Inhibition of Nucleic Acid Synthesis in P-388 Lymphocytic Leukemia Tumor Cells by Helenalin and Bis(helenalinyl)malonate In Vivo

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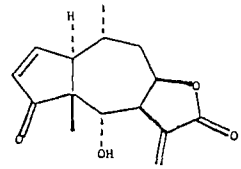
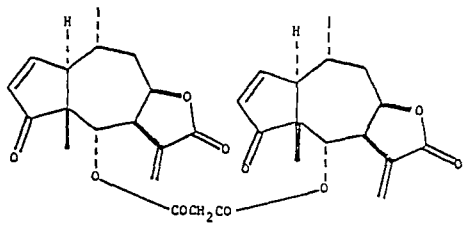
Abstract □ Although the parent sesquiterpene lactone, helenalin, and its derivative, bis(helenalinyl)malonate, are structurally related chemically, they demonstrate differences in their antineoplastic activity, with bis(helenalinyl)malonate being much more active against P-388 lymphocytic leukemia cell growth (T/C% = 261) compared with helenalin (T/C% = 162). Previous studies have shown that both agents strongly inhibit protein synthesis in vivo by >70% after 3 d of administration and in vitro by 50% at a 100 μM concentration of drug. This inhibition of protein synthesis of P-388 cells may be partially responsible for the cytotoxicity of the drug. These agents also inhibit nucleic acid synthesis in vivo, with DNA synthesis being suppressed by >90% after 2 d of administration of drugs at the therapeutic dose. Of the sulfhydryl-bearing enzymes involved in nucleic acid synthesis that were assayed, only the activities of inosine-5'-monophosphate (IMP) dehydrogenase and the ribonucleotide reductase complex were inhibited by >50% by these sulfhydryl-reactive drugs, which would account for the observed inhibition of nucleic acid synthesis in the P-388 cells. The inhibition of the activities of these enzymes lowered the deoxyribonucleotide levels in P-388 cells, which would explain the overall suppression of DNA synthesis by the sesquiterpene lactones.

Many sesquiterpene lactones have significant antineoplastic or cytotoxic activity. Preliminary evidence from the laboratory of Kupchan[1] suggested that the cytotoxicity of the sesquiterpene lactones was related to the presence of a conjugated α-methylene-γ-lactone moiety. Further SAR studies by Lee and co-workers[2,3] demonstrated that antineoplastic activity was not necessarily exclusively due to an α-methylene-γ-lactone, but that the O=C—C=CH_2 system, whether it be in a lactone or ketone form,[2] played a role. Helenalin, administered at the therapeutic effective dose of 33.3 mg/kg/d ip for 7 d, yielded 99% inhibition of growth of Ehrlich ascites carcinoma cells in mice, a T/C% of 316 survival at a dose of 2.5 mg/kg/d administered for 14 d in the Walker 256 carcinosarcoma screen, and a T/C% of 142 at a dose of 25 mg/kg/d administered for 11 d in the Lewis lung carcinoma screen. A dose–response curve in the P-388 lymphocytic leukemia screen resulted in helenalin affording a T/C% of 162 at the optimal dose of 8 mg/kg/d administered for 14 d,[4] and in bis(helenalinyl)malonate affording a T/C% of 261 at the optimum dose of 15 mg/kg/d.

Helenalin and bis(helenalinyl)malonate suppress DNA, RNA, and protein synthesis in P-388 cells.[5] Ultraviolet spectral studies indicated that there was no reaction of helenalin with DNA.[6,7] Studies in Ehrlich ascites cells showed that helenalin reduced the enzymatic activity of nuclear DNA polymerase and thymidylate synthetase;[3] both enzymes are susceptible to thiol alkylation. Thus, the present study deals with the mechanism of action of helenalin and bis(helenalinyl)malonate in P-388 lymphocytic leukemia cells in vivo, with particular emphasis on thiol-bearing enzymes that participate in DNA synthesis.



HELENALIN



BIS(HELENALINYL) MALONATE

Experimental Section

Materials—All biochemicals were purchased from Sigma Chemical Company (St. Louis, MO). [8-^{14}C]Inosine-5'-monophosphate was purchased from Amersham (Arlington Heights, IL). All other radiochemicals were purchased from New England Nuclear (Boston, MA). Radioactivity was determined in Fisher Scintiverse scintillation fluid, with a Packard Tricarb scintillation counter, and corrected for quenching by internal standardization techniques. Helenalin was obtained from the chloroform extract of the dried whole plant of *Helenium microcephalum*, as described by Lee et al.[2] Bis(helenalinyl)malonate was prepared by refluxing helenalin and malonyl chloride in dry benzene according to the method of Lee et al.[4]

Determination of T/C%—All experiments were performed with BDF_1 male mice (~25 g) that were inoculated ip with 1×10^6 P-388 lymphocytic leukemia cells[8] which had been suspended in sterile isotonic saline on day 0. The test compounds were suspended in 0.05% polysorbate 80:water by homogenization, and administered ip on days 6, 7, and 8. The average survival time of each group was recorded, and the T/C% values were calculated by the following method:

$$T/C\% = \frac{\text{average days survival of treated}}{\text{average days survival of control}} \times 100$$

In Vitro DNA, RNA, and Protein Synthesis—For the in vitro procedures, animals inoculated with tumor were sacrificed on day 8, and the tumor cells were harvested from the peritoneal cavity. These studies were conducted with 1×10^6 whole or homogenized cells. Homogenized cells were prepared by homogenizing 1×10^6 P-388

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cells in the P-388 ascites fluid on ice with ten strokes of the Potter-Elvehjem pestle. The cell preparation was incubated with 25, 50, or 100 μM drug, in the presence of 1 μCi of [methyl-^{3}H]thymidine (84 Ci/mmol), [6-^{3}H]uridine (22.4 Ci/mmol), or L-[4,5-^{3}H(N)]leucine (56.5 Ci/mmol), for 60 min.

In Vivo DNA, RNA, and Protein Synthesis—In vivo studies were performed by treating test animals on days 6, 7, and 8 with drug (ip), after tumor inoculation.[9] One hour prior to sacrifice, the animals were injected ip with 10 μCi of [methyl-^{3}H]thymidine (84 Ci/mmol), 10 μCi of [6-^{3}H]uridine (22.4 Ci/mmol), or 10 μCi of L-[4,5-^{3}H(N)]leucine (56.5 Ci/mmol). Following sacrifice, the P-388 cells were collected. Incorporation of [^{3}H]thymidine into DNA was determined by the modified method of Chae et al.[10] Results are reported as dpm of [^{3}H]thymidine incorporated into DNA per mg of DNA as determined by the diphenylamine reaction.[10] Incorporation of [^{3}H]uridine into RNA was determined according to the method of Wilson et al.[8] The concentration of hydrolyzed RNA was assayed by the orcinol method.[11] Results are reported as dpm of [^{3}H]uridine incorporated into RNA per mg of RNA. Incorporation of [^{3}H]leucine into protein was determined by the method of Booth and Sartorelli.[12] Results are reported as dpm of [^{3}H]leucine incorporated per mg of protein as determined by the Lowry technique.[13]

Enzyme Assays—The DNA polymerase-α activity was determined in a cytoplasmic extract isolated by the method of Eichler et al.[14] The polymerase assay was that of Sawada et al.[15] with [^{3}H]TTP. Messenger, ribosomal, and transfer RNA polymerase enzymes were isolated with different concentrations of ammonium sulfate,[16,17] and the individual RNA polymerase activities were determined using [^{3}H]UTP. Ribonucleotide reductase activity was measured with [^{14}C]CDP, with and without dithioerythritol.[18] Thioredoxin activity was measured by the spectrophotometric method of Holmgren.[19] The deoxyribonucleotides were separated from ribonucleotides by TLC on PEI plates. Thymidine, TMP, and TDP kinase activities were measured spectrophotometrically as the disappearance of NADH at 340 nm.[20] Carbamyl phosphate synthetase activity was determined by the method of Kalman et al.,[21] and citrulline was determined colorimetrically.[22] Aspartate transcarbamylase activity was determined by the method of Kalman et al.,[21] and carbamyl aspartate was determined colorimetrically.[23] The OMP decarboxylase activity was assayed with a 16,300 g × 20 min supernatant and [^{14}C]OMP. Thymidylate synthetase activity was analyzed by the method of Kampf et al.[24] The ^3H_2O measured was proportional to the amount of TMP formed from [^{3}H]dUMP. Dihydrofolate reductase activity was determined by the spectrophotometric method of Ho et al.[25] Formate incorporation into purines was determined with [^{14}C]formic acid. Adenine and guanine were separated by TLC.[26] The PRPP amidotransferase activity was determined by the method of Spassova et al.,[27] and IMP dehydrogenase activity was determined with [^{14}C]IMP, where XMP was separated on PEI plates by TLC.[28] The cAMP levels in P-388 cells were determined with a commercial radioimmunoassay kit from Becton Dickinson. Protein was determined for all of the enzymatic assays by the Lowry technique.[13]

Ribonucleoside phosphates and deoxyribonucleoside triphosphates were extracted by the method of Baghara and Finch.[29] The ribonucleoside phosphates were determined by the HPLC method of McKeag and Brown.[30] Deoxyribonucleoside triphosphates were determined by the method of Hunting and Henderson,[31] with calf thymus DNA, *E. coli* DNA polymerase I, and either 0.4 μCi of [^{3}H-methyl]dTTP or [5-^{3}H]dCTP and nonlimiting amounts of the three deoxyribonucleoside triphosphates which were not being currently assayed.

The P-388 tissue culture cells were treated with colcemid (demecolcine) (0.025 g/mol), which arrests the cells in the metaphase stage. The cells were washed in cold minimal essential medium (MEM) with 10% fetal calf serum (FCS) and cultured in fresh warm medium. Drugs were added at concentrations of 10 or 100 μg/mL at 0, 30, and 60 min. There was a 30-min lag before the cells again began to grow, but then the cell number increased over the next 3.5 h.

The effects of sesquiterpene lactones on DNA strand scission were determined by the in vitro method of Suzuki et al.,[32] Pera et al.,[33] and Woynarowski et al.[34] The BDF_1 mice bearing P-388 lymphocytic leukemia cells were injected ip with 10 μCi of [methyl-^{3}H]thymidine (84.0 Ci/mmol) 24 h prior to sacrifice. After harvesting the P-388 cells from the peritoneal cavity, 5 × 10^6 cells were centrifuged at 600 g × 10 min in phosphate buffered saline (PBS), washed, and suspended in 1 mL of PBS. Drugs (100 μM) were added, and the suspension was incubated at 37 °C for 2 h and centrifuged. The pellet was washed and suspended in 1 mL of PBS. Lysis buffer (0.5 mL; 0.5 M NaOH, 0.02 M EDTA, 0.01% Triton X-100, and 2.5% sucrose) was layered onto a 5–20% alkaline sucrose gradient (5 mL; 0.3 M NaOH, 0.7 KCl, and 0.01 M EDTA), followed by 0.2 mL of the cell preparation. After incubating for 30 min at room temperature, the gradient was centrifuged at 25,000 rpm at 20 °C for 60 min (Beckman rotor SW 60). Fractions (0.2 mL) were collected from the top of

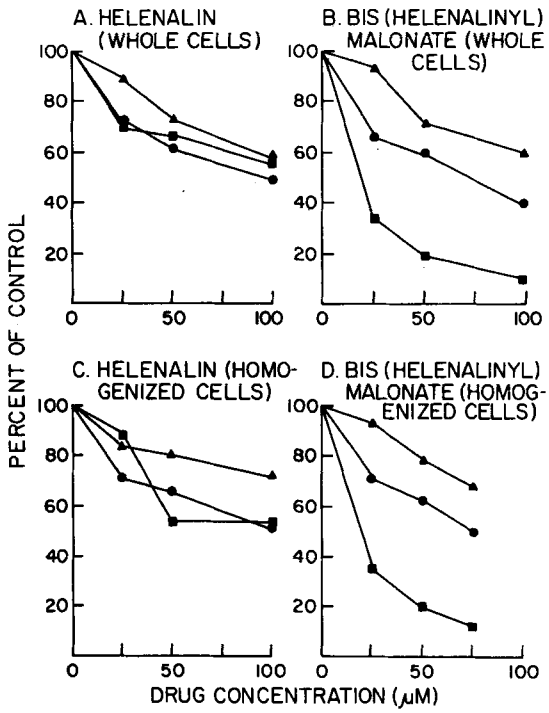

Figure 1—*Effects of drugs on in vitro incorporation of radiolabeled precursors into nucleic acids and protein of P-388 lymphocytic leukemia whole* (A & B) *and homogenized* (C & D) *cells. For whole cell uptake, 10^6 P-388 cells were used in 1 mL of MEM (10% fetal calf serum). Cell homogenates and assays were prepared as described in the Experimental Section. Key:* (■–■) *[^{3}H]thymidine incorporation into DNA:* (▲–▲) *[^{3}H]uridine incorporation into RNA:* (●–●) *[^{3}H]leucine incorporation into protein.*

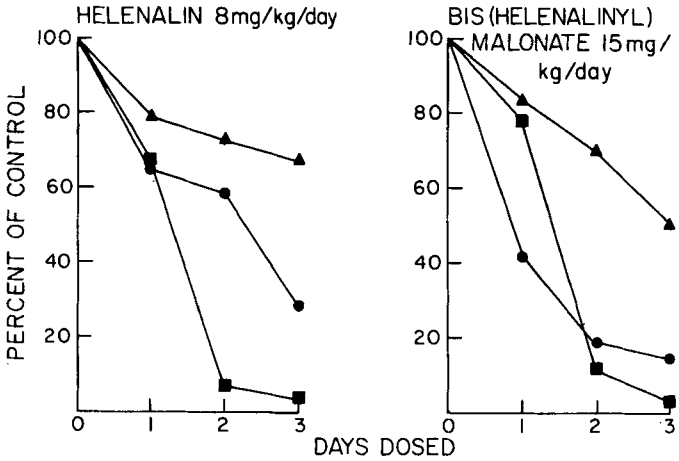

Figure 2—*Comparison of the effects of in vivo administration of helenalin and bis(helenalinyl)malonate with different numbers of doses on RNA, DNA, and protein synthesis in P-388 lymphocytic leukemia cells. Dosing was on day 8 (1 d), days 7 and 8 (2 d), or days 6, 7, and 8 (3 d) at 8 mg/kg/d (helenalin) or 15 mg/kg/d [bis(helenalinyl)malonate]. Assays for in vivo RNA, DNA, and protein synthesis were performed on day 8 as described in the Experimental Section. Key:* (■–■) *[^{3}H]thymidine incorporation into DNA;* (▲–▲) *[^{3}H]uridine incorporation into RNA;* (●–●) *[^{3}H]leucine incorporation into protein.*

the gradient and neutralized with 0.2 mL of 0.3 M HCl before radioactivity was measured. The DNA viscosity and thermal denaturation studies were carried out by previously described literature methods[35] with 100 μM of helenalin or bis(helenalinyl)malonate.

Statistics—The mean and standard deviation are designated as x ± SD. The number of animals or test samples per group is designated by the letter n. The probable level of significance (p) between test samples and control samples was determined by the *t* test with raw data. Standard deviations in Figures 1–6 were not >7% of control.

Results

Helenalin proved to be active in vivo against P-388 cell growth at a dose of 8 mg/kg/d, resulting in a T/C% of 162 for 14 d. Bis(helenalinyl)malonate was more effective with a T/C% of 261 at a dose of 15 mg/kg/d for 14 d. The LD_{50} of helenalin was ~43 mg/kg and that of bis(helenalinyl)malonate was ~92 mg/kg in mice. Three daily doses of the drug at the therapeutic dose reduced the P-388 cell number/mL by 80% for helenalin and 90% for bis(helenalinyl)malonate. Both drugs markedly suppressed DNA synthesis in vitro (Figure 1) and in vivo after 3 d of administration (Figure 2), and exhibited a dose–response effect. The suppression of protein synthesis was significant but lagged behind the suppression of DNA synthesis by day 3. The RNA synthesis was only marginally inhibited by the agents. Enzymatic studies in vivo after 3 d of administration of the drugs demonstrated that RNA and DNA polymerase isoenzyme activities (Table I) were not affected by the drugs. Also, thymidine, TMP or TDP kinases, thymidylate synthetase, dihydrofolate reductase, carbamyl phosphate synthetase, aspartate transcarbamylase, and OMP decarboxylase activities were not inhibited after 3 d of administration of drug. Purine de novo synthesis was suppressed 60% by helenalin and 75% by bis(helenalinyl)malonate. The inhibition of this synthetic pathway was not at the regulatory site of PRPP amido transferase, but appeared to be at IMP dehydrogenase, with helenalin causing 55% inhibition and bis(helenalinyl)malonate causing 71% inhibition. When bis(helenalinyl)malonate at a dose of 3, 8, or 15 mg/kg/d for 3 d was examined for a dose–response effect, it was observed that DNA, RNA, protein synthesis, cell number/mL, and IMP dehydrogenase activity (Figure 3) were reduced with increased dose. However, the inhibition of IMP dehydrogenase activity did not directly parallel DNA synthesis inhibition; consequently, other sites in DNA synthesis were examined for drug inhibition. Helenalin and bis(helenalinyl)malonate had no effect on DNA strand scission, suggesting that DNA was not the target of the drug (Figure 4). Neither drug caused any change in DNA viscosity or DNA thermal denaturation after in vitro incubation at 100 μM. The drugs at high concentration did interfere during metaphase, suggesting a partial block (Figure 5). Kinetic studies (Figure 6) did suggest that the inhibition of IMP dehydrogenase activity by both agents over periods of 1, 2, and 3 d correlated positively with the inhibition of de novo purine synthesis. When cytoplasmic DNA polymerase-α activity was examined, marginal inhibition by helenalin (32%) and bis(helenalinyl)malonate (30%) was observed in vivo (Figure 6). The β-isoenzyme principally in the nuclear preparation was not inhibited by either drug (Table I). When ribonucleotide reductase activity was measured in the presence of dithioerythritol, the drugs afforded no inhibition (Table I); nevertheless, if dithioerythritol was removed, a kinetic effect was observed for the inhibition of ribonucleoside reductase activity (Figure 7). Thioredoxin activity, a hydrogen donor system for the reductase enzyme, was inhibited in a manner that paralleled the reductase

Table I—Effects of In Vivo Administration of Helenalin and Bis(helenalinyl)malonate on P-388-UNC Lymphocytic Leukemia Cell Metabolism in BDF_1 Male Mice

Enzymatic Activity Assayed (n = 5)	Percent of Control		
	Control x ± SD	Helenalin (8 mg/kg/d) x ± SD	Bis(helenalinyl)malonate (15 mg/kg/d) x ± SD
Nuclear DNA polymerase (β-isoenzyme)	100 ± 10[a]	100 ± 10	106 ± 12
Messenger RNA polymerase	100 ± 2[b]	98 ± 6	97 ± 5
Transfer RNA polymerase	100 ± 5[c]	92 ± 8	87 ± 15
Ribosomal RNA polymerase	100 ± 6[d]	109 ± 14	92 ± 10
Thymidine kinase	100 ± 6[e]	112 ± 12	115 ± 12
TMP kinase	100 ± 4[f]	114 ± 20	131 ± 9
TDP kinase	100 ± 3[g]	89 ± 6[s]	98 ± 8
Thymidylate synthetase	100 ± 3[g]	130 ± 10	105 ± 3
Ribonucleotide diphosphate reductase	100 ± 5[h]	105 ± 7	103 ± 7
Dihydrofolate reductase	100 ± 6[i]	91 ± 7	92 ± 6
[^{14}C]Formate incorporation into purines	100 ± 4[j]	40 ± 5[s]	25 ± 4[s]
PRPP amidotransferase	100 ± 1[k]	99 ± 2	96 ± 5
IMP dehydrogenase	100 ± 6[l]	45 ± 16	29 ± 2[s]
Carbamoyl phosphate synthetase	100 ± 7[m]	105 ± 1	102 ± 2
Aspartate transcarbamylase	100 ± 5[o]	99 ± 2	100 ± 5
OMP decarboxylase	100 ± 6[p]	98 ± 4	97 ± 6
cAMP levels	100 ± 27[q]	117 ± 30	131 ± 30
Number of tumor cells/mL	100 ± 8[r]	20 ± 3[s]	9 ± 4[s]

[a] 3979 dpm [^{3}H]TTP incorporated into DNA/h/mg protein. [b] 8600 dpm [^{3}H]UTP incorporated into mRNA/h/mg protein. [c] 3288 dpm [^{3}H]UTP incorporated into tRNA/h/mg protein. [d] 3009 dpm [^{3}H]UTP incorporated into rRNA/h/mg protein. [e] 0.065 optical density units decrease at 340 nm/h/mg protein. [f] 0.055 optical density units decrease at 340 nm/h/mg protein. [g] 0.075 optical density units decrease at 340 nm/h/mg protein. [h] 543831 dpm [^{3}H]H_2O formed/h/mg protein. [i] 21437 dpm [^{3}H]dCDP formed/h/mg protein. [j] 0.489 optical density units decrease at 340 nm/h/mg protein. [k] 14 438 dpm [^{14}C]formate incorporated into purine/h/mg protein. [l] 0.086 optical density units increase at 340 nm/h/mg protein. [m] 42151 dmp XMP formed/h/mg protein. [n] 0.257 μmol citrulline formed/h/mg protein. [o] 0.422 mol *N*-carbamoyl aspartate formed/h/mg protein. [p] 121730 dpm [^{14}C]CO_2 formed/h/mg protein. [q] 1.384 mol cAMP formed/h/10^6 cells. [r] 1.174×10^8 cells/mL. [s] $p \leq 0.001$.

inhibition by the drugs over a 3-d period. When deoxyribonucleotide pools were examined for the same 3 d, significant reductions in dATP, dGTP, and dCTP were observed on day 2 after helenalin administration, and dATP and dGTP were suppressed on day 3 (Table II). Bis(helenalinyl)malonate afforded reductions in dGTP, dCTP, and dTTP on days 1, 2, and 3. Ribonucleotide pool levels for the same period of time showed significant reductions of XMP, on days 1 and 2, by both drugs. In vivo drug treatment resulted in elevations in the pool levels of GMP, GDP, GTP, and CDP on day 3, and UDP on day 2 (Table III).

Discussion

It is evident that bis(helenalinyl)malonate (T/C% = 261) is more potent than helenalin (T/C% = 162) in vivo; this correlates with the reported lower ED_{50} values in vitro of 17.8 μM compared with 31.8 μM, respectively.[4] There are several possible hypotheses available to explain the difference in potency of the drugs. The difference may be due to the metabolic fate of bis(helenalinyl)malonate. Nonspecific esterase activity is well documented, and two helenalin moieties may result from each molecule of bis(helenalinyl)malonate. Another hypothesis is that the high in vivo antileukemic activity of bis(helenalinyl)malonate might be due to a greater cellular uptake of the bisester compound than the parent helenalin. However, in vitro studies in whole

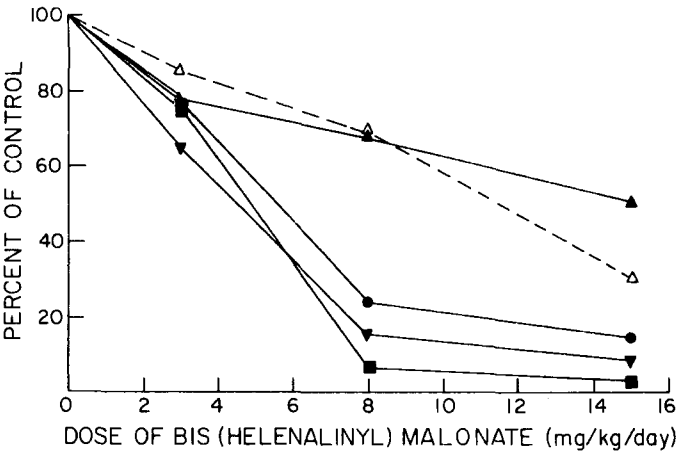

Figure 3—*Comparison of the effects of in vivo administration of bis(helenalinyl)malonate at different doses on P-388 lymphocytic leukemia cell growth and nucleic acid synthesis. Bis(helenalinyl)malonate was administered at 3, 8, and 15 mg/kg/d on days 6, 7, and 8. Cell homogenates were prepared and assays were performed on the appropriate cell fraction on day 8 as described in the Experimental Section. Key: (■–■) [^{3}H]thymidine incorporation into DNA; (△–△) IMP dehydrogenase activity; (▼–▼) P-388 cell number/mL of ascites fluid; (●–●) [^{3}H]leucine incorporation into protein; (▲–▲) [^{3}H]uridine incorporation into RNA.*

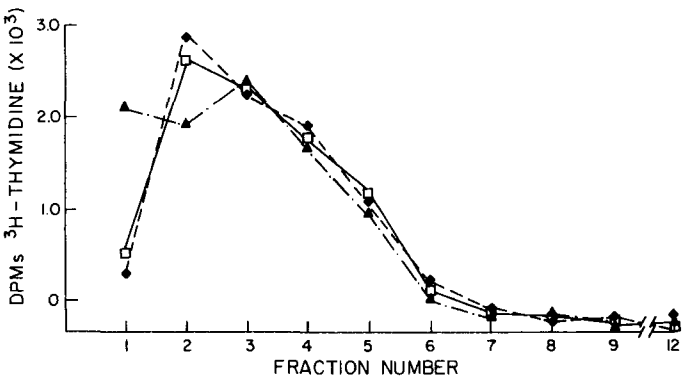

Figure 4—*Effects of helenalin and bis(helenalinyl)malonate (100 μM) on P-388 DNA strand scission. Assays were performed as described in the Experimental Section. Key: (◆–◆) control; (□–□) helenalin; (▲–▲) bis(helenalinyl)malonate.*

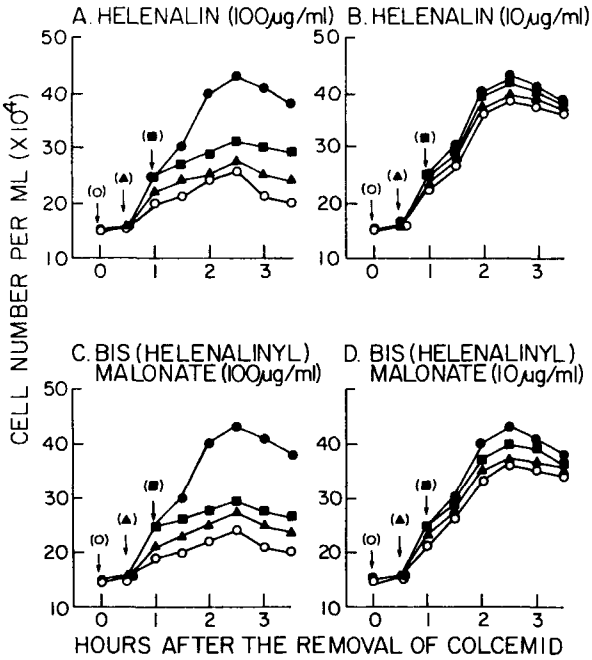

Figure 5—*Effect of helenalin and bis(helenalinyl)malonate on growth of P-388 cells synchronized with colcemid. The P-388 cells (2 × 10^5) were incubated with 0.025 μg/mL of colcemid for 7 h. The cells were then washed with cold MEM + 10% FCS and suspended in fresh warm medium. Key: (●–●) control without drugs present; drugs (10 and 100 μg/mL) were added to the tissue culture cells at zero (○–○), 0.5 (▲–▲), and 1 h (■–■) after the removal of colcemid. The cell number was determined using a hemocytometer over the next 3.5 h. Adriamycin at 2 μg/mL was used as a standard control drug. (A) Helenalin, 100 μg/mL. (B) Helenalin, 10 μg/mL. (C) Bis(helenalinyl)malonate, 100 μg/mL. (D) Bis(helenalinyl)malonate, 10 μg/mL.*

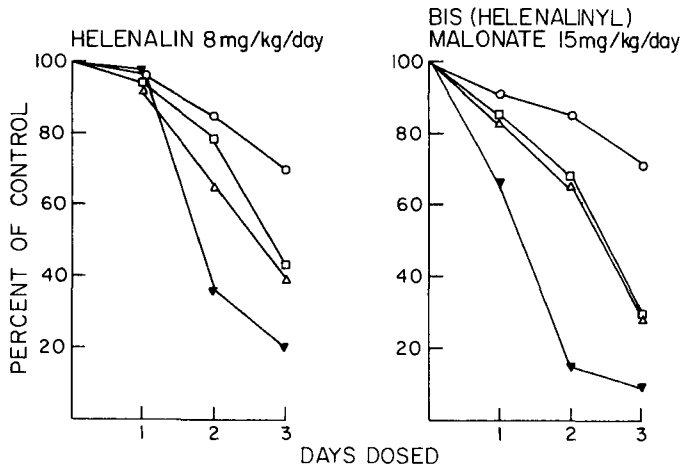

Figure 6—*Effects of helenalin (8 mg/kg) and bis(helenalinyl)malonate (15 mg/kg) on purine synthesis, IMP dehydrogenase activity, cell number, and DNA Polymerase-α activity of P-388 cells in vivo. Cell homogenates were prepared and assays were performed on the appropriate cell fraction on day 8 as described in the Experimental Section. Key: (△–△) [^{14}C]formate incorporation into purine; (□–□) IMP dehydrogenase activity; (○–○) DNA polymerase-α activity; (▼–▼) cell number/mL.*

cell versus homogenized cell preparations did not support this idea because bis(helenalinyl)malonate was also more effective than helenalin in homogenized cell preparations. Finally, the difference in potency could be explained on the basis of the strength of interaction of the agents with individual enzymes. For example, bis(helenalinyl)malonate has been demonstrated to bind much more strongly to purified IMP dehydrogenase enzyme from P-388 cells[36] than does helenalin. Woynarowski and Konopa[26] have shown that some sesquiterpene lactones inhibit the cell uptake of [^{3}H]thymidine, in addition to their direct effect on the inhibition of DNA synthesis; Roberts has recently confirmed this observation with P-388 cells in vitro.[37] However, the present study showed that the inhibition of precursor incorporation into macromolecules followed a similar dose–response pattern in both whole cells and cell homogenates, indicating that both of the sesquiterpene lactones inhibit macromolecular synthesis directly and that the inhibition of precursor

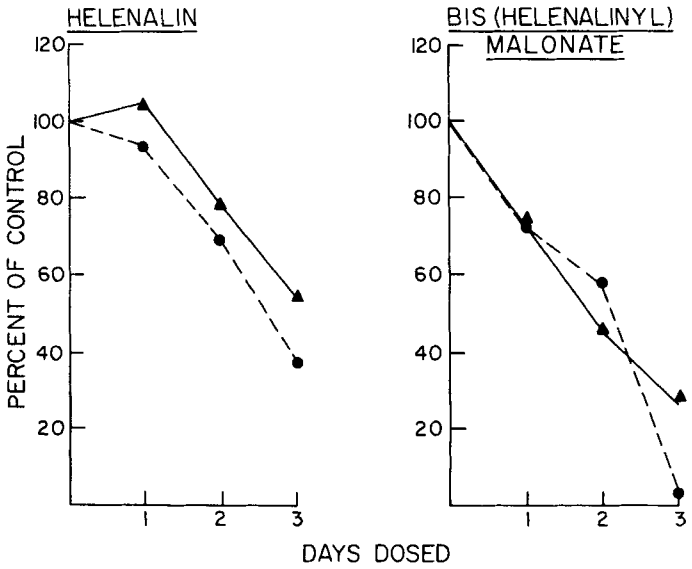

Figure 7—*Effects of helenalin (8 mg/kg) and bis(helenalinyl)malonate (15 mg/kg) on ribonucleotide diphosphate reductase and thioredoxin activities of P-388 cell supernatants (5000 g × 10 min), as described in the Experimental Section, were used for assays after administration of the in vivo dose. Ribonucleoside diphosphate reductase activity (▲–▲) was determined with CDP (5-^{3}H; 16.2 Ci/mmol) for 30 min at 37°C; the control value was 1592 dpm/mg protein. The control value for thioredoxin activity (●–●) was 6.84 × 10^{-3} OD units/min/mg protein.*

Table II—Effects of Helenalin and Bis(helenalinyl)malonate on Deoxyribonucleoside Triphosphate Pools of P-388 Cells In Vivo after Three Days of Administration[a]

	Percent of Control (x ± SD)			
Drug	dATP	dGTP	dCTP	dTTP
Control	100 ± 4[b]	100 ± 3[c]	100 ± 5[d]	100 ± 7[e]
Helenalin, 8 mg/kg/d				
Day 1	59 ± 6[f]	86 ± 6	64 ± 4[f]	102 ± 8
Day 2	30 ± 6[f]	49 ± 3[f]	74 ± 8[f]	119 ± 6
Day 3	65 ± 9[f]	43 ± 3[f]	114 ± 7	119 ± 7
Bis(helenalinyl)malonate, 15 mg/kg/d				
Day 1	78 ± 6[f]	97 ± 9	57 ± 7[f]	55 ± 2[f]
Day 2	88 ± 5	38 ± 6[f]	60 ± 7[f]	60 ± 5[f]
Day 3	102 ± 7	81 ± 6[f]	54 ± 4[f]	54 ± 7[f]

[a] n = 4. [b] Control values: 7.8 pmol dATP/10^6 cells. [c] Control values: 11.7 pmol dGTP/10^6 cells. [d] Control values: 12.32 pmol dCTP/10^6 cells. [e] Control values: 13.1 pmol dTTP/10^6 cells. [f] $p \leq 0.001$.

uptake of [^{3}H]thymidine into whole cells is proportional to the inhibition of DNA synthesis of P-388 cells. In vivo P-388 DNA synthesis inhibition by the sesquiterpene lactones followed a concentration- and time-dependent response.

Colcemid treatment of synchronized P-388 cells, which then were allowed to grow for 3.5 h, demonstrated little effect at 10 μg/mL, but at 100 μg/mL, both sesquiterpene lactones interfered with cell growth immediately after the metaphase blockage, suggesting that at very high concentrations the sesquiterpene lactones can affect events necessary for growth during the latter stages of the cell cycle in synchronized cells. Nevertheless, 100 μg/mL is 10–20 times the ED_{50} value necessary for inhibition of P-388 cell growth.

Studies in HeLa cells by Woynarowski et al.[34] have demonstrated that some cytotoxic and antitumor sesquiterpene lactones preferentially inhibited DNA synthesis subsequent to formation of the deoxyribonucleoside triphosphates, and that this inhibition seemed to play a role in the reduction of growth by the compounds studied. Further studies demonstrated that some of these sesquiterpene lactones (e.g., parthenolide) induced single strand breaks in DNA.[34] Previous studies[7] have demonstrated that there is no interaction over a 24-h period between helenalin and dGMP, dGTP, dAMP, dATP, or DNA, when observed spectrophotometrically over the ranges of 200–360 or 300–360 nm. Furthermore, NMR data confirmed the absence of reaction between helenalin and dGMP.[7] The present alkaline sucrose-gradient studies of P-388 [^{3}H]thymidine-labeled DNA (Figure 4) demonstrated that at 100 μM neither helenalin nor bis(helenalinyl)malonate caused appreciable DNA polynucleotide strand scission. Furthermore, DNA thermal denaturation and DNA viscosity studies demonstrate no effect of helenalin or bis(helenalinyl)-malonate at 100 μM, indicating that the DNA template was

Table III—Effects of Helenalin and Bis(helenalinyl)malonate on Ribonucleotide Pools of P-388 Cell after Three Days of Administration[a]

	Percent of Control (x)[b]					
Drug	XMP[c]	AMP[d]	ADP[e]	ATP[f]	GMP[g]	GDP[h]
Control	100	100	100	100	100	100
Helenalin						
Day 1	38°	103	60	86	46°	78
Day 2	34°	94	102	186°	149°	149°
Day 3	92	164°	198°	145°	258°	258°
Bis(helenalinyl)malonate						
Day 1	84	119	158°	136°	232°	212°
Day 2	30°	22°	39°	45°	169°	124°
Day 3	—	—	71°	49°	227°	119

Drug	GTP[i]	CMP[j]	CDP[k]	CTP[l]	UDP[m]	UTP[n]
Control	100	100	100	100	100	100
Helenalin						
Day 1	89	—	77°	77°	78°	52°
Day 2	61°	26°	94	161°	214°	243°
Day 3	102	122	222°	224°	232°	312°
Bis(helenalinyl)malonate						
Day 1	115	65°	212°	153°	192°	173°
Day 2	67°	12°	124°	102	149°	96
Day 3	124°	44°	119	—	—	88

[a] n = 4. [b] Standard deviations were <15%. [c] Control = 7.9 nmol XMP/10^8 cells. [d] Control = 30 nmol AMP/10^8 cells. [e] Control = 13.6 nmol ADP/10^8 cells. [f] Control = 128.0 nmol ATP/10^8 cells. [g] Control = 35.3 nmol GMP/10^8 cells. [h] Control = 26.1 nmol GDP/10^8 cells. [i] Control = 39.5 nmol GTP/10^8 cells. [j] Control = 60.9 nmol CMP/10^8 cells. [k] Control = 2.5 nmol CDP/10^8 cells. [l] Control = 42.7 nmol CTP/10^8 cells. [m] Control = 3.3 nmol UDP/10^8 cells. [n] Control = 23.3 nmol UTP/10^8 cells. ° $p \leq 0.05$.

not a target of the drug at its therapeutic dose.

Even though the principal compound of Woynarowski's study was parthenolide, a sesquiterpene lactone like helenalin, the compounds differ[34] greatly in structural features. Although both compounds contain an α-methylene-γ-lactone moiety, parthenolide also contains an epoxide moiety that is a structural characteristic of many compounds that cause DNA strand scission. This group may be the structural difference between the two compounds that determines DNA strand hydrolysis. Furthermore, when evaluated in the Ames *Salmonella typhimurium* test for mutagens and potential carcinogens, helenalin was not observed to be mutagenic,[38] while pathenolide was clearly mutagenic[38] in the same assay. Thus, there is essentially no evidence that the pseudoguaianolide type of sesquiterpene lactones interacts with DNA bases of P-388 cells, particularly since template activity for mRNA synthesis was not affected by the drugs.

The activity of DNA polymerase-α, the enzyme believed to carry out DNA replication in eucaryotic cells, was strongly inhibited by the sulfhydryl reagents *p*-chloromercuribenzoate and *N*-ethylmaleimide.[39] Nuclear DNA polymerase activity, probably principally the β-isoenzyme, has been reported to be markedly suppressed by helenalin in Ehrlich ascites carcinoma in vivo and in vitro.[7] This was not confirmed in the present study with P-388 cells (Table I). A number of sesquiter pene lactones have been shown to inhibit the activity of DNA polymerase-α in vitro in P-388 cells.[40] While some inhibition of DNA polymerase-α can be demonstrated by helenalin and bis(helenalinyl)malonate in vivo, it would appear that the magnitude of inhibition is not sufficient to account for the observed inhibition of DNA synthesis in P-388 cells (Figure 6).

Enzymes important to the pyrimidine synthetic pathway were examined, and the sesquiterpene lactones did not affect the synthesis of UMP and CMP. Thymidylate synthetase bears essential sulfhydryl groups that will react with *p*-chloromercuribenzoate, resulting in loss of catalytic activity.[41] Helenalin has previously been shown to markedly decrease thymidylate synthetase activity in Ehrlich ascites tumor cells in vitro at excessively high concentrations[3] of drug and in human KB cells.[42] However, helenalin and bis(helenalinyl)malonate demonstrated no inhibition of this enzymatic activity in P-388 lymphocytic leukemia cells in vivo at the therapeutic doses. This may be due to differences in the enzymes from the carcinoma and the leukemic cells, demonstrating a tissue specificity of the drugs.

The de novo biosynthetic pathway of purines was significantly suppressed by helenalin and bis(helenalinyl)malonate in vivo. Bis(helenalinyl)malonate, the more potent antileukemic agent, inhibited purine biosynthesis to a greater degree than helenalin. The inhibition of the purine biosynthetic pathway by the sesquiterpene lactones would presumably lead to decreased levels of the purine nucleotides and a subsequent decrease in nucleic acid synthesis. The activity of IMP dehydrogenase was also markedly reduced by in vivo administration of either drug in both a dose- and time-dependent fashion. The inhibition of IMP dehydrogenase also demonstrated a pattern of inhibition similar to that observed for the inhibition of formate incorporation into purines over the 3-d period. The fact that IMP dehydrogenase activity was inhibited by the sesquiterpene lactone was reflected in the reduction in XMP levels of P-388 cells, especially since levels of most of the other ribonucleotides are elevated due to the inhibition of the ribonucleoside diphosphate reductase pathway allowing accumulation of di- and triphosphate ribonucleotides.

In normal, nonproliferative tissues, the activity of IMP dehydrogenase is low, and increased enzyme activity is correlated with cell proliferation in both normal and malignant cells; IMP dehydrogenase is one of the rate-limiting steps of purine synthesis and is inhibited by a number of known antineoplastic agents (e.g., the nucleoside 5'-monophosphates of 6-chloropurine, 6-mercaptopurine, and 6-thioguanine).[43,44] Thus, inhibition of this enzyme step is a feasible mode of action of antineoplastic agents such as the sesquiterpene lactones.

The ribonucleoside diphosphate reductase pathway is an enzyme complex responsible for converting ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates for subsequent DNA synthesis. The ribonucleoside diphosphate reductase enzyme requires hydrogen donors for activity and, in the reduced state, the sulfhydryl groups of the enzymes may be a possible target for thiol-reactive agents such as helenalin and bis(helenalinyl)malonate. This assay utilizes an added sulfhydryl compound (dithioerythritol) as reductant, which provides the hydrogen ion for the reductase reaction. Under these conditions, helenalin and bis(helenalinyl)malonate did not inhibit ribonucleoside diphosphate reductase activity. However, measurement of the ribonucleoside reductase activity without dithioerythritol demonstrated >40% inhibition by helenalin on day 3 and >70% inhibition by bis(helenalinyl)malonate, suggesting that one or more of the endogenous hydrogen donors of this complex (i.e., the thioredoxin and glutaredoxin system) had been inactivated by the sesquiterpene lactones.

The thioredoxin activity was inhibited in a manner parallel to the overall ribonucleotide reductase activity, demonstrating >60% inhibition by helenalin and 95% by bis(helenalinyl)malonate on day 3. Subsequent in vitro studies have shown that the sesquiterpene lactones also inhibit glutaredoxin in KB[42] and P-388[45] tissue culture cells. Inactivation of thioredoxin by the sesquiterpene lactones would indirectly inhibit the ribonucleoside diphosphate reductase pathway, leading to a depletion of the deoxyribonucleoside triphosphate pools needed for DNA synthesis. The reduction in deoxyribonucleoside triphosphate pools of P-388 cells in vivo as a result of the reduction of riboncleotide reductase was observed. This reduction in d NTP) pools would be consistent with the observed reduction in DNA synthesis afforded by the elevations in drug levels on day 3, and may be due to blocking of DNA polymerase-α, thus allowing d(NTP) accumulation. The accumulation of ribonucleotide compounds, particularly the ribonucleoside diphosphates, was also evident; this finding is consistent with suppression of ribonucleotide reductase by the drugs.

Previous studies have established that sesquiterpene lactones are capable of inactivating a number of thiol containing enzymes.[6-8,46-48] Thus, it appears likely that the inhibition of IMP dehydrogenase and thioredoxin of the ribonucleoside reductase system by helenalin and bis(helenalinyl)malonate occurs via thiol addition. Because of the reported inhibition of IMP dehydrogenase activity by thiol-reactive agents, it is proposed that helenalin and bis(helenalinyl)malonate alkylate the enzyme sulfhydryl groups, involving the α-methylene-γ-lactone and/or α,β-unsaturated cyclopentenone moieties of the sesquiterpene lactones, by a Michael-type addition, thus inactivating the enzyme. Selectivity for the thiol groups of enzymes involved in DNA synthesis was evident since these drugs did not inhibit other thiol-bearing enzymes in the pathway. These two agents are the only sesquiterpene lactones that have been reported to date to exert their antineoplastic activity by selectively inhibiting thiol-bearing enzymes.

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