited by 53% *in vitro* and 59% *in vivo* by daphnoretin.

Carbamyl phosphate synthetase activity was 0.128 mg of carbamyl phosphate formed/hr/mg of protein for the control. Aspartate carbamyl transferase activity for 10-day Ehrlich ascites cells was 7.526 mg of car-

Table II—The *In Vitro* Effects of Daphnoretin at 340 μM Concentration on Ehrlich Ascites Cell Metabolism

Biochemical Parameter or Enzyme (n=6)	Percent Control: Control $\bar{x} \pm SD$	Percent Control: Treated $\bar{x} \pm SD$
DNA polymerase	100 ± 14	107 ± 9
mRNA polymerase	100 ± 13	90 ± 8
rRNA polymerase	100 ± 6	181 ± 10[a]
tRNA polymerase	100 ± 10	86 ± 7
Ribonucleotide reductase	100 ± 8	69 ± 4[a]
[^{14}C]Formic acid incorporation into purines	100 ± 7	68 ± 9[a]
Phosphoribosyl pyrophosphate amido transferase	100 ± 11	61 ± 6[a]
Inosinic acid dehydrogenase	100 ± 6	107 ± 7
Dihydrofolate reductase	100 ± 9	47 ± 7[a]
Carbamyl phosphate synthetase	100 ± 10	99 ± 8
Aspartate transcarbamylase	100 ± 12	100 ± 10
Orotidine monophosphate decarboxylase	100 ± 9	64 ± 5[a]
Thymidylate synthetase	100 ± 13	105 ± 7
Thymidine kinase	100 ± 9	75 ± 6[a]
Thymidylate monophosphate kinase	100 ± 8	26 ± 8[a]
Thymidylate diphosphate kinase	100 ± 10	83 ± 7
Acid deoxyribonuclease	100 ± 14	71 ± 13[a]
Acid ribonuclease	100 ± 19	73 ± 16
Acid cathepsin	100 ± 26	64 ± 22
[^{32}P]Phosphorylation of nonhistone proteins	100 ± 12	135 ± 8[a]
Oxidative phosphorylation processes		
Substrates:		
Succinate: State 4 respiration	100 ± 4	65 ± 6[a]
Succinate: State 3 respiration	100 ± 4	64 ± 4[a]
α-Ketoglutarate: State 4 respiration	100 ± 5	62 ± 7[a]
α-Ketoglutarate: State 3 respiration	100 ± 6	73 ± 5[a]

[a] $p \leq 0.001$.

bamyl aspartate formed/hr/mg of protein. Neither of these enzyme activities was affected by daphnoretin *in vitro* or *in vivo*. Orotidine monophosphate decarboxylase activity for the control was 10,775 dpm of $^{14}CO_2$ generated in 15 min/mg of protein. In the *in vitro* studies, decarboxylase activity was inhibited by 36%, and after drug administration *in vivo* the activity was reduced by 31%. Thymidylate synthetase activity for the control was 103,328 dpm/mg of protein which was unaffected by drug treatment. Thymidine kinase activity for the control was 0.531 optical density units/hr/mg of protein which was suppressed by 25% *in vitro* and 53% *in vivo*. Thymidylate monophosphate kinase activity for the control was 0.305 optical density units/hr/mg of protein inhibited by 74% *in vitro* and by 87% *in vivo*. Thymidylate diphosphate kinase activity for 10-day Ehrlich ascites cells was 0.238 optical density units/hr/mg of protein which was suppressed 17% *in vitro*, but daphnoretin had no effect *in vivo*. Thymidine incorporation studies into nucleotide showed that daphnoretin reduced the thymidylate monophosphate pools by 16%, thymidylate diphosphate pools by 53%, and thymidylate triphosphate levels by 45%. Deoxyribonuclease hydrolytic activity for day 10 Ehrlich ascites cells was 247 μg of DNA hydrolyzed/hr/mg of protein which was inhibited 29% *in vitro* by daphnoretin. Ribonuclease hydrolytic activity for control tumor cells was 43 μg of RNA hydrolyzed/hr/mg of protein which was suppressed 27% in the presence of drug. Cathepsin activity for the control was 869 μg of protein hydrolyzed/hr/mg of protein which was reduced 36% in the presence of daphnoretin.

The basal respiration (State 4) of day 10 Ehrlich ascites tumor cells with succinate as substrate was 5.273 μl of oxygen consumed/hr/mg of protein. Daphnoretin inhibited *in vitro* State 4 respiration 35% at 0.340 mM. Adenosine diphosphate stimulated (State 3) respiration was 8.752 μl of oxygen consumed/hr/mg of protein which was reduced 36% in the presence of daphnoretin. Using α-ketoglutarate as substrate resulted in

Table III—The *In Vivo* Effects of Daphnoretin at 6 mg/kg/day on Ehrlich Ascites Cell Metabolism

Biochemical Parameters or Enzymes (n=6)	Percent Control: Control 0.05% Polysorbate 80 $\bar{x} \pm SD$	Percent Control: Daphnoretin 6 mg/kg/day on Day 7, 8, and 9 $\bar{x} \pm SD$
[^{3}H]Thymidine incorporation into DNA	100 ± 8	63 ± 6[a]
[^{3}H]Uridine incorporation into RNA	100 ± 12	58 ± 4[a]
[^{3}H]Leucine incorporation into protein	100 ± 13	23 ± 3[a]
Number cells × 10^6/ml ascites fluid	100 ± 10	40 ± 6[a]
DNA polymerase	100 ± 6	84 ± 5[a]
mRNA polymerase	100 ± 12	83 ± 8[b]
rRNA polymerase	100 ± 8	194 ± 9[a]
tRNA polymerase	100 ± 9	90 ± 10[a]
Ribonucleotide reductase	100 ± 8	61 ± 7[a]
[^{14}C]Formic acid incorporation into purines	100 ± 12	70 ± 8[a]
Phosphoribosyl pyrophosphate amido transferase	100 ± 9	78 ± 3[a]
Inosinic acid dehydrogenase	100 ± 10	81 ± 9[a]
Dihydrofolate reductase	100 ± 12	41 ± 9[a]
Carbamyl phosphate synthetase	100 ± 10	104 ± 11
Aspartate transcarbamylase	100 ± 9	98 ± 8[a]
Orotidine monophosphate decarboxylase	100 ± 10	69 ± 7[a]
Thymidylate synthetase	100 ± 9	112 ± 11[a]
Thymidine kinase	100 ± 9	46 ± 8[a]
Thymidylate monophosphate kinase	100 ± 9	13 ± 4[a]
Thymidylate diphosphate kinase	100 ± 7	103 ± 9[a]
[^{32}P]Phosphorylation of histones	100 ± 15	64 ± 8[a]
[^{32}P]Phosphorylation of nonhistones	100 ± 13	206 ± 11[a]
Cyclic adenosine monophosphate levels	100 ± 10	117 ± 8

[a] $p \leq 0.001$. [b] $p \leq 0.005$.

a State 4 respiration of 3.569 μl of oxygen consumed/hr/mg of protein and in State 3 respiration of 5.156 μl of oxygen consumed/hr/mg of protein. In the presence of daphnoretin, State 4 respiration was reduced by 38% and State 3 was reduced by 27%. Histone phosphorylation of chromatin proteins for the control was at the rate of 3650 dpm/mg of isolated chromatin protein which was inhibited 36% after *in vivo* administration of daphnoretin. Nonhistone phosphorylation of chromatin proteins for the control was 28,593 dpm/mg of chromatin protein isolated, which was elevated 35% *in vitro* and 106% *in vivo*. Cyclic adenosine monophosphate levels for 10-day Ehrlich ascites cells was 3.65 pmoles/10^6 cells which was elevated 17% after daphnoretin administration for 3 days. The *in vitro* UV studies demonstrated that the spectrum of DNA and daphnoretin when present together caused a hyperchromic shift of DNA. However, there were no new peaks nor any shift to a higher wavelength, which probably suggests nonspecific binding of daphnoretin to DNA.

DISCUSSION

Daphnoretin significantly inhibited Ehrlich ascites tumor growth in CF_1 mice. DNA synthesis was inhibited at a low dose of daphnoretin, whereas protein synthesis was inhibited at a higher concentration. However, protein synthesis was inhibited to a greater degree than DNA synthesis after *in vivo* drug administration. The major enzymatic sites of inhibition by daphnoretin which could reduce DNA synthesis were thymidine kinase, thymidylate monophosphate kinase, ribonucleotide reductase, and dihydrofolate reductase. The reduction in enzymatic activity at any of these biochemical sites would be of sufficient magnitude to account for the degree of suppression of total DNA synthesis. Other minor sites of enzyme reduction are orotidine monophosphate decarboxylase, phosphoribosyl amidotransferase, and inosinic acid dehydrogenase as well as the incorporation of formic acid into purines and phosphorylation of histones. The magnitude of inhibition of the regulatory enzyme phosphoribosyl amido transferase of the purine pathway is of the same magnitude of reduction observed in the formate incorporation.

Daphnoretin also suppressed oxidative phosphorylation processes at 0.340 m*M*. Using the substrate succinate, which is a flavine adenine dinucleotide-linked dehydrogenase, daphnoretin suppressed basal and adenosine diphosphate stimulated respiration. The same was also true when using α-ketoglutarate, a nadide-linked dehydrogenase. Loss of energy from oxidative phosphorylation processes could account for the reduction of kinase activity of tumor cells since kinase reactions require ATP.

Daphnoretin reduced the catalytic enzyme activities responsible for the hydrolysis of nucleic acids and proteins. Elevation of the activities of these acid hydrolytic enzymes by the drug could account for the reduction of incorporation of radiolabeled precursors into nucleic acid or proteins. However, no elevation was observed with daphnoretin.

High cellular proliferation is associated with an increase in histone phosphorylation. As can be seen, daphnoretin reduced phosphorylation of chromatin histones (32). Although daphnoretin caused only a slight increase in cyclic adenosine monophosphate levels, elevated levels are associated with cessation of cellular division and reversal of tumor morphology to a normal state (33). A detailed study on the mechanism of protein synthesis inhibition has been reported (30).

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ACKNOWLEDGMENTS

Supported by American Cancer Society Grant CH-19 and National Cancer Institute Grant CA-26466.

The authors thank Larry Carpenter, Mary Dorsey, Jerry McKee, and Melba Gibson for technical assistance on this project.