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Antitumor Agents XLVII: The Effects of Bisbrusatolyl Malonate on P-388 Lymphocytic Leukemia Cell Metabolism

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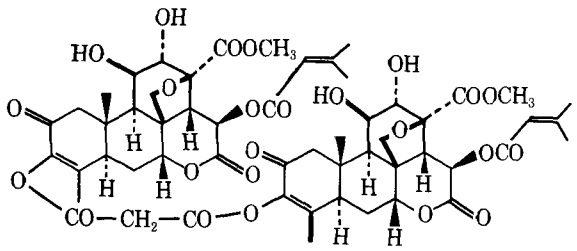
Abstract □ Bisbrusatolyl malonate, which was shown previously to be active against P-388 lymphocytic leukemia cell growth, was investigated for inhibitory effects on nucleic acid and protein synthesis. DNA and RNA synthesis as well as protein synthesis were markedly inhibited at 10, 25, and 50 μmole final concentrations *in vitro*. The major sites of inhibition of nucleic acid synthesis appeared to be DNA polymerase, messenger and transfer RNA polymerases, orotidine-5′-monophosphate decarboxylase, phosphoribosyl pyrophosphate amino transferase, and dihydrofolate reductase. Moderate inhibition of nucleotide kinase activities and oxidative phosphorylation processes occurred after drug treatment. Cyclic adenosine monophosphate levels were reduced. Protein synthesis was inhibited during the elongation step of peptide synthesis. The data suggested that bisbrusatolyl malonate interfered with the peptide bond formation. However, the ongoing polypeptide synthesis must be completed before the drug can bind to the ribosome effectively.

Keyphrases □ Bisbrusatolyl malonate—effects on P-388 lymphocytic leukemia cell metabolism □ Antitumor agents—effects of bisbrusatolyl malonate on P-388 lymphocytic leukemia cell metabolism □ RNA—synthesis, effect of bisbrusatolyl malonate, P-388 lymphocytic leukemia cells □ DNA—synthesis, effect of bisbrusatolyl malonate, P-388 lymphocytic leukemia cells □ Protein synthesis—effect of bisbrusatolyl malonate, P-388 lymphocytic leukemia cells

Antineoplastic activity against P-388 lymphocytic leukemia growth was established previously for bisbrusatolyl esters (1, 2). One of these derivatives, bisbrusatolyl malonate at 0.6 mg/kg/day ip, gave T/C % values of 271, 197, and 188 in BDF_1 male mice (2) in a P-388 tumor model sensitive to quassinoids (2). This agent was shown to suppress *in vitro* DNA synthesis by 45%, RNA synthesis by 48%, and protein synthesis by 83% at 10 μM concentration after 90 min incubation. A number of enzymes involved in nucleic acid metabolism, *e.g.*, DNA polymerase and dihydrofolate reductase, were inhibited *in vitro* by this ester (2). A detailed study of the effects of bisbrusatolyl malonate on P-388 lymphocytic leukemia cellular metabolism was conducted and its effects on nucleic acid and protein metabolism are now reported.

EXPERIMENTAL

Bisbrusatolyl malonate (I) was synthesized and characterized previously in the literature (2). The P-388 lymphocytic leukemia tumor line was maintained in DBA/2 male mice (~20 g). For the *in vitro* studies,



I

P-388 cells were harvested from the peritoneal cavity 10 days after administering 10^6 P-388 lymphocytic leukemia cells intraperitoneally into BDF_1 male mice (~20 g) on day 0 (3). In the *in vivo* studies, BDF_1 male mice were inoculated with 10^6 P-388 cells intraperitoneally, and on days 7, 8, and 9 the mice were administered 0.6 mg/kg/day bisbrusatolyl malonate intraperitoneally. The biochemical studies were performed on cells harvested from the peritoneal cavity on day 10.

The *in vitro* incorporation studies (4) were conducted using 1 μCi [6-^{3}H]thymidine (21.8 Ci/mmole), [6-^{3}H]uridine (22.4 Ci/mmole), or [4,5-^{3}H(N)]L-leucine (56.5 Ci/mmole) with 10^6 P-388 cells in minimum essential medium, 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 by vacuum suction. RNA and protein assays were terminated with trichloroacetic acid and collected on nitrocellulose membranes by vacuum suction. The acid-insoluble precipitates on the filter papers were placed in scintillation vials and counted. *In vivo* thymidine incorporation into DNA was determined by injecting into the animal 10 μCi of [^{3}H]-methylthymidine (24.7 Ci/mmole) intraperitoneally 1 hr before sacrifice. The DNA was isolated (5) and the [^{3}H]thymidine content determined in scintillation fluid[1] 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 dpm/mg of DNA isolated. Uridine incorporation into RNA was determined in an analogous manner with 10 μCi of [^{3}H]uridine (20 Ci/mmole) and the RNA extracted (6). Leucine incorporation into protein was determined by the method of Sartorelli (7) with 10 μCi[^{3}H]L-leucine (56.5 Ci/mmole). The control values for DNA synthesis were 202,098 dpm/mg of DNA, 235,360 dpm/mg of RNA for RNA synthesis, and 99,102 dpm/mg of protein isolated for leucine incorporation.

On day 10 after *in vivo* administration of the drug, the number of tumor cells per milliliter and the 0.4% trypan blue uptake were determined with a hemocytometer (8). The *in vitro* UV binding studies were conducted with I (50 μg/ml) incubated with DNA (38 μg/ml) in 0.1 *M* phosphate

[1] Fisher Scintiverse.

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Table I—*In Vitro* Effects of Bisbrusatolyl Malonate on Nucleic Acid and Protein Synthesis of P-388 Lymphocytic Leukemia Cells after 60-min Incubation

	% Control, whole cells		
	DNA Synthesis	RNA Synthesis	Protein Synthesis
Control (0.05% polysorbate 80)	100 ± 6[a]	100 ± 5[b]	100 ± 5[c]
Bisbrusatolyl malonate			
10 μM	58 ± 3[d]	78 ± 5[d]	52 ± 6[d]
25 μM	54 ± 7[d]	65 ± 6[d]	18 ± 3[d]
50 μM	57 ± 6[d]	92 ± 7	8 ± 2[d]
	% Control homogenized cells		
	DNA Synthesis	RNA Synthesis	Protein Synthesis
Control (0.05% polysorbate 80)	100 ± 7	100 ± 8	100 ± 6
Bisbrusatolyl malonate			
10 μM	59 ± 6[d]	72 ± 5	48 ± 4[d]

[a] 37659 dpm/10^6 cells/hr. [b] 42981 dpm/10^6 cells/hr. [c] 77421 dpm/10^6 cells/h. [d] $p \leq 0.001$.

buffer (pH 7.2) from 0–24 hr and measured over the range of 200–340 nm (8).

The *in vitro* enzymatic studies were conducted with I present in a final concentration of 10 μM. The *in vitro* and *in vivo* enzymatic assays have been described previously (4). Nuclear DNA polymerase activity was determined on isolated P-388 cell nuclei (9) using the incubation medium of Sawada *et al.* (10), except that [^{3}H]methylthymidine triphosphate (78.1 Ci/mmole) was used. The insoluble nucleic acids were collected on glass fiber papers. 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 (11), and the individual polymerase activities were measured using [5,6-^{3}H]-uridine-5-triphosphate (23.2 Ci/mmole). Insoluble ribonucleic acids were collected on nitrocellulose filters (11, 12). Control values for messenger, ribosomal, and transfer RNA polymerase activities were 1807, 2987, and 2270 dpm/mg of nucleic protein, respectively. Ribonucleotide reductase activity was measured by a previous method (13) using [^{14}C]cytidine-5′-diphosphate (25 Ci/mmole). The deoxyribonucleotides were separated from ribonucleotides by polyethyleneiminecellulose TLC. The control value for the reductase was 22,392 dpm/mg of protein. Deoxythymidine kinase, deoxythymidylate monophosphate kinase, and deoxythymidylate diphosphate kinase activities were measured by spectrophotometric assay (14) based on the disappearance of nadide at 340 nm. Control values using a postnuclear supernate (600×*g* for 10 min) were a change in absorbance of 0.365, 0.182, and 0.368 o.d. units/hr/mg of protein, respectively. Enzymes of the pyrimidine synthetic pathway were also measured. Carbamyl phosphate synthetase activity was determined by a previous method (15). The colorimetric determination of citrulline was also performed according to a previous method (16), resulting in 3.26 mg of citrulline formed/hr/μg of protein. Aspartate carbamyl transferase activity was carried out in the presence of aspartate transcarbamylase (15) and the colorimetric determination (17) of the carbamyl aspartate that formed resulted in 0.301 μmole formed/hr/mg of protein. Orotidine-5′-phosphate decarboxylase activity was measured using a 16,300×*g* (20 min) supernate by a technique (18) using [^{14}C]carbonyl orotidine-5′-monophosphate (34.9 mCi/mmole). The control value was 39,059 dpm/mg of protein. Thymidylate synthetase activity was determined by a method (19) utilizing a postmitochondrial supernate (9000×*g* for 10 min) with 5 μCi of [5-^{3}H]deoxyuridine monophosphate (11 Ci/mmole), giving a control value of 12,374 dpm/hr/mg of protein. Dihydrofolate reductase activity was determined using a 600×*g* (10 min) supernate by a spectrophotometric method (20) based on the disappearance of reduced nadide resulting in a value of 0.761 o.d. unit/hr/mg of protein for 10-day P-388 cells. [^{14}C]-Formate incorporation into purines was measured by a method (21) using 0.5 μCi of [^{14}C]formic acid (4.95 mCi/mmole). Purine separation was achieved by silica gel TLC eluting with *n*-butanol–acetic acid–water (4:1:5). Using guanine and adenine standards, the appropriate spots were scraped and the radioactivity determined. The control value for purine synthesis was 10,621 dpm/mg of protein. Phosphoribosyl pyrophosphate amino transferase activity was determined by a spectrophotometric method (22) at 340 nm using a 600×*g* (10 min) supernate, resulting in a control value of 0.806 o.d. unit/hr/mg of protein. Inosinic acid dehydrogenase activity was determined by a spectrophotometric method (23) at 340 nm. The control value for 10-day P-388 cells was 0.133 o.d. unit/min/μg of protein for a 600×*g* (10 min) supernate. Deoxyribonuclease activity was measured at pH 5.0 by a modification of the deDuve method (24). Ribonuclease and acid cathepsin activities were determined at pH 5.0 by a previous method (25). The control deoxyribonuclease activity was 1.72 μg of DNA hydrolyzed/min/mg of protein; ribonuclease activity was 7.24 μg RNA hydrolyzed/min/mg of protein. Cathepsin activity was 65.7 μg of azocasein hydrolyzed/min/mg of protein for 10-day P-388 cells.

Table II—*In Vitro* Effects of Bisbrusatolyl Malonate on Enzymatic Activities of P-388 Lymphocytic Leukemia Cells

Enzyme (n = 6)	% Control: Control (0.05% Polysorbate 80)	% Control: Bisbrusatolyl Malonate, 10 μM
Deoxyribonucleic acid polymerase	100 ± 12	43 ± 8[a]
Messenger ribonucleic acid polymerase	100 ± 8	75 ± 11[b]
Ribosomal ribonucleic acid polymerase	100 ± 3	84 ± 10[b]
Transfer ribonucleic acid polymerase	100 ± 3	66 ± 7[a]
Ribonucleotide reductase	100 ± 12	88 ± 9
d-Thymidine kinase	100 ± 7	72 ± 5[a]
d-Thymidine monophosphate kinase	100 ± 13	50 ± 6[a]
d-Thymidine diphosphate kinase	100 ± 6	79 ± 9[b]
Carbamyl phosphate synthetase	100 ± 3	85 ± 8[b]
Aspartate carbamoyltransferase	100 ± 9	107 ± 12
Orotidine-5′-phosphate decarboxylase	100 ± 11	69 ± 4[a]
Thymidylate synthetase	100 ± 11	82 ± 6[c]
Dihydrofolate reductase	100 ± 13	71 ± 10[b]
^{14}C-Formate incorporation into purines	100 ± 6	45 ± 6[a]
Phosphoribosyl pyrophosphate aminotransferase	100 ± 3	69 ± 5[a]
Inosinic acid dehydrogenase	100 ± 12	94 ± 10
Deoxyribonuclease	100 ± 8	33 ± 7[a]
Ribonuclease	100 ± 5	16 ± 7[a]
Cathepsin	100 ± 7	72 ± 6[a]
Histone phosphorylation	100 ± 5	111 ± 6
Nonhistone phosphorylation	100 ± 5	89 ± 4[b]
S-Adenosyl-L-methionine methyl transferase	100 ± 6	53 ± 7
Oxidative phosphorylation processes		
Succinate		
State 4	100 ± 4	84 ± 3[a]
State 3	100 ± 3	76 ± 5[a]
α-ketoglutarate		
State 4	100 ± 5	71 ± 3[a]
State 3	100 ± 4	78 ± 3[a]

$p \leq 0.005$. [c] $p \leq 0.010$.

Histone phosphorylation was determined by injecting 10 μCi of [γ-^{32}P]adenosine triphosphate (27 Ci/mmole) intraperitoneally. The nuclei were isolated 1 hr later and the histone chromatin protein extracted (26). Nonhistone chromatin phosphorylation by nucleic protein kinase was determined on isolated nuclei (9), utilizing 2 mmoles of [γ-^{32}P]adenosine triphosphate. Chromatin protein was collected on nitrocellulose membrane filters (27) and cyclic adenosine triphosphate levels were determined with a commercial radioimmunoassay kit[2] using iodine 125. Methyl transferase activity was determined by a method (28) using 0.05 μCi of [$^{14}CH_3$]*S*-adenosyl-L-methionine (53 mCi/mmole). The control value for histone phosphorylation was 1211 dpm/mg of chromatin protein isolated; nonhistone phosphorylation was 2391 dpm/mg of protein, and cyclic adenosine phosphate was 1.25 pmoles/10^6 cells. Methyl transferase activity for the control was 217 dpm/mg of protein. Oxidative phosphorylation studies were carried out on 9-day P-388 cells (29). Oxygen consumption was measured with a Clark electrode connected to an oxygraph. The reaction vessel contained 55 μmoles of sucrose, 22 μmoles of monobasic potassium phosphate, 22 μmoles of potassium chloride, 90 μmoles of succinate or 60 μmoles of α-ketoglutarate as substrate, and 22 μmoles of adenosine triphosphate. The final I concentration was 10 μmoles. After the basal metabolic level (state 4) was reached, 0.257 μmole of adenosine diphosphate was added to the vessel to obtain the adenosine diphosphate-stimulated respiration rate (state 3). Basal respiration (state 4) using succinate as substrate resulted in 11.44 μl O_2 consumed/min/mg of protein and 10.0/μl of O_2 consumed/min/mg of protein using α-ketoglutarate. The adenosine diphosphate stimulated respiration rate for succinate was 20.76, and for α-ketoglutarate 10.17 μl of O_2 consumed/min/mg of protein.

The studies on the effects of I on protein synthesis were conducted on P-388 cells harvested on day 10. P-388 lymphocytic leukemia lysates were

[2] Becton Dickinson.

Table III—Effects of Bisbrusatolyl Malonate on P-388 Lymphocytic Leukemia Cells *In Vivo*

	Control	Bisbrusatolyl Malonate, 0.6 mg/kg/day, days 7, 8, 9
Incorporation Studies		
Thymidine into DNA	100 ± 9	41 ± 5[a]
Uridine into RNA	100 ± 8	53 ± 9[a]
Leucine into protein	100 ± 4	34 ± 7[a]
Enzymatic Studies		
DNA polymerase	100 ± 8	44 ± 7[a]
Messenger RNA polymerase	100 ± 6	61 ± 5[a]
Ribosomal RNA polymerase	100 ± 6	83 ± 6[b]
Transfer RNA polymerase	100 ± 7	50 ± 7[a]
Ribonucleotide reductase	100 ± 6	108 ± 8
d-Thymidine kinase	100 ± 5	68 ± 4
d-Thymidine monophosphate kinase	100 ± 8	63 ± 6[a]
d-Thymidine diphosphate kinase	100 ± 10	77 ± 8[b]
Carbamyl phosphate synthetase	100 ± 8	102 ± 7
Aspartate carbamyl transferase	100 ± 12	100 ± 8
Orotidine monophosphate decarboxylase	100 ± 10	27 ± 3[a]
Thymidylate synthetase	100 ± 6	89 ± 5
Dihydrofolate reductase	100 ± 11	12 ± 7[a]
[^{14}C]Formate incorporation into purines	100 ± 12	55 ± 11[a]
Phosphoribosyl pyrophosphate aminotransferase	100 ± 4	59 ± 7[a]
Inosinic acid dehydrogenase	100 ± 10	100 ± 8
Histone phosphorylation	100 ± 12	136 ± 13[b]
Non-histone phosphorylation	100 ± 4	79 ± 7[a]
Cyclic adenosine monophosphate levels	100 ± 8	20 ± 5[a]
Oxidative Phosphorylation Studies		
Succinate		
State 4	100 ± 4	75 ± 7[a]
State 3	100 ± 4	66 ± 4[a]
α-ketoglutarate		
State 4	100 ± 3	72 ± 5[a]
State 3	100 ± 5	70 ± 2[a]
Number cells × 10^6/ml ascites fluid	100 ± 6	44 ± 5[a]

[a] $p \leq 0.001$. [b] $p \leq 0.005$. [c] $p \leq 0.010$.

prepared by the method of Kruh *et al.* (30). The following were isolated from P-388 lysates by literature techniques: run-off ribosomes (31), pH 5 enzyme (30), and uncharged transfer RNA (32).The P-388 lymphocytic leukemia cell initiation factors for protein synthesis were prepared by the method of Majumdar (33) O^{3}H methionyl transfer RNA was prepared from P-388 cell transfer RNA (tRNA) by a previous method (34). The effect of I on endogenous protein synthesis (35) of P-388 lysates was carried out in a reaction mixture (0.5 ml) containing 10 m*M* tromethamine hydrochloride (pH 7.6), 76 m*M* KCl, 1 m*M* adenosine triphosphate, 0.2 m*M* guanosine triphosphate, 15 m*M* phosphocreatine, 2 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 0.1 m*M* of each of the 19 essential amino acids, 0.9 mg/ml creatine phosphokinase, and 20 μCi [^{3}H]leucine (56.5 Ci/mmole). An aliquot of the reaction mixture was incubated at 30°. After 90 sec of incubation, I or the standards pyrocatechol violet or emetine was added to a final concentration of 100 μ*M*. At 1-min intervals, 50-μl aliquots were removed from the reaction tubes and spotted on filter papers[3] and then treated for 10 min in boiling 5% trichloroacetic acid, followed by 10 min in cold 5% trichloroacetic acid and washed with cold 5% trichloroacetic acid, ether–ethanol (1:1), and ether. The filter papers were dried and counted in scintillation fluid.

The effects of I, pyrocatechol violet, and emetine on the ribosome profile (35) of P-388 cell lysate were assayed using the described reaction medium (500 μl). Following drug addition (100 μ*M* final concentration) the reaction was incubated for 4 min at 37°. The reaction was terminated in ice and gradient buffer [1 ml of tromethamine hydrochloride (pH 7.6) 10 m*M* KCl, and 1.5 m*M* $MgCl_2 \cdot 6H_2O$] was added. The mixture was layered over 36 ml of 10–25% linear sucrose gradient (35), prepared in gradient buffer, and centrifuged for 165 min at 25,000 rpm in a swinging bucket rotor[4] at 4°. The absorbance profile at 260 nm was determined using a flow cell (light path 0.2 cm) attached to a spectrophotometer[5].

The reaction mixture (50 μl) for the polyuridine directed polyphenylalanine synthesis (36) contained 50 m*M* tromethamine hydrochloride (pH 7.6), 12.5 m*M* magnesium acetate, 80 m*M* KCl, 5 m*M* phospho-

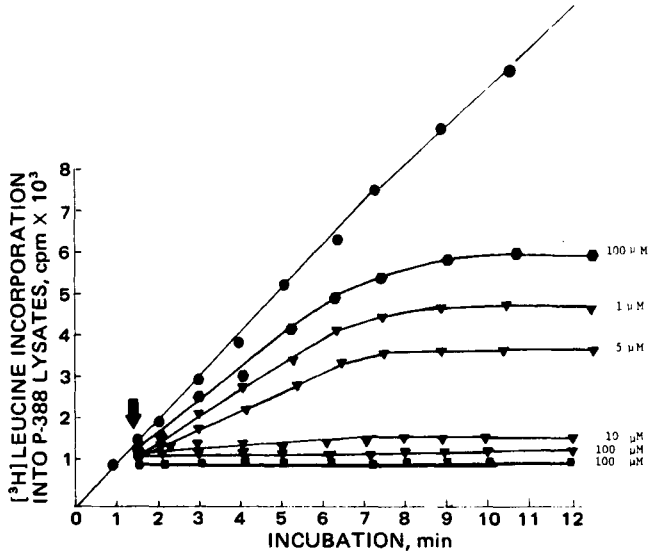

Figure 1—*Effects of bisbrusatolyl malonate on the protein synthesis of P-388 lysates using endogenous mRNA. Key:* ●, *control;* ●, *pyrocatechol violet;* ▼, *bisbrusatolyl malonate; and* ■, *emetine.*

creatine, 0.05 mg/ml creatine phosphokinase, 0.36 mg/ml polyuridine[6] (A_{280}/A_{260} = 0.34), 0.5 μCi [^{14}C]phenylalanine (536 mCi/mmole) 75 μg uncharged P-388 cell transfer RNA, 70 μg of P-388 cell pH 5 enzyme preparation, and 0.9 A_{260} of P-388 cell run-off ribosomes. After incubation for 20 min at 30°, a 35-μl aliquot was spotted on filter paper[3] and processed as described previously.

The reaction medium (200 μl) used to measure the formation of the 80S initiation complex and the methionyl puromycin reaction (37) contained 15 m*M* tromethamine hydrochloride (pH 7.6), 80 m*M* KCl, 1 m*M* adenosine triphosphate, 0.5 m*M* guanosine triphosphate, 20 m*M* phosphocreatine, 0.2 mg/ml creatine phosphokinase, 3 m*M* magnesium acetate, 0.1 m*M* ethylenediaminetetraacetic acid, 1 m*M* dithiothreitol, 0.1 m*M* of each of the 19 essential amino acids, 3 mg of P-388 cell lysates, 100 μg/ml chlortetracycline[7], 3 × 10^5 cpm [^{3}H]methionine-transfer ribonucleic acid$_f$ (Met-tRNA$_f$), and 20 μg/ml polyadenosine–uridine–guanosine (poly AUG) and 5 μl of drug. The incubation was carried out at 23° and after 2 min aliquots were withdrawn to analyze for 80S complex formation. Puromycin (10 μg/ml) was then added to the reaction medium. The incubation was continued for another 6 min and aliquots were withdrawn to analyze for reaction of the 80S complex with puromycin. All aliquots (50 μl) were diluted to 250 μl with buffer [20 m*M* tromethamine hydrochloride (pH 7.6), 80 m*M* KCl, 3 m*M* magnesium acetate, 1 m*M* dithiothreitol, and 0.1 m*M* ethylenediaminetetraacetic acid], layered on 11.8 ml of a 15–30% linear sucrose gradient, and centrifuged for 3 hr at 36,000 rpm in a swinging bucket rotor[8]. Fractions (0.4 ml) were collected and precipitated with 10% trichloroacetic acid on filter papers and counted.

The reaction mixtures (75 μl) for the ternary complex formation (37) contained 21.4 m*M* tromethamine hydrochloride (pH 8.0), 80 m*M* KCl,

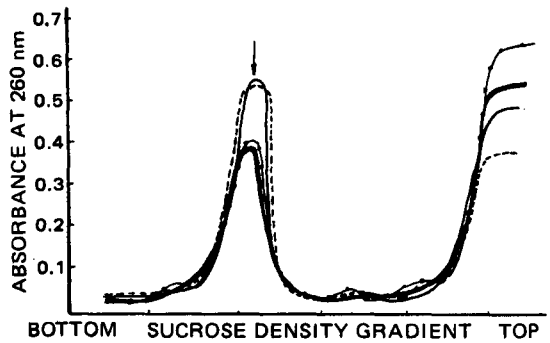

Figure 2—*P-388 ribosome profile. Key: – – –, bisbrusatolyl malonate; ——, pyrocatechol violet; ——, emetine, and – · – · –, control.*

[3] Whatman No. 3.
[4] Beckman SW27.
[5] Gilford.
[6] Miles Laboratory, Inc.
[7] Sigma Chemical Co.
[8] Beckman SW40 rotor.

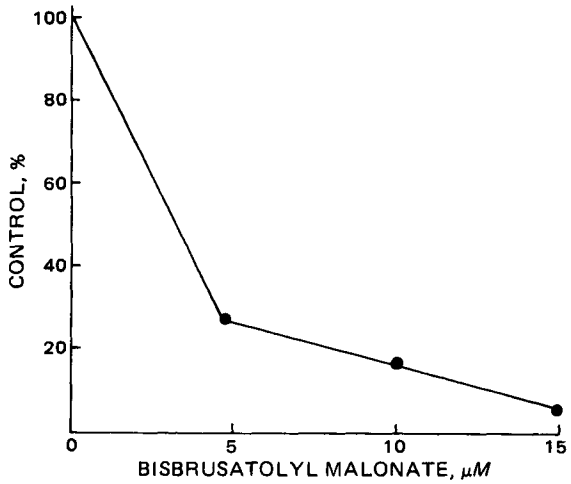

Figure 3—*Effect of various concentrations of bisbrusatolyl malonate on polyuridine directed poly[^{14}C]phenylalanine synthesis in P-388 run-off ribosomes.*

0.26 m*M* guanosine triphosphate, 21.4 m*M* dithiothreitol, 10 μg of bovine serum albumin, 5 pmoles of P-388 cell [^{3}H]methionine-transfer ribonucleic acid$_f$ (Met-tRNA$_f$, 1 × 10^4 cpm), 100 A_{260}/ml of crude P-388 cell initiation factors, and 10 μl of drug or standard. The incubation was conducted for 5 min at 37° and terminated by the addition of 3 ml of cold buffer [21.4 m*M* tromethamine hydrochloride (pH 8.0), 80 m*M* KCl, 2.14 m*M* dithiothreitol]. The samples were filtered through 0.45 μm filters[9], washed twice in buffer, and counted.

The reaction mixture (75 μl) for the 80S initiation complex (38) formation contained 1.9 m*M* magnesium acetate, 5 A_{260}/ml polyadenosine–uridine–guanosine[5], and 100 A_{260}/ml of 80S P-388 cell ribosomes in addition to the components necessary for the ternary complex formation reaction. Incubation was 10 min at 37° which was then cooled to 4° and titrated to 5 m*M* with magnesium acetate. After 5 min at 4°, the samples were diluted with cold buffer [21.4 m*M* tromethamine hydrochloride (pH 8.0) 80 m*M* KCl, 5 m*M* magnesium acetate, and 2.14 m*M* dithiothreitol] and filtered as indicated for the ternary complex formation experiment.

Amino acid-transfer RNA activation steps were determined by a previous method (39). The reaction medium contained 0.1 m*M* tromethamine hydrochloride (pH 7.4), 0.2 m*M* adenosine triphosphate, 0.3 mg/ml pH 5 enzyme from P-388 cells, and 2.5 μCi/ml of [^{14}C]phenylalanine (536 mCi/mmole), [^{3}H]leucine (56.5 Ci/mmole), or [^{3}H]methionine (80.0 Ci/mmole) in a total volume of 1 ml. After incubation at 37° for 20 min, 2 ml of ice cold 10% trichloroacetic acid was added and the activated amino acid-transfer RNA collected on nitrocellulose filters.

RESULTS AND DISCUSSION

In vitro incubation of I at 10-, 25-, and 50-μ*M* concentrations indicated that DNA synthesis was inhibited by relatively the same degree (42–46%) for all three concentrations employed (Table I). RNA synthesis was inhibited at 10 and 25 μ*M* but not at 50 μ*M*. The inhibition of protein synthesis *in vitro* followed a dose response curve for the doses employed in this study. Homogenized and whole cells demonstrated approximately the same degree of inhibition of DNA, RNA, and protein synthesis at 10 μ*M* final concentration of drug indicating that I inhibition of macromolecular synthesis was not due to the inhibition of radiolabeled precursor transport across the tumor cell membrane. The *in vitro* effects of I at 10 μ*M* on several of the enzymes required for nucleic acid synthesis (Table II) indicated that the major inhibition sites are at DNA polymerase, thymidine monophosphate kinase, purine synthesis, and methyl transferase, all of which were suppressed ~50% by I *in vitro*. Minor sites of *in vitro* inhibition included RNA polymerase, thymidine kinase, orotidine monophosphate decarboxylase, dihydrofolate reductase, phosphoribosyl pyrophosphate amino transferase, and oxidative phosphorylation processes, both basal and adenosine diphosphate stimulated respiration using succinate or α-ketoglutarate as substrate.

Examination of the effects of I on lysosomal hydrolytic enzymatic activities indicated that lysosomal membranes were stabilized by the drug. Thus, nucleases and proteolytic enzymatic activities of the lysosomes of the P-388 cells would not play a decreased role in nucleic acid and protein turnover, thereby resulting in erroneous incorporation values of radiolabeled precursors into synthetic macromolecules.

In vivo administration (Table III) of I at 0.6 mg/kg on days 7, 8, and 9 showed that the incorporation of thymidine, uridine, and leucine into DNA, RNA, and protein were drastically reduced (*i.e.*, at least 50% for nucleic acids and 66% for protein synthesis). Examination of the enzymes involved in nucleic acid synthesis indicated that DNA polymerase activity was inhibited 56%, messenger RNA polymerase activity was inhibited by 39%, and transfer RNA polymerase was inhibited by 50%. The suppression of the nucleic acid polymerase activities would indicate that template activity is interfered with by the drug binding in some manner. However, the UV spectrophotometric studies indicated only marginal interaction between DNA and I after incubation for 24 hr. Nucleotide kinase activity was inhibited moderately (23–37%). Orotidine-5′-monophosphate decarboxylase was inhibited significantly, inhibition being far greater *in vivo* (73%) than *in vitro* (31%). This difference may be due to a metabolite or may reflect slow binding between enzyme and drug. No other enzyme in the pyrimidine pathway was inhibited by I. Purine synthesis was inhibited 45%; one of the key regulatory sites of purine synthesis, phosphoribosyl pyrophosphate amino transferase was inhibited 41%. However, another enzyme that plays a significant role in one carbon transfer for both pyrimidine and purines, dihydrofolate reductase, was 88% inhibited *in vivo* whereas *in vitro* it was only 29% inhibited. Cyclic adenosine triphosphate levels were inhibited by 80%. Oxidative phosphorylation after *in vivo* administration of the drug was moderately inhibited (34–25%). Although a moderate loss of energy from this process could influence all synthetic pathways of the tumor cell. The number of tumor cells per milliliter was reduced by 56% after dosing for 3 days with I at 0.6 mg/kg.

In comparison to brusatol's effects on P-388 lymphocytic leukemia cell metabolism at 100 μg/kg/day (4), I was found to inhibit DNA polymerase and dihydrofolate reductase activities more effectively than brusatol both *in vivo* and *in vitro*. Furthermore, I inhibited thymidine nucleotide kinases enzymatic activities where no inhibition of the kinases was observed

Table IV—Effects of Bisbrusatolyl Malonate on Ternary and 80S Complex Formation

	Concentration, μ*M*	Complex Formation, pmole	Percent of Control
Ternary Complex Formulation			
Control		2.10	100
+ Emetine	100	2.02	96
+ Pyrocatechol violet	100	0.21	10
+ Bisbrusatolyl malonate	25	1.81	86
80S Initiation Complex Formation			
Control		1.82	100
+ Emetine	100	1.49	82
+ Pyrocatechol violet	100	0.36	20
+ Bisbrusatolyl malonate	25	1.51	83

Table V—Effects of Bisbrusatolyl Malonate on Amino Acid Transfer RNA Activation in P-388 Lymphocytic Leukemia Cells

Inhibitor	Concentration	Amino Acid Transfer RNA Formation, pmol	Percent of Control
[^{14}C]Phenylalanine-transfer RNA			
Control	—	1.05	100
+ Emetine	100	0.97	92
+ Pyrocatechol violet	100	0.98	93
+ Bisbrusatolyl malonate	50	0.97	92
]^{3}H]Leucyl-transfer RNA			
Control	—	1.83	100
+ Emetine	100	1.74	95
+ Pyrocatechol violet	100	1.72	94
+ Bisbrusatolyl malonate	50	1.76	96
[^{3}H]Methionyl-transfer RNA			
Control	—	1.62	100
+ Emetine	100	1.54	95
+ Pyrocatechol violet	100	1.57	97
+ Bisbrusatolyl malonate	50	1.52	94

[9] Millipore.

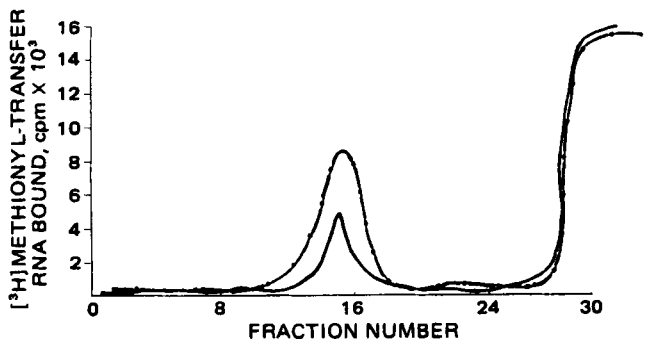

Figure 4—*Formation of the 80S initiation complex of P-388 cell system (linear sucrose gradient centrifugation). Key: ——, control; - · - · -, bisbrusatolyl malonate.*

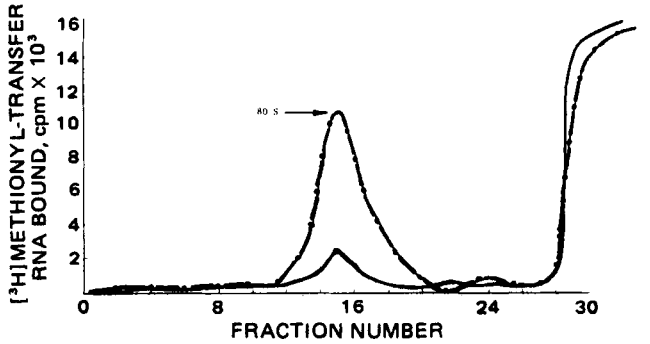

Figure 5—*Formation of the methionyl puromycin reaction of P-388 cell system (linear sucrose gradient centrifugation). Key: ——, control and puromycin; - · - · -, bisbrusatolyl malonate and puromycin.*

with brusatol. Brusatol caused a massive increase in cyclic adenosine monophosphate levels which was not observed for I. Brusatol caused a greater magnitude of inhibition of nucleic acid and protein synthesis in P-388 cells than I; however, this greater suppression did not correlate with increased antileukemic activity (T/C % = 158) for brusatol.

When the effect on protein synthesis of the P-388 cells was examined in detail, I clearly mimicked the action of brusatol and bruceantin. Previous studies with bruceantin in a yeast system (40) and brusatol in a rabbit reticulocyte system (41) have shown that these drugs bind preferentially to the free 80S subunit and subsequently inhibit any peptidyl transferase reaction. Thus, they allowed elongation of preformed polypeptide chains and formation of a new 80S initiation complex, but prevented utilization of that 80S initiation complex in the elongation steps of protein synthesis. The malonate ester has a similar mode of action.

Figure 1 shows that at a 1 μM concentration there is a lag of 6–7 min before inhibition by I becomes fully effective. This behavior is similiar to the initiation inhibitor pyrocatechol violet and suggests that preformed polypeptide chains are being completed prior to the onset of inhibition. This hypothesis is further confirmed by examining the ribosome profile following drug treatment of lysates prelabeled with [^{3}H]leucine (Fig. 2). At 1 μM, I causes accumulation of an 80S peak (presumably the 80S initiation complex). Since no radioactivity is associated with this peak, it is evident that 1 μM I also allows completion and release of the prelabeled polypeptide chain. These results are comparable to what one might expect to see from an initiation inhibitor (pyrocatechol violet). However, at higher concentrations (10 and 100 μM) I mimics the elongation inhibitors emetine and tetracycline. That is, inhibition occurs without any lag (Fig. 1) and results in freezing the preformed polypeptide chain on the ribosome (Fig. 2). This observation is consistent with previous data showing that at low concentrations, bruceantin binds only to free 80S subunits; however, at sufficiently high concentrations, it will also bind directly to the 80S elongation complex (41).

Further evidence that I acts as an elongation inhibitor comes from studies on polyuridine directed polyphenylalanine synthesis. Figure 3 shows the inhibition curve for polyphenylalanine synthesis carried out with purified run-off ribosomes isolated from 10-day P-388 cells. Clearly, the drug is effective as an inhibitor of polyuridine directed and endogenous protein synthesis. Since polyuridine directed protein synthesis does not require normal initiation or termination reactions, these data strongly suggest that I is an elongation inhibitor.

Confirmation of this hypothesis comes from detailed studies of the individual initiation and elongation steps. The initiation steps of protein synthesis were evaluated using a fractionated P-388 cell system. Bisbrusatolyl malonate (Table IV) had little or no effect on the formation of either the ^{3}H-ternary complex[10] or the 80S ^{3}H-initiation complex[11]. Bisbrusatolyl malonate also had no effect on the amino acid transfer RNA activation reactions with phenylalanine, leucine, and methionine (Table V).

Finally, the formation of the 80S initiation complex[11] and peptide bond formation were assayed as described previously (41). Addition of polyadenine–uridine–guanosine (poly AUG) to the P-388 lysate resulted in the formation of a detectable 80S initiation complex which was not inhibited by the addition of I (Fig. 4). Addition of puromycin to this reaction mixture caused formation of methionyl puromycin and consequent release of [^{3}H]methionine from the 80S complex (Fig. 5). This reaction was completely blocked by I.

[10] eIF·GTP[^{3}H]methionyl-transfer RNA.
[11] 80S·AUG·eIF·GTP[^{3}H]methionyl-transfer RNA.

In conclusion, I does not significantly affect the individual initiation steps leading to the formation of stable 80S initiation complex in P-388 cells. However, it does inhibit both polyuridine directed polyphenylalanine synthesis and the formation of the first peptide bond between puromycin and the [^{3}H]methionyl transfer RNA bound to the initiation complex. The data derived from these experiments suggests that I is a potent inhibitor of the peptidyl transferase reaction. The apparent runoff of polyribosomes to the 80S ribosomes suggests that the ribosome must complete its elongation and termination cycles before this drug can bind and usually must complete the initiation steps before the bound drug becomes an effective inhibitor. This pattern of inhibition is almost identical to that observed previously with the other quassinoids, bruceantin and brusatol (40), in rabbit reticulocytes and yeast cells.

In P-388 lymphocytic leukemia cells, I is a potent inhibitor of nucleic acids synthesis by inhibiting key sites in pyrimidine and purine synthesis and DNA and RNA polymerase activities. In addition, it is a potent inhibitor of protein synthesis by inhibiting the elongation step of polypeptide synthesis.

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NOTES

Improved and Rapid High-Performance Liquid Chromatographic Assay for 13-*cis*-Retinoic Acid or All-*trans*-retinoic Acid

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Abstract □ A rapid, specific, and sensitive reversed-phase high-performance liquid chromatographic (HPLC) assay for the quantitative determination of all-*trans*- retinoic acid (I) or 13-*cis*-retinoic acid (II) in rat serum without extraction or lyophilization is described. Chromatographic separation from retinol, serum components, and retinol acetate standard was achieved on octadecylsilane-coated particles with acetonitrile–1% ammonium acetate as the eluent. Serum samples (100 μl) containing as little as 10 ng of retinoid were analyzed. Serum level profiles of rats dosed with the retinoids demonstrated the utility of the assay and indicated elimination half-lives of 0.58 and 0.92 hr for I and II, respectively.

Keyphrases □ High-performance liquid chromatography—assay for 13-*cis*-retinoic acid and all-*trans*-retinoic acid □ 13-*cis*-Retinoic acid—high-performance liquid chromatographic analysis □ All-*trans*-retinoic acid—high-performance liquid chromatographic analysis

High-performance liquid chromatographic (HPLC) assay procedures for all-*trans*-retinoic acid (I) or 13-*cis*-retinoic acid (II) in serum samples differ markedly in sample preparation. One previously reported method employed lyophilization followed by extraction with methanol before analysis by reversed-phase chromatography. By this method as little as 50 ng of retinoic acid was detected in 0.5 ml of human plasma samples (1). Another report (2) showed a sensitivity limit of 25 ng/ml of plasma with reversed-phase chromatography after extraction of samples containing 1 ml of plasma with a mixture of hexane, methylene chloride, and isopropanol. In another study (3), 0.5 ml of serum was extracted with ethyl acetate, followed by evaporation and dissolution of the residue in mobile phase. Normal-phase adsorption chromatography was employed to achieve a sensitivity of 10–20 ng/ml of serum. A fourth method (4) required no extraction step and had a detection limit of 100 ng of retinoic acid when 400 μl of serum was mixed with methanol and centrifuged for 20 min and the supernatant liquid was analyzed directly by reversed-phase chromatography.

The present study describes a simple timesaving method for determining serum I or II by reversed-phase liquid chromatography with no extraction or lyophilization steps. Only 100 μl of serum is required, and many samples can be analyzed in a short time. Sensitivity compares favorably with other reported assay procedures.

EXPERIMENTAL

Reagents—All-*trans*-retinoic acid[1] (I), 13-*cis*-retinoic acid[2] (II), and all-*trans*-retinol acetate[3] (III) were used as received. All other chemicals

[1] Eastman Kodak, Rochester, N.Y.
[2] Hoffmann-La Roche, Nutley, N.J.
[3] Sigma Chemical Co., St. Louis, Mo.

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