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# Antitumor Agents LV: Effects of Genkwadaphnin and Yuanhuacine on Nucleic Acid Synthesis of P-388 Lymphocytic Leukemia Cells


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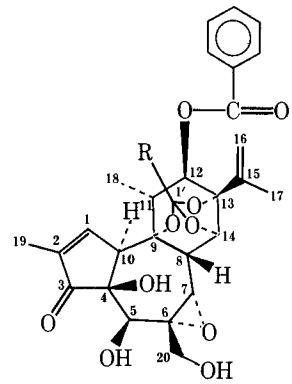
**Abstract** □ The diterpene esters, genkwadaphnin and yuanhuacine, have been shown to possess significant antileukemic activity in the P-388 screen. The major metabolic effects of the diterpene esters were on DNA and protein synthesis. The effects on DNA synthesis *in vitro* were evoked at a lower concentration than that required for protein synthesis inhibition. The sites in DNA synthesis which were inhibited were DNA polymerase and purine synthesis. In the latter pathway the enzyme activities inhibited were phosphoribosyl aminotransferase, inosinic acid dehydrogenase, and dihydrofolate reductase. *In vivo* administration of the diterpene esters at 0.8 mg/kg afforded identical types of effects on purine and DNA synthesis and in addition suppressed histone phosphorylation and reduced the number of surviving tumor cells. The *in vivo* effects on purine and DNA synthesis were evident as early as 6 and 24 hr after administration of a single dose of the diterpene esters.



**Keyphrases** □ Antitumor agents—nucleic acid synthesis by genkwadaphnin and yuanhuacine of P-388 lymphocytic leukemia cells, diterpene esters □ Genkwadaphnin—antitumor agents, nucleic acid synthesis by yuanhuacine of P-388 lymphocytic leukemia cells, diterpene esters □ Yuanhuacine—antitumor agents, nucleic acid synthesis by genkwadaphnin of P-388 lymphocytic leukemia cells, diterpene esters


Genkwadaphnin (I) and yuanhuacine (II) are two *ortho* esters bearing daphnane type diterpenes which possess an isopropylene side chain at $C_{13}$. Compounds belonging to the daphnane diterpene esters have previously been shown to have antileukemic activity as opposed to tigliane diterpene esters, *e.g.*, phorbol esters, which are known to be carcinogenic promoting agents (1). Compound II (odoracin or gnidilatidin) has previously been isolated from *Daphne genkwa*, *Daphne odorata*, *Gnidia latifolia*, and *Gnidia glaucus* Fres, and like other diterpene esters, such as 12-hydroxydaphnetoxin, gnidimacrin, gnidimacrin-20-palmitate, gnidilatidin-20-palmitate, gnididin, gniditrin, and gnidicin, has been demonstrated to have antileukemic activity in the 20- to 100-μg/kg dose range in rodents (2–6). The isolation and chemical characterization of a new diterpene ester (I) has recently been reported (7) which also has demonstrated antileukemic activity. The effects of daphnane diterpene esters on nucleic acid and protein synthesis in P-388 lymphocytic leukemia is now reported to establish a mode of action in P-388 lymphocytic leukemia cells to explain their *in vivo* antileukemic activity.



I: R = —C₆H₅

II: R = —CH=CH—CH=CH—$(CH_2)_4$—$CH_3$

## EXPERIMENTAL

The air-dried flowers of *Daphne genkwa*[1] (9 kg) (known as Yuán-Huā in Chinese folklore; Thymelaeaceae) were extracted with methanol. Guided by the *in vivo* P-388 lymphocytic leukemia rodent screen as conducted by the NCI protocol (6), the resulting active residue was dis-

[1] The plant material utilized in this investigation was identified as *Daphne genkwa* Sieb and Zucc (Thymelaeaceae) by H. C. Huang (7). A voucher sample (No. HCH-DG-771022) representing material collected for this investigation is available for inspection at the Herbarium of the School of Pharmacy, Kaohsiung Medical College, Kaohsiung, Taiwan, Republic of China.


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Table I—The Antineoplastic Activity of Genkwadaphnin and Yuanhuacine in the P-388 Lymphocytic Leukemia Screen in $BDF_1$ Male Mice

| Compound ($n$ = 6) | Dose, mg/kg/day | Average Survival Days, (T/C) | T/C %[a] |
|---|---|---|---|
| 0.05% Polysorbate 80 | | 11.0/11.0 | 100 |
| Genkwadaphnin | 0.4 | 15.5/11.0 | 141 |
| | 0.8 | 19.0/11.0 | 173 |
| Yuanhuacine | 0.4 | 16.0/11.0 | 145 |
| | 0.8 | 16.6/11.0 | 151 |
| 5-Fluorouracil | 12.5 | 19.6/11.0 | 178 |

[a] (Number of days survived by the treated group/number of days survived by the control group) × 100.

solved in a methanol–water mixture (1:1) and then partitioned into $n$-hexane and ether successively. Column chromatography of the active ethereal extract (55 g) on silica gel[2] (500 g) in chloroform afforded two fractions (fraction A, 540 mg and fraction B, 820 mg), which possessed P-388 antileukemic activity. Subsequent purification of fraction B by preparative TLC led to the isolation of the new antileukemic principle I, as reported previously (7).

Column chromatography of fraction A (540 mg) on modified dextran beads[3] (3 g) in chloroform afforded a fraction (420 mg), which was further purified by preparative TLC[4] eluted with $n$-hexane–2-propanol (10:1) and then ether–$n$-hexane (5:1) to yield compound II (132.5 mg). Final purification of II was achieved by high-performance liquid chromatography (HPLC)[5] to give 82.3 mg of pure II: $[\alpha]_D^{25}$ + 62.4 ($c$ = 0.242, $CHCl_3$) [previously reported: $[\alpha]_D^{24}$ + 34.3° ($c$ = 0.184, $CHCl_3$) for odoracin (8), $[\alpha]_D^{23}$ + 28° ($c$ = 0.16, $CHCl_3$) for gnidilatidin (2), and $[\alpha]_D^{32}$ + 61.7° ($c$ = 1.07, $CHCl_3$) for yuanhuacine (9)]; $UV_{max}$ (ethanol) 227 nm ($\epsilon$, 44,300) [previously reported $\lambda_{max}$ (ethanol) 232 nm ($\epsilon$ 36,000) and $\lambda_{max}$ 232 nm ($\epsilon$ 52,706); MS $m/z$ 648.2939 ($M^+$, Calc. for $C_{37}H_{44}O_{10}$: 648.2935 (9)].

The IR, $^1H$-NMR, and $^{13}C$-NMR spectra of II were identical to those of odoracin (8). Since the structure of odoracin is also identical to that of gnidilatidin (2) or yuanhuacine (9), yuanhuacine is thereby used as the name of II, since the work dealing with the isolation and structural determination of II was submitted for publication earlier than odoracin or gnidilatidin.

The P-388 lymphocytic leukemia tumor line was maintained in DBA/2 male mice (~20 g) and the antineoplastic activity was established by using the NCI protocol (6). For the *in vitro* studies, P-388 cells were harvested from the peritoneal cavity 10 days after administering $10^6$ P-388 ip lymphocytic leukemia cells into $BDF_1$ male mice (~20 g) on day 0 (10).

In the *in vivo* studies, $BDF_1$ male mice were inoculated on day 0 with $10^6$ P-388 cells ip, and on days 7, 8, and 9 the mice were administered 0.8 mg/kg, the optimum dose for antineoplastic activity, of I or II intraperitoneally. The biochemical studies were performed on cells harvested from the peritoneal cavity on day 10. Alternately, mice were injected with a single dose of drug at 0.8 mg/kg, the optimum dose for antineoplastic activity, on day 7 and sacrificed either 6 or 24 hr later.

The *in vitro* incorporation studies were conducted using 1 $\mu$Ci of thymidine (6-$^3H$, 21.8 Ci/mmole), uridine (6-$^3H$, 22.4 Ci/mmole), or L-leucine (4.5-$^3H$(N), 56.5 Ci/mmole) with $10^6$ P-388 whole cells or homogenized cells in minimum essential growth medium[6], pH 7.2, in a total volume of 1 ml, incubated for 60 min at 37°. Thymidine incorporation into DNA was terminated with perchloric acid containing pyrophosphate, which was filtered on glass fiber paper (GF/F) by vacuum suction. RNA and protein assays were terminated with trichloroacetic acid and collected on nitrocellulose membranes by vacuum suction. The acid-insoluble nucleic acid or protein precipitated on the filter papers was placed in scintillation vials and counted[7]. The control value for *in vitro* DNA synthesis for 10-day P-388 cells was 31,777 dpm/mg of DNA, for RNA 38,615 dpm/mg, and for protein 76,518 dpm/mg (11). *In vivo* thymidine incorporation into DNA was determined by injecting into the animal 1 hr before sacrifice 10 $\mu$Ci ip of [$^3H$](methyl)thymidine (21.5 Ci/mmole).

[2] SilicAR, CC-7.
[3] Sephadex LH-20.
[4] Silica Gel GF, 1000 $\mu$m, 6 plates.
[5] Waters Associates model ALC/GPC 244 Liquid Chromatograph using a Whatman Partisil M9 (500 × 12.8 mm) column and a mixture of $n$-hexane–isopropanol (5:1) in a recycle mode with a flow rate of 4 ml/min.
[6] Eagle MEM × 1 supplemented with 10% fetal calf serum, penicillin, and streptomycin.
[7] Fisher Scintiverse.

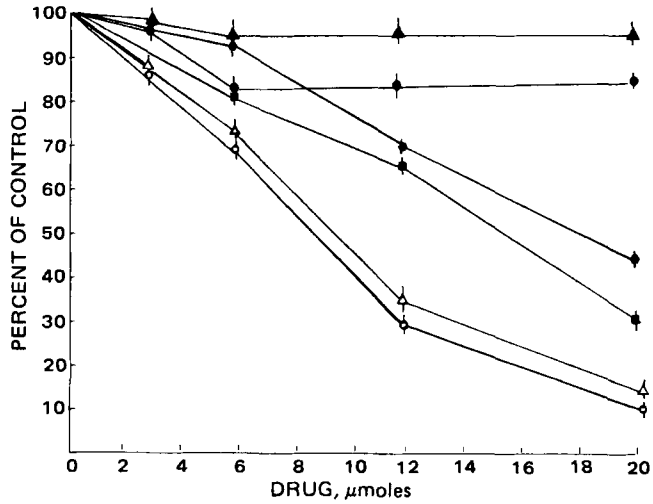


**Figure 1**—*The* in vitro *effects of genkwadaphnin (I) and yuanhuacine (II) on nucleic acid and protein synthesis of P-388 lymphocytic leukemia cells (60-min incubation)* (n = 5). *Key: [$^3H$]uridine into RNA, (▲) yuanhuacine, (●) genkwadaphnin; [$^3H$]leucine into protein, (■) yuanhuacine, (□) genkwadaphnin; [$^3H$]thymidine into DNA, (△) yuanhuacine, (○) genkwadaphnin; (|) standard deviation.*

The DNA was isolated (12), and the tritium content determined in scintillation fluid[7] and corrected for quenching. The DNA concentration was determined by the diphenylamine reaction using calf thymus DNA as the standard. The results were expressed as disintegrations per minute per milliliter of DNA isolated. Uridine incorporation into RNA was determined in an analogous manner with 10 $\mu$Ci of 6-[$^3H$]uridine (22.4 Ci/mmole) and the RNA extracted (13). Leucine incorporation into protein was determined by a previous method (14) with 10 $\mu$Ci [$^3H$]4,5($N$)-L-leucine (52.0 Ci/mmole). The control values for DNA incorporation were 202,098 dpm/mg DNA, for RNA incorporation 356,926 dpm/mg RNA, and for leucine incorporation 319,300 dpm/mg of protein isolated.

After *in vivo* administration of the drug, on day 10, the number of tumor cells per milliliter and the 0.4% trypan blue uptake were determined with a hemocytometer (15). The *in vitro* UV binding studies were conducted with diterpene esters (5 $\mu$g/ml) incubated with DNA (38 $\mu$g/ml) in 0.1 $M$ phosphate buffer, pH 7.2 from 0–24 hr and measured over the range of 200–340 nm (15).

The *in vitro* enzymatic studies were conducted with diterpene esters present in a final concentration of 12 $\mu M$[8]. The *in vitro* and *in vivo* enzymatic assays have been described previously (10). Briefly, nuclear DNA polymerase activity was determined on isolated P-388 cell nuclei (16) using a previous incubation medium (17), except that [$^3H$]methyldeoxyribothymidine triphosphate (82.4 Ci/mmole) was used. The insoluble nucleic acids were collected on glass fiber papers (GF/F). The control value was 24,568 dpm/mg of nuclear protein. Messenger, ribosomal, and transfer RNA polymerase enzymes were isolated using different concentrations of ammonium sulfate (18), namely, 0.3 $M$, 0.04 $M$, and 0.00 $M$, respectively, and the individual polymerase activities were measured using 5-[$^3H$]uridine-5-triphosphate (23.2 Ci/mmole).

Insoluble RNAs were collected on nitrocellulose filters (18, 19). Control values for messenger, ribosomal, and transfer RNA polymerase activities were, respectively, 21,434; 25,408; and 2,082 dpm/mg of nucleic protein. Ribonucleotide reductase activity was measured by a previous method (20) using 5-[$^3H$]cytidine-5′-diphosphate (21.5 Ci/mmole). The deoxyribonucleotides were separated from ribonucleotides by poly(ethyleneimine)cellulose TLC. The control value for the reductase was 577,720 dpm/mg of protein. Deoxythymidine kinase, deoxythymidylate monophosphate kinase, and deoxythymidylate diphosphate kinase activities were measured by spectrophotometric assay (21) based on the disappearance of nicotinamide dinucleotide at 340 nm. Control values using a postnuclear supernatant fraction (600×$g$, 10 min) were a change in absorbance of 0.385, 0.386, and 0.378 optical density units/20 min/mg of protein, respectively. Thymidine incorporation into the nucleotides was evaluated by using a previous medium (21). The reaction was extracted with ether and the aqueous layer plated on poly(ethylene)imine cellulose F plates eluted with 0.5 $N$ formic acid–lithium chloride (1:1).

[8] Concentration of drug was selected because the micromolar range would be between $ID_{50}$ values required to inhibit DNA and protein synthesis.

Table II—The *In Vitro* Effects of Genkwadaphnin and Yuanhuacine (12 $\mu M$) on P-388 Lymphocytic Leukemia Cell Metabolism

| Biochemical Parameter ($n$ = 6) | Control | Percent Control | |
|---|---|---|---|
| | | Genkwadaphnin | Yuanhuacine |
| [$^3$H]Thymidine incorporation into DNA | 100 ±8 | 23 ±4[a] | 33 ±5[a] |
| [$^3$H]Uridine incorporation into RNA | 100 ±10 | 84 ±8 | 92 ±5 |
| [$^3$H]Leucine incorporation into protein | 100 ±9 | 66 ±7[a] | 70 ± 8 |
| [$^{14}$C]Formate into purines | 100 ±6 | 51 ±8[a] | 76 ±9[a] |
| DNA polymerase | 100 ±6 | 71 ±9[a] | 84 ±4[a] |
| tRNA polymerase | 100 ±5 | 92 ±6 | 95 ±3 |
| rRNA polymerase | 100 ±6 | 81 ±3[a] | 106 ±4 |
| mRNA polymerase | 100 ±6 | 109 ±8 | 126 ± 5 |
| Carbamyl phosphate synthetase | 100 ±9 | 114 ±8 | 110 ±11 |
| Aspartate transcarbamylase | 100 ±7 | 113 ±4 | 89 ± 9 |
| Orotidine monophosphate decarboxylase | 100 ±8 | 89 ±5 | 96 ±6 |
| Thymidylate synthetase | 100 ±3 | 95 ±4 | 99 ±2 |
| Thymidine kinase | 100 ±7 | 98 ±5 | 102 ±6 |
| Thymidylate monophosphate kinase | 100 ±6 | 99 ±7 | 110 ±8 |
| Thymidylate diphosphate kinase | 100 ±4 | 97 ±4 | 93 ±6 |
| Phosphoribosyl aminotransferase | 100 ±7 | 68 ±7[a] | 74 ± 9[a] |
| Inosinic acid dehydrogenase | 100 ±5 | 66 ±8[a] | 67 ±6[a] |
| Dihydrofolate reductase | 100 ±12 | 55 ±8[a] | 61 ±9[a] |
| Ribonucleotide reductase | 100 ±7 | 96 ±5 | 114 ±7 |
| Nonhistone phosphorylation | 100 ±6 | 100 ±4 | 88 ±6 |

[a] $p \leq 0.001$.

Table III—The *In Vivo* Effects of Genkwadaphnin and Yuanhuacine on P-388 Lymphocytic Leukemia Cell Metabolism After 3 Days of Dosing at 0.8 mg/kg/day

| Biochemical Parameter ($n$ = 6) | Control | Percent Control, Day 10 | |
|---|---|---|---|
| | | Genkwadaphnin | Yuanhuacine |
| [$^3$H]Thymidine incorporation into DNA | 100 ±6 | 14 ±3[a] | 19 ±4[a] |
| [$^3$H]Uridine incorporation into RNA | 100 ±11 | 23 ±5[a] | 68 ±8[a] |
| [$^3$H]Leucine incorporation into protein | 100 ±8 | 40 ±5[a] | 51 ±5[a] |
| [$^{14}$C]Formate into purines | 100 ±9 | 60 ±6[a] | 66 ±4[a] |
| DNA polymerase | 100 ±6 | 43 ±6[a] | 40 ±5[a] |
| tRNA polymerase | 100 ±8 | 118 ±6 | 111 ±9 |
| rRNA polymerase | 100 ±4 | 86 ±8[a] | 81 ±5[a] |
| mRNA polymerase | 100 ±8 | 115 ±10 | 81 ±9[a] |
| Carbamyl phosphate synthetase | 100 ±7 | 91 ±8 | 94 ±8 |
| Aspartate transcarbamylase | 100 ±12 | 84 ±6 | 78 ±13 |
| Orotidine monophosphate decarboxylase | 100 ±6 | 99 ±5 | 117 ±17 |
| Thymidylate synthetase | 100 ±7 | 91 ±10 | 98 ±5 |
| Thymidine kinase | 100 ±8 | 118 ±9[a] | 105 ±6 |
| Thymidylate monophosphate kinase | 100 ±8 | 125 ±9[a] | 98 ±8 |
| Thymidylate diphosphate kinase | 100 ±7 | 109 ±10 | 93 ±8 |
| Phosphoribosyl aminotransferase | 100 ±8 | 70 ±7[a] | 69 ±11[a] |
| Inosinic acid dehydrogenase | 100 ±8 | 58 ±5[a] | 51 ±5[a] |
| Dihydrofolate reductase | 100 ±7 | 35 ±4[a] | 51 ±6[a] |
| Ribonucleotide reductase | 100 ±8 | 108 ±16 | 94 ±10 |
| Histone phosphorylation | 100 ±11 | 65 ±4[a] | 51 ±5[a] |
| Number of cells per milliliter | 100 ±3 | 16 ±2[a] | 27 ±5[a] |

[a] $p \leq 0.001$.

Areas that correlated with $R_f$ values of thymidine monophosphate, diphosphate, and triphosphate standards were scraped and counted[9]. Enzymes of the pyrimidine synthetic pathway were also measured. Carbamyl phosphate synthetase activity was determined by a previous method (22). The colorimetric determination of citrulline was performed according to another method (23), resulting in 3.26 mg of citrulline formed/hr/$\mu$g of protein.

Aspartate carbamyl transferase activity was carried out in the presence of aspartate transcarbamylase (22), and the colorimetric determination of the carbamyl aspartate formed was according to a previous method (24), resulting in 0.301 $\mu$mole formed/hr/mg of protein. Orotidine monophosphate decarboxylase activity was measured using a (16,300×$g$, 20 min) supernatant fraction by a previous technique (25) using [$^{14}$C]-(*carboxyl*)*orotidine monophosphate* (34.9 mCi/mmole). The control value was 41,760 dpm/mg of protein. Thymidylate synthetase activity was determined by a previous method (26), utilizing a postmitochondrial (9000×$g$, 10 min) supernatant fraction with 5 $\mu$Ci of 5-[$^3$H]deoxyuridine monophosphate (14 Ci/mmole), affording a control value of 14,500 dpm/hr/mg of protein.

Dihydrofolate reductase activity was determined using a (600×$g$, 10 min) supernatant fraction by a spectrophotometric method (27) based on the disappearance of reduced nicotinamide adenine dinucleotide resulting in a value of 0.761 optical density U/hr/mg of protein for 10-day P-388 cells. [$^{14}$C]Formate incorporation into purines was measured by a method used previously (28), using 0.5 $\mu$Ci of [$^{14}$C]formic acid (52.0 mCi/mmole). Purine separation was achieved by silica gel TLC eluting with $n$-butanol–acetic acid–water (4:1:5). Using the standards, guanine and adenine, the appropriate spots were scraped and the radioactivity determined. The control value for purine synthesis was 23,682 dpm/mg of protein. Phosphoribosyl pyrophosphate aminotransferase activity was determined by a previous spectrophotometric method (29) at 340 nm using a (600×$g$, 10 min) supernatant fraction resulting in a control value of 0.806 optical density U/hr/mg of protein. Inosinic acid dehydrogenase activity was determined by a previous method (30) using 8-[$^{14}$C]inosine-5′-monophosphate (61 mCi/mmole). The control value was 5745 dpm/mg of protein.

Histone phosphorylation was determined by injecting 10 $\mu$Ci ip of $\gamma$-[$^{32}$P]adenosine triphosphate (27 Ci/mmole). The nuclei were isolated 1 hr later, and the histone chromatin protein was extracted by a method used previously (31). Nonhistone chromatin phosphorylation by nucleic acid kinase was determined on isolated nuclei (16) utilizing 2 nmoles of $\gamma$-[$^{32}$P]adenosine triphosphate. Chromatin protein was collected on nitrocellulose membrane filters (32). The control value for histone phosphorylation was 2612 dpm/mg of chromatin protein isolated. Nonhistone phosphorylation was 18,616 dpm/mg of protein. Protein was determined by a previous method (33).

Data are expressed in the tables as percent of control with standard

[9] Packard Scintillation Counter in Fisher scintillation fluid and corrected for quenching.

**Table IV—The *In Vivo* Effects of Genkwadaphnin and Yuanhuacine on P-388 Lymphocytic Leukemia Cell Metabolism 24 hr After a Single Dose at 0.8 mg/kg**

| Biochemical Parameter ($n$ = 6) | Control | Percent Control, Day 8 | |
|---|---|---|---|
| | | Genkwadaphnin | Yuanhuacine |
| [$^3$H]Thymidine incorporation into DNA | 100 ±7 | 17 ±4[a] | 16 ±5[a] |
| [$^3$H]Uridine incorporation into RNA | 100 ±12 | 82 ±9 | 84 ±8 |
| [$^3$H]Leucine incorporation into protein | 100 ±9 | 80 ±8[a] | 103 ±8 |
| [$^{14}$C]Formate incorporation into purines | 100 ±7 | 69 ±5[a] | 75 ±6[a] |
| DNA polymerase | 100 ±5 | 75 ±5[a] | 70 ±6[a] |
| tRNA polymerase | 100 ±6 | 95 ±5 | 84 ±7[a] |
| rRNA polymerase | 100 ±7 | 95 ±6 | 63 ±9[a] |
| mRNA polymerase | 100 ±8 | 111 ±10 | 96 ±8 |
| Orotidine monophosphate decarboxylase | 100 ±7 | 99 ±8 | 117 ±9 |
| Phosphoribosyl aminotransferase | 100 ±8 | 48 ±9[a] | 46 ±11[a] |
| Inosinic acid dehydrogenase | 100 ±7 | 58 ±8[a] | 53 ±8[a] |
| Dihydrofolate reductase | 100 ±7 | 54 ±5[a] | 53 ±5[a] |
| Ribonucleotide reductase | 100 ±9 | 126 ±10[a] | 91 ±8 |

[a] $p \leq 0.001$.

**Table V—The *In Vivo* Effects of Genkwadaphnin and Yuanhuacine on P-388 Lymphocytic Leukemia Cell Metabolism 6 hr After a Single Dose at 0.8 mg/kg**

| Biochemical Parameter ($n$ = 6) | Control | Percent Control, Day 7 | |
|---|---|---|---|
| | | Genkwadaphnin | Yuanhuacine |
| [$^3$H]Thymidine incorporation into DNA | 100 ±8 | 13 ±5[a] | 35 ±8[a] |
| [$^{14}$C]Formate incorporation into purines | 100 ±7 | 67 ±6[a] | 73 ±7[a] |
| DNA polymerase | 100 ±7 | 71 ±6[a] | 69 ±7[a] |
| Phosphoribosyl aminotransferase | 100 ±9 | 47 ±5[a] | 80 ±7[a] |
| Inosinic acid dehydrogenase | 100 ±8 | 50 ±6[a] | 49 ±7[a] |
| Dihydrofolate reductase | 100 ±6 | 77 ±5[a] | 68 ±4[a] |

[a] $p \leq 0.001$.

deviation. The number of animals per group is represented by $n$. The probable ($p$) significant differences were determined by Student's $t$ test.

## RESULTS

Genkwadaphnin (I) and yuanhuacine (II) both demonstrated significant antineoplastic activity against the growth of P-388 lymphocytic leukemia. Compound I at 0.8 mg/kg/day afforded a T/C % value of 173, whereas II afforded a T/C % value of 151 (Table I). The antileukemic activity of the diterpene esters was comparable to the standard, 5-fluorouracil (T/C = 178% at 12.5 mg/kg) in their ability to inhibit P-388 tumor growth.

The *in vitro* studies involving whole cells and homogenized cells showed pellucidly that the major effect of the diterpene esters was on DNA synthesis, with I producing a 77% suppression and II producing a 67% suppression at 12 $\mu M$ concentrations (Fig. 1). The effects of diterpene esters on DNA synthesis were identical in the studies on whole cells and homogenized cells, indicating that the effects of diterpene esters were not on the membrane transport of the radiolabeled precursor, but rather on cellular synthesis. The $ID_{50}$ for the inhibition of DNA was 5.62 $\mu M$ for I and 7.11 $\mu M$ for II. The studies demonstrated that the diterpene esters inhibited *in vitro* protein synthesis at a higher concentration, compared with the concentration required to inhibit DNA synthesis, affording an $ID_{50}$ of ~14.8 $\mu M$ for I and an $ID_{50}$ of 18.5 $\mu M$ for II.

Further *in vitro* studies (Table II) showed that the principal pathway in nucleic acid synthesis which was suppressed by the diterpene esters was the purine pathway. Formate incorporation into purines was reduced 49% by I and 24% by II. A number of enzyme sites in purine synthesis appeared to be blocked by the diterpene esters. Phosphoribosyl aminotransferase activity was reduced 32% by I and 26% by II. Inosinic acid dehydrogenase activity was reduced 34% by I and 33% by II. Dihydrofolate reductase activity was suppressed 45% by I and 39% by II *in vitro*. Marginal inhibition of DNA polymerase activity was observed with 29% reduction by I and 16% by II. *In vitro* the diterpene esters had little effect on the enzymatic activities of the pyrimidine pathway, on the thymidylate kinases, or on the RNA polymerases.

In the *in vivo* studies, after dosing for 3 days at 0.8 mg/kg/day ip, I reduced the number of cells per milliliter from 206 to 33 × $10^6$, and II reduced this number from 206 to 56 × $10^6$ (Table III). The diterpene esters, *in vivo*, suppressed DNA synthesis significantly, with I causing an 86% reduction and II producing an 81% reduction after 3 days of dosing. Inhibition of RNA synthesis was observed *in vivo* with I causing a 77% reduction and II causing a 32% reduction. Likewise, protein synthesis was also reduced *in vivo* with I administration resulting in a 60% reduction and II a 49% reduction. *In vivo* purine synthesis was suppressed 40% by I and 34% by II. As observed in the *in vitro* studies, key enzymes of the purine pathway were blocked by the diterpene esters. Phosphoribosyl aminotransferase activity was reduced 30% by I and 31% by II after 3 days of administration. Inosinic acid dehydrogenase activity was suppressed *in vivo* 42% by I and 49% by II. Dihydrofolate reductase activity was inhibited 65% by I and 49% by II therapy. DNA polymerase activity *in vivo* was suppressed 57% by I and 60% by II administration. Phosphorylation of the chromatin basic histones was reduced by the administration of the diterpene esters, such that I caused 35% suppression and II caused 49% reduction. *In vivo* administration of the diterpene esters had little effect on the pyrimidine synthetic pathway or on the RNA polymerase activities.

Examination of the studies (Table IV) 24 hr after a single dose of the agents showed that DNA synthesis was inhibited significantly, with I resulting in 83% inhibition and II resulting in 84% inhibition. However, RNA synthesis and protein synthesis produced <20% inhibition 24 hr after a single dose of the diterpene esters. Formate incorporation into purines was reduced 31% by I and 25% by II administration. Phosphoribosyl aminotransferase activity was inhibited 52% by I and 54% by II therapy. Similarly, inosinic acid dehydrogenase activity was reduced 42% by I and 47% by II and dihydrofolate reductase activity was inhibited 46% by I and 47% by II after a single dose. DNA polymerase activity was inhibited 25% by I and 30% by II administration.

*In vivo* administration of a single dose of daphnane diterpene esters (Table V) with sacrifice of the mice 6 hr later demonstrated that DNA synthesis was suppressed 87% by I and 65% by II. Formate incorporation into purines after 6 hr demonstrated that I caused 33% reduction and II caused 27% reduction. Phosphoribosyl aminotransferase activity was reduced 53% by I and 20% by II administration. Inosinic acid dehydrogenase activity was reduced ~50% by a single dose of the diterpene esters. Dihydrofolate reductase activity was reduced 23% by I and reduced 32% by II therapy. DNA polymerase activity was reduced ~30% by the diterpene esters using a single dose.

The *in vitro* UV studies with DNA indicated that there were no hyperchromic effects or shift of absorbance to a higher wavelength. These studies indicate the diterpene esters did not bind to DNA or interfere with template activity for nucleic acid synthesis.

## DISCUSSION

The diterpene esters, I and II, had significant effects on cellular metabolism which would explain their antineoplastic activity in the P-388 screen. The major effect is the suppression of DNA synthesis of P-388

lymphocytic leukemia cells. The observed inhibition of key regulatory steps of the purine pathway are of a sufficient magnitude to account for the reductions observed in DNA synthesis. In addition, other sites of inhibition by the diterpenes were DNA polymerase and dihydrofolate reductase activity. The inhibition DNA synthesis occurs within 6 hr, suggesting that it is not directly related to the abilities of the agents to suppress protein synthesis.

The effects on P-388 cellular metabolism induced by the daphnane diterpene esters are in direct contrast to the reported cellular effects of the tigliane diterpenes, croton oil, and 12-*O*-tetradecanoyl-phorbol-13-acetate. These esters are hyperplasia-inducing agents (34) and induce cell proliferation (35, 36) augmenting carcinogenesis. It has been suggested that they act on cellular or intercellular membranes causing irritation and inflammation (37). Both crude extracts and esters have been observed to stimulate DNA and RNA synthesis, which was positively related to their ability to induce tumors (38). Stimulation of protein synthesis also occurs after the increase in RNA synthesis but before stimulation of DNA synthesis (38, 39). Stimulation of phosphorylation of histones by the croton esters (40) has been observed. Increased histone phosphorylation is related to the ability of the croton oil esters to promote tumors and stimulate nucleic acid synthesis. Further studies have indicated that the croton oil esters inhibit the DNA repair enzyme system (41).

Plants of the Euphorbiaceae family are known for their toxic effects on cells (42). However, these plants historically were used in herbal medicine to treat human cancer and warts (42). An antileukemic phorbol ester has been isolated from *Croton tiglium* and an antileukemic ingenol ester has been isolated from *Euphorbia eoula* (43). Two of the more potent diterpene esters are the macrocyclic diterpenoid gnidimacrin and its C-20 palmitate (44). It has been suggested that the C-20 and C-12 ester groups are necessary for antileukemic activity and that these groups enable the agent to pass through the cellular membrane (2, 44), thus facilitating its antineoplastic effect on cell metabolism. Compounds I and II have an ester in the C-12 position but not the C-20 which contains a hydroxy group.

Studies on structural requirements for the antileukemic activity such as the effect of the cyclopentenone ring, 6,7-epoxide group, the ortho esters and the configuration of A/B rings of the daphnane nucleus are currently in progress.

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