Antitumor Agents XXXIV: Mechanism of Action of Bruceoside A and Brusatol on Nucleic Acid Metabolism of P-388 Lymphocytic Leukemia Cells

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Abstract □ The quassinoids bruceantin, brucein D, brucein E, bruceoside A, and brusatol significantly inhibited P-388 lymphocytic leukemic cell RNA and protein synthesis in tissue culture. However, DNA synthesis inhibition seemed to correlate more directly with the antineoplastic activity of these compounds in the *in vivo* P-388 survival system. *In vitro*, brusatol and bruceoside A marginally inhibited 10-day P-388 lymphocytic leukemia DNA polymerase, RNA polymerase, thymidylate synthetase, dihydrofolate reductase, phosphoribosyl pyrophosphate aminotransferase, and cathepsin protease activities. *In vivo* studies demonstrated similar inhibition and elevated cyclic AMP levels, correlating positively with the antineoplastic activity of individual compounds. Purine synthesis was inhibited drastically by brusatol *in vivo*, and one key inhibition site in purine synthesis was at phosphoribosyl pyrophosphate aminotransferase, the regulatory enzyme. Histone phosphorylation and ribonucleotide reductase activity also were inhibited marginally by brusatol.

Keyphrases □ Antineoplastic agents—bruceoside A and brusatol, effect on cultured P-388 lymphocytic leukemia cell nucleic acid metabolism □ Quassinoids—antineoplastic activity, effect on cultured P-388 lymphocytic leukemia cell nucleic acid metabolism □ Leukemia, lymphocytic—*in vitro* P-388 cells, effect of quassinoids on nucleic acid metabolism □ Nucleic acid synthesis, tumor—effect of quassinoids on P-388 lymphocytic leukemia cells

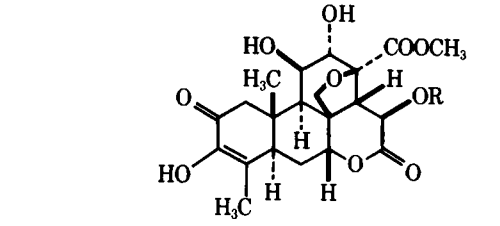
The structure of bruceantin (I), a quassinoid isolated from *Brucea antidysenterica*, was elucidated previously (1). Subsequently, bruceantin was active at 1 mg/kg/day against P-388 lymphocytic leukemia (T/C = 197) and at 2 mg/kg/day against B16 melanoma (T/C = 178) in mice. Brucein D (II), brucein E (III), and bruceoside A (IV) were isolated from *Brucea javanica* and characterized by Lee *et al.* (2)[1].

Studies with HeLa cells (3) demonstrated that bruceantin inhibited protein synthesis >90% at 2 μM, whereas DNA synthesis was only inhibited ~60%. RNA synthesis was marginally inhibited (~15%). Protein synthesis was blocked 79% in rabbit reticulocytes by bruceantin at 0.1 mM during the initiation events of translocation. In *Saccharomyces cereviseae*, bruceantin did not inhibit the initiation event but blocked peptide bond formation during chain elongation by binding to the free ribosome P site, resulting in 90% inhibition at 0.01 mM (4).

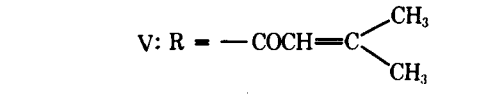
This paper reports the effects of brusatol and bruceoside A on P-388 lymphocytic leukemia cell metabolism.

EXPERIMENTAL

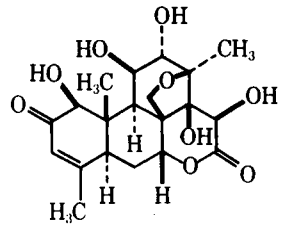
Materials—Bruceantin (I) was obtained in small quantities from the National Cancer Institute. Brucein D (II), brucein E (III), and bruceoside A (IV) were extracted from *B. javanica* according to the exact literature technique (2), and brusatol (V) was obtained by treating bruceoside A with 3 N H_2SO_4–methanol (1:1) to hydrolyze the glycosidic linkage (2).



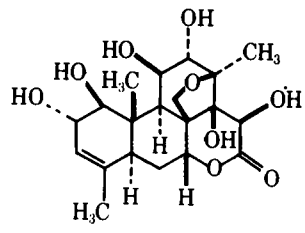
I: R = —COCH=C(CH$_3$)—CH(CH$_3$)$_2$



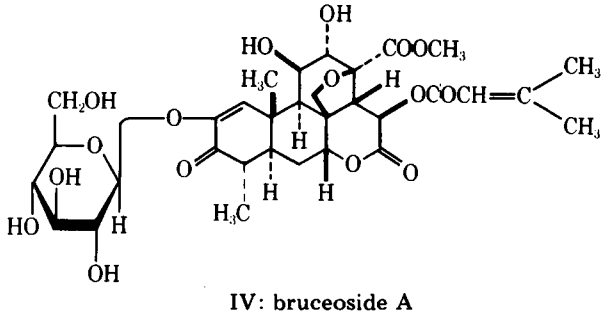
V: R = —COCH=C(CH$_3$)$_2$



II: brucein D



III: brucein E



IV: bruceoside A

P-388 Lymphocytic Leukemia Antitumor Screen—DBA/2 male mice (~25 g) were administered 10^6 P-388 lymphocytic leukemia cells intraperitoneally on Day 0. Test compounds were homogenized in 0.05% polysorbate 80 and administered on Days 1–14 intraperitoneally. The average days survived for each group was determined, and T/C (treated/control) values were calculated (5). Fluorouracil was used as a positive standard.

P-388 Lymphocytic Leukemia Cell Metabolism Studies—DBA/2 male mice were innoculated with 10^6 P-388 lymphocytic leukemia cells on Day 0. On Days 7, 8, and 9, brusatol, 100 μg/kg/day, or bruceoside A, at 5 mg/kg/day, was administered intraperitoneally. On Day 10, P-388 cells were harvested from the peritoneal cavity. The number of tumor cells per milliliter and the 0.4% trypan blue uptake were determined with a hemocytometer (6). Thymidine incorporation into DNA was determined by a literature method (7). One hour prior to the animal's sacrifice, 10 μCi of [^{14}C-*methyl*]-thymidine (47.5 mCi/mmole) was injected intraperitoneally.

[1] See K. H. Lee, Y. Imakura, Y. Sumida, R. Y. Wu, I. H. Hall, and H. C. Huang, *J. Org. Chem.*, in press.

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Table I—P-388 Lymphocytic Leukemia Screen in DBA/2 Male Mice (~25 g)

Compound (n = 6)	Dose, μg/kg/day	Average Days Survived	T/C[a]
I[b]	1,000	22.7/11.5	197
	500	16.8/11.5	146
	250	15.0/11.5	130
	125	14.3/11.5	124
II	125	10.3/9.5	108
III	500[c]	8.5/9.5	89
	250	10.8/9.5	114
	125	10.8/9.5	114
IV	6,000	15.6/10.0	156
	250	11.5/9.5	121
	125	10.0/9.5	105
V	500[c]	12.5/9.5	132
	250	14.5/9.5	153
	125	15.0/9.5	158
Fluorouracil	25,000	19.7/10.6	186

[a] T/C ≥ 125 is required for significant activity. [b] Bruceantin was tested by the National Cancer Institute in CDF_1 mice. [c] Toxic at this dose.

The DNA was isolated, and the carbon-14 content was determined in two parts of toluene, one part of octoxynol, 0.4% 2,5-diphenyloxazole, and 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene scintillation fluid and corrected for quenching. The DNA concentration was determined by UV spectrophotometry at 260 nm with calf thymus DNA as a standard. Uridine incorporation into RNA was determined in an analogous manner with 10 μCi of 5-^{3}H-uridine (24.2 Ci/mmole). RNA was extracted by a literature method (8). Leucine incorporation into protein was determined by the Sartorelli (9) method with 5 μCi of ^{14}C-leucine (54.4 Ci/mmole).

The P-388 tissue culture cells were maintained according to literature methods (10). Fetal calf serum (10%) was added to the medium 24 hr prior to studies. The P-388 cells were diluted to 1.6×10^6 cells/ml, and ^{14}C-thymidine (0.086 μCi), ^{3}H-uridine (0.08 μCi), or ^{14}C-leucine (0.0845 μCi) was added to 1 ml of cells (3). The synthesis rates, after 1 hr, of DNA, RNA, or protein were measured as the uptake of labeled precursors into cold trichloroacetic acid-insoluble material. DNA was collected on glass fiber paper (GF/F) by vacuum suction. RNA and protein were collected on nitrocellulose membrane filters.

The P-388 lymphocytic cells from 10-day DBA/2 mice also were collected for *in vitro* studies. The drug effects on the transport of precursor radiolabeled thymidine, uridine, or leucine across membranes were determined using whole cells and cell homogenates. Labeled macromolecules were collected on the appropriate filters after 1 hr of incubation (3).

In the *in vitro* studies, bruceantin and bruceoside A were present at 0.015 m*M* concentrations and brusatol was present at 0.015 and 0.005 m*M* final concentrations.

Emzymatic Studies—Nuclear DNA polymerase activity was determined on isolated P-388 lymphocytic leukemia cell nuclei (11). The incubation medium was that of Sawada *et al.* (12), except that 2-^{14}C-deoxyribothymidine triphosphate (45 mCi/mmole) was used; the insoluble nucleic acids were collected on glass fiber paper (GF/F). Messenger RNA polymerase activity was measured by literature methods (13, 14) with 5,6-^{3}H-uridine 5′-triphosphate (35 Ci/mmole). Insoluble RNA was collected on nitrocellulose filters.

Deoxythymidine kinase, deoxythymidylate monophosphate kinase, and deoxythymidylate diphosphate kinase activities were measured by a literature method (15) based on the disappearance of 0.1 μmole of reduced nadide (nicotinamide adenine dinucleotide) at 340 nm in 2 min. Thymidylate synthetase activity was assayed by a literature method (16), utilizing a postmitochondrial (9000×*g* for 10 min) supernate and 5 μCi of 5′-^{3}H-deoxyuridine monophosphate (11 Ci/mmole). Dihydrofolate reductase activity was determined by a literature method (17) based on the disappearance of 0.1 μmole of reduced nadide phosphate at 340 nm for 1 hr. *S*-Adenosylmethionine transferase activity was determined by a literature method (18) with 0.05 μCi of $^{14}CH_3$-*S*-adenosyl-L-methionine (53 mCi/mmole).

Ribonucleotide reductase activity was determined by the method of Moore and Hurlbert (19). Deoxyribonucleotides were separated from ribonucleotides by poly(ethyleneimine) cellulose TLC (20). ^{14}C-Formate incorporation into purines was measured by a reported method (21), using 0.5 μCi of ^{14}C-formic acid (4.95 mCi/mmole). Purine separation was carried out on silica gel TLC plates eluted with *n*-butanol–acetic acid–water (4:1:5). The plates were scraped and radioactivity was determined.

Table II—Relative Incorporation of Radiolabeled Precursors into P-388 Tissue Culture Cells during 1 hr with the Incubation Test Drugs (0.015 m*M*)

Compound	Percent Control (dpm)		
	^{14}C-Thymidine Incorporation into DNA	^{3}H-Uridine Incorporation into RNA	^{14}C-Leucine Incorporation into Protein
I (0.015 m*M*)	63 ± 7[a]	68 ± 9[b]	38 ± 5[a]
II (0.015 m*M*)	81 ± 5	45 ± 5[a]	26 ± 9[a]
III (0.015 m*M*)	76 ± 8	43 ± 3[a]	35 ± 13[a]
IV (0.015 m*M*)	67 ± 9[a]	45 ± 5[a]	35 ± 15[a]
V (0.015 m*M*)	16 ± 9[a]	38 ± 3[a]	14 ± 8[a]
0.05% Polysorbate 80	100 ± 7	100 ± 3	100 ± 14

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

Phosphoribosyl pyrophosphate aminotransferase activity was determined by a method (22) based on the reduction of 0.6 μmole of nadide at 340 nm for 30 min.

Carbamyl phosphate synthetase activity was determined with ornithine and ornithine transcarbamylase present by the method of Kalman *et al.* (23). The colorimetric determination of citrulline was performed according to the Archibald method (24). Aspartate carbamyl transferase activity was carried out by a literature method (23) in the presence of aspartate transcarbamylase. The colorimetric determination of carbamyl aspartate was carried out according to a literature method (25).

To determine if purine synthesis inhibition was the reason for decreased ^{14}C-thymidine incorporation into DNA, P-388 lymphocytic leukemia cells were incubated *in vitro* with 0.05 μCi of [^{14}C-*methyl*]-thymidine and deoxyguanosine triphosphate, deoxycytosine triphosphate, and deoxyadenosine triphosphate in buffer for 1 hr. A second experiment was performed after dosing with brusatol on Days 7, 8, and 9 at 100 μg/kg/day to determine if the actual purine content had been altered *in vivo*. Purines were extracted (21) and determined by UV analysis at 258 nm.

Histone phosphorylation was determined by injecting intraperitoneally 10 μCi of γ-^{32}P-adenosine triphosphate (18.8 Ci/mmole). The nuclei were isolated 1 hr later, and the histone chromatin protein was extracted by a literature method (26). Nonhistone chromatin phosphorylation by nuclei protein kinase was determined on isolated nuclei (11), utilizing 2 nmoles of γ-^{32}P-adenosine triphosphate. Chromatin protein was collected on nitrocellulose membrane filters (27).

Cyclic AMP levels were determined by a literature method (28) with the ^{3}H(G)-adenosine 3′,5′-cyclic phosphate (39.8 Ci/mmole) radioimmunoassay. The drug effect on hydrolytic enzymatic activity was measured also. Deoxyribonuclease activity was measured at pH 5.0 by a modification of the deDuve method (29). Ribonuclease activity and acid cathepsin activity were determined at pH 5.0 by a literature method (30).

In vitro UV binding studies (6) were conducted with brusatol or bruceoside A (7.8 μg/ml) with DNA or deoxyguanosine monophosphate (38 μg/ml) in 0.1 *M* phosphate buffer, pH 7.2, over 200–340 nm for 24 hr. Protein concentration was determined by the method of Lowry *et al.* (31).

RESULTS

Bruceantin was active against the P-388 lymphocytic leukemia cell survival system in the 250–1000-μg/kg/day range. Brucein D and brucein E were inactive. Bruceoside A was active against the P-388 survival system at 6 mg/kg/day, and brusatol was active at 125–500 μg/kg/day (Table I). Incorporation of radiolabeled precursors into P-388 tissue culture cell macromolecules showed that thymidine, uridine, and leucine were incorporated at the rates of 5559, 3381, and 1646 dpm/10^6 cells, respectively, for the control.

All five compounds reduced protein and RNA syntheses significantly at 0.015 m*M* (Table II). Brusatol caused the highest inhibition, resulting in 86% suppression of protein synthesis and 62% suppression of RNA synthesis of P-388 lymphocytic leukemia tissue culture cells. DNA synthesis was inhibited 37% by bruceantin, 19% by brucein D, 24% by brucein E, 33% by bruceoside A, and 84% by brusatol at 0.15 m*M*. Since brucein D and brucein E were inactive in the P-388 lymphocytic leukemia survival system, it appeared that suppression of RNA and protein was not the critical event resulting in tumor cell death. DNA synthesis inhibition appeared to correlate more directly with antitumor activity. For this reason, it was decided to examine specific events in DNA synthesis.

Table III—*In Vitro* Effect of Brusatol (0.005 m*M*) and Bruceantin (0.015 m*M*) on Whole Cells and Homogenates of 10-Day P-388 Lymphocytic Leukemia Cells (n = 5)

	Whole Cells			Homogenized		
	Control	Brusatol[a]	Bruceantin[a]	Control	Brusatol[a]	Bruceantin[a]
Thymidine incorporation into DNA	100 ± 12	62 ± 5[b]	63 ± 7[b]	100 ± 9	46 ± 7[b]	50 ± 8[b]
Uridine incorporation into RNA	100 ± 15	56 ± 7[b]	68 ± 9[c]	100 ± 8	55 ± 5[b]	—
Leucine incorporation into protein	100 ± 14	40 ± 6[b]	38 ± 5[b]	100 ± 5	25 ± 3[b]	33 ± 4[b]
Thymidine incorporation in presence of deoxyriboguanine, cytosine, and adenosine triphosphates	—	—	—	100 ± 11	86 ± 10	—

[a] Percent of control. [b] p = 0.001. [c] p = 0.005.

Table IV—*In Vitro* Effects of Test Drugs on 10-Day P-388 Lymphocytic Leukemia Cell Metabolism of DBA/2 Mice at 0.015 *M* Final Concentration

	Percent Control					
Enzyme Assayed	Control	Bruceantin	Brucein D	Brucein E	Bruceoside A	Brusatol
Thymidine kinase	100 ± 5	—	—	—	104 ± 5	107 ± 6
Thymidylate monophosphate kinase	100 ± 4	—	102 ± 5	97 ± 5	105 ± 11	106 ± 5
Thymidylate diphosphate kinase	100 ± 5	—	95 ± 7	93 ± 8	98 ± 7	97 ± 4
DNA polymerase	100 ± 10	—	105 ± 17	88 ± 12	85 ± 10	79 ± 3[a]
Thymidylate synthetase	100 ± 6	—	103 ± 10	99 ± 13	87 ± 6[b]	68 ± 18[c]
Dihydrofolate reductase	100 ± 12	—	73 ± 18[b]	75 ± 15[b]	69 ± 17[c]	64 ± 11[d]
Deoxyribonuclease	100 ± 3	—	81 ± 5[d]	42 ± 4[d]	100 ± 3	99 ± 5
Ribonuclease	100 ± 9	—	55 ± 8[d]	63 ± 7[d]	96 ± 9	73 ± 6[d]
Cathepsin	100 ± 21	—	6 ± 3[d]	18 ± 5[d]	7 ± 6[d]	38 ± 13[d]
RNA polymerase	100 ± 18	—	—	83 ± 15	68 ± 12[b]	88 ± 17
Phosphoribosyl pyrophosphate aminotransferase	100 ± 8	96 ± 4	103 ± 9	104 ± 13	100 ± 8	61 ± 7[d]

[a] p = 0.005. [b] p = 0.025. [c] p = 0.010. [d] p = 0.001.

Whole cells harvested from 10-day P-388 lymphocytic leukemia-bearing mice and cells homogenized four times showed that brusatol at 0.005 m*M* and bruceantin at 0.015 m*M* did not retard ^{14}C-thymidine transport across the tumor membrane. In fact, both brusatol and bruceantin were more effective inhibitors of protein and DNA syntheses in homogenates (Table III). Untreated 10-day P-388 lymphocytic leukemia cells demonstrated changes in absorbance of 0.500 optical density (O.D.) unit/min/mg of protein for thymidine kinase activity, of 0.489 O.D. unit/min/mg of protein for thymidylate monophosphate kinase activity, and of 0.566 O.D. unit/min/mg of protein for thymidylate diphosphate kinase activity. Compounds II–V at 0.015 m*M* had no effect on the nucleotide kinases tested (Table IV). DNA polymerase activity for 10-day P-388 cells was 27,368 dpm/mg of nuclear protein. Brusatol caused 21% inhibition of polymerase activity. Thymidylate synthetase activity in 10-day P-388 cells was 1,645,840 dpm/mg of protein, which brusatol reduced 32%. Dihydrofolate reductase activity for 10-day P-388 cells was 2192 O.D. units/hr/mg of protein. At 0.015 m*M*, dihydrofolate reductase activity was reduced 27% by brucein D, 25% by brucein E, 31% by bruceoside A, and 36% by brusatol. At 0.005 m*M*, brusatol and methotrexate reduced dihydrofolate reductase activity by 28 and 59%, respectively.

Hydrolytic enzymatic activity was not increased due to the drugs; therefore, increased hydrolytic activity of deoxyribonuclease, ribonuclease, or cathepsin protease could not account for the reduction of radiolabeled precursors into macromolecules of P-388 cells. Deoxyribonuclease activity for 10-day cells was 1.718 μg of DNA hydrolyzed/min/mg of protein. Brucein D reduced the activity by 19% and brucein E reduced it by 58%. Ribonuclease activity for 10-day P-388 cells was 7.236 μg of RNA hydrolyzed/min/mg of protein. Brucein D reduced the activity by 45%, brucein E by 37%, and brusatol by 27%. Cathepsin activity for 10-day P-388 cells was 65.7 μg of azocasein hydrolyzed/min/mg of protein, which was suppressed significantly by brusatol, bruceoside A, brucein D, and brucein E.

In vitro messenger RNA polymerase activity also was measured in 10-day P-388 cells and was 76,088 dpm/mg of protein. The presence of brucein E reduced RNA polymerase activity by 17%; bruceoside A and brusatol reduced it by 32 and 12%, respectively. Phosphoribosyl pyrophosphate aminotransferase was inhibited by brusatol by 39%.

In the *in vivo* experiments, DBA/2 mice were treated with brusatol and bruceoside A on Days 7, 8, and 9 and the cells were harvested on Day 10. ^{14}C-Thymidine incorporation into DNA for the control was 75,098 dpm/mg of DNA, which was reduced 77% by brusatol and 48% by bruceoside A (Table V). ^{3}H-Uridine incorporation into RNA for the control was 386,004 dpm/mg of RNA, which was reduced 68% by brusatol and 55% by bruceoside A. ^{14}C-Leucine incorporation for the control was 22,666 dpm/mg of protein, which was reduced 86% by brusatol and 41% by bruceoside A. The number of tumor cells at Day 10 for the control was 142 × 10^6 cells/ml, which was reduced 79% by brusatol and 65% by bruceoside A after 3 days of administration.

Cyclic AMP in the control animals was 1.01 pmoles/10^6 P-388 cells. *In vivo* brusatol treatment increased cyclic AMP to 12.69 pmoles/10^6 cells. A single dose of brusatol (100 μg/kg) on Day 10 increased cyclic AMP by 167% after 1 hr. Bruceoside A for 3 days increased cyclic AMP only to 2.74 pmoles/10^6 cells. Bruceantin caused an increase to 5.49 pmoles of cyclic AMP/10^6 cells. Histone phosphorylation by ATP for the 10-day P-388 cells was 241 cpm/mg of protein, which was reduced 36% by brusatol. Nonhistone phosphorylation for the 10-day P-388 cells was 6246 cpm/mg of protein, which was reduced 8% by brusatol.

^{14}C-Formate incorporation into purine for the 10-day P-388 cells was 19,807 dpm/mg of protein. Brusatol treatment reduced purine synthesis 50%, whereas bruceoside A reduced it only 4%. Phosphoribosyl pyrophosphate aminotransferase activity for the 10-day P-388 cells was 0.285 O.D. unit/min/mg of protein, which was reduced 43% by brusatol. Carbamyl phosphate synthetase activity for 10-day P-388 cells was 1.8 mg of carbamyl phosphate formed/hr/mg of protein, which was unaffected by brusatol. Aspartate carbamyl transferase activity for 10-day P-388 cells was 44.4 mg of carbamyl aspartate formed/hr/mg of protein, which was reduced 7% by brusatol.

In vivo dihydrofolate reductase activity was reduced 30% by brusatol. Ribonucleotide reductase activity of 10-day P-388 cells was 517,053 dpm/mg of protein, which was reduced 14% by brusatol. *S*-Adenosyl-L-methionine transferase activity of 10-day P-388 cells was 251 dpm/mg of protein, which was unaffected by brusatol. Purine and pyrimidine base concentration after 3 days of brusatol treatment was reduced 17%. *In vitro* incorporation of ^{14}C-thymidine into DNA of 10-day P-388 cells in the presence of deoxyguanosine triphosphate, deoxyadenosine triphosphate, deoxycytidine triphosphate, and 0.005 *M* brusatol was inhibited only 14% (Table III). *In vivo* DNA polymerase activity was inhibited 16% by brusatol.

UV spectral studies demonstrated that brusatol had absorption peaks at 219 and 279 nm. Bruceoside A had an absorption peak at 270 nm. DNA had absorption peaks at 260 and 220 nm. Deoxyguanosine monophosphate had absorption peaks at 270 and 253 nm. Since these spectra overlapped, it was difficult to determine if binding occurred between nucleotides and drugs. However, when the drug was present, the absorption was higher for DNA and for deoxyguanosine monophosphate than would be expected for two single species in solution. The areas under the curves indicated that a combination of brusatol and DNA resulted in a 29% higher absorption than the calculated absorbances of both species added together. Brusatol and deoxyguanosine monophosphate combinations resulted in a 40% higher absorbance than the calculated

Table V—*In Vivo* Effects of Brusatol and Bruceoside A on P-388 Lymphocytic Leukemia Cell Metabolism of DBA/2 Mice ($n = 6$)

	Control	Percent Control: Brutasol, 100 μg/kg/day	Percent Control: Bruceoside A, 5 mg/kg/day
^{14}C-Thymidine incorporation into DNA	100 ± 17	23 ± 15[a]	52 ± 7[a]
^{3}H-Uridine incorporation into RNA	100 ± 16	32 ± 11[a]	45 ± 10[a]
^{14}C-Leucine incorporation into protein	100 ± 26	14 ± 2[a]	59 ± 21[b]
Number of tumor cells × 10^6/ml	100 ± 6	21 ± 5[a]	35 ± 9[a]
3′,5′-Cyclic adenosine monophosphate levels	100 ± 8	1269 ± 60[a]	271 ± 2[a]
γ-^{32}P-Adenosine triphosphate incorporation into histone proteins	100 ± 20	64 ± 17[b]	—
γ-^{32}P-Adenosine triphosphate incorporation into nonhistone proteins	100 ± 11	92 ± 27	99 ± 10
^{14}C-Formate incorporation into purines	100 ± 10	50 ± 12[a]	96 ± 24
Phosphoribosyl pyrophosphate aminotransferase activity	100 ± 12	57 ± 11[a]	—
Carbamyl phosphate synthetase activity	100 ± 7	107 ± 6	—
Aspartate carbamyl transferase activity	100 ± 5	93 ± 16	—
Dihydrofolate reductase activity	100 ± 12	70 ± 11[c]	—
Deoxynucleic acid polymerase activity	100 ± 7	84 ± 5[c]	—
Ribonucleotide reductase activity	100 ± 5	86 ± 3[a]	—
S-Adenosyl-L-methionine transferase activity	100 ± 10	100 ± 13	—
Purine concentration	100 ± 3	83 ± 2[a]	—

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

absorbances. With DNA and deoxyguanosine monophosphate, bruceoside A likewise showed 73 and 18% higher absorbances, respectively, than the calculated absorbances of both species added together.

The Student t test was used to calculate the probable (p) significant difference in Tables II–V. Data are expressed as percent of control of the average mean and standard deviation; n equals the number of animals per group.

DISCUSSION

The effects of five quassinoids on P-388 lymphocytic leukemia metabolism are diverse. Although bruceantin, brucein D, brucein E, bruceoside A, and brusatol cause drastic inhibition of protein and RNA synthesis of cultured P-388 leukemic cells, these effects could not be correlated directly with cell death. Of these agents, Liao *et al.* (3) observed that compounds that did not contain the quassinoid ester side chain caused less protein synthesis reduction in HeLa cells and rabbit reticulocytes. These investigators postulated that the ester side chain at C-15 was necessary for drug transport across the cell membrane. Studies with P-388 cells do not support this postulate; brucein D and brucein E both inhibited protein synthesis. Actually the P-388 tumor cell membrane appears to retard transport of brusatol and bruceantin into the cell.

The inhibition of DNA synthesis showed the strongest correlation with antitumor activity in the P-388 lymphocytic leukemia survival system. DNA template activity, as observed through DNA polymerase activity, both *in vitro* and *in vivo* and RNA polymerase activity *in vitro* were inhibited marginally by the quassinoids. The nucleotide UV spectral studies showed absorbance increases in the presence of brusatol or bruceoside A above the calculated absorbance of the two individual species in solution, which may reflect partial or limited drug binding to the template and suppression of polymerase template activity. *In vitro* thymidylate synthetase inhibition correlated with antitumor activity for the quassinoids. Dihydrofolate reductase also was inhibited marginally by the quassinoids, with brusatol having the most activity. However, brusatol was only one-half as active as methotrexate at the same concentration. The nucleotide kinases did not appear to be a site of metabolism inhibition by the quassinoids.

In vivo quassinoid effects on P-388 lymphocytic leukemia cells at Day 10 were similar to the effects on DNA, RNA, and protein syntheses after 3 days of dosing. Brusatol required a dose of 100 μg/kg/day to bring about the same magnitude of inhibition as an *in vitro* concentration of 0.015 mM, whereas bruceoside A required a dose of 5 mg/kg/day. The *in vivo* doses correlated positively with doses required for antineoplastic activity in the P-388 survival system. The observation that brusatol was more active *in vivo* than bruceoside A raised the question whether brusatol is bioactivated *in vivo* to the active species, which is not fully seen in the *in vitro* studies.

Elevated cyclic AMP levels correlated with antineoplastic activity, brusatol > bruceantin > bruceoside A. Elevated levels of cyclic AMP reverse malignant states to a more normal cell (33, 34) and are correlated with less phosphorylation of the regulatory H-1 histone fraction, thereby reducing cell proliferation (35). Brusatol treatment moderately reduced histone phosphorylation, whereas nonhistone phosphorylation was unaffected. Cancer cells are also known to have a high proteolytic activity on the membrane surface. The quassinoids effectively blocked P-388 cathepsin proteolytic activity. Purine synthesis was significantly inhibited by brusatol but not by bruceoside A. The regulatory enzyme of purine synthesis, phosphoribosyl pyrophosphate aminotransferase, was a major site of brusatol inhibition. The enzyme was less affected by brusatol *in vitro* at 0.015 mM, again possibly indicating *in vivo* drug bioactivation.

Early steps in pyrimidine synthesis were not affected by brusatol. One carbon transfer from *S*-adenosyl-L-methionine also was unaffected by brusatol. Purine in the P-388 cells after 3 days of treatment also was reduced. A drastic purine reduction was not seen, which may have been due to the salvage pathway of purine from already synthesized nucleic acid. When DNA polymerase was assayed in the presence of added triphosphate deoxyribonucleotide, only marginal inhibition by brusatol *in vitro* was observed. One means of determining if the lack of purine for DNA synthesis was the prime factor in inhibition of ^{14}C-thymidine incorporation was to add nucleotide to the medium. In the presence of ample purine and pyrimidine, thymidine incorporation was inhibited only 14%, which probably reflects brusatol inhibition of DNA polymerase. Lack of purine would also explain the reduction in RNA synthesis. The reduction of ribose to deoxyribose nucleotide also was marginally inhibited in brusatol.

From these studies, it can be concluded that brusatol and bruceoside A have multiple sites of action on P-388 lymphocytic cells. Brusatol appeared to be more potent and effectively blocked a number of metabolic sites necessary for nucleic acid synthesis. Quassinoid effects on protein synthesis are currently being investigated. Preliminary data support the observation of Fresno *et al.* (4) that the elongation process of protein synthesis is the site of brusatol and bruceoside A inhibition.

REFERENCES

(1) S. M. Kupchan, R. W. Britton, J. A. Lacadie, M. F. Ziegler, and C. W. Sigel, *J. Org. Chem.*, **40**, 648 (1975).

(2) K. H. Lee, Y. Imakura, and H. C. Huang, *J. Chem. Soc. Chem. Commun.*, **1977**, 69.

(3) L.-L. Liao, S. M. Kupchan, and S. B. Howitz, *Mol. Pharmacol.*, **12**, 167 (1976).

(4) M. Fresno, A. Gonales, D. Vazquez, and A. Jimenez, *Biochim. Biophys. Acta*, **518**, 104 (1978).

(5) R. I. Geran, N. H. Greenberg, M. M. MacDonald, A. M. Schumacher, and B. J. Abbott, *Cancer Chemother. Rep.*, **3**, 9 (1972).

(6) I. H. Hall, K. H. Lee, E. C. Mar, C. O. Starnes, and T. G. Waddell, *J. Med. Chem.*, **20**, 333 (1977).

(7) C. B. Chae, J. L. Irvin, and C. Piantadosi, *Proc. Am. Assoc. Cancer Res.*, **9**, 44 (1968).

(8) R. G. Wilson, R. H. Bodner, and G. E. MacHorter, *Biochim. Biophys. Acta*, **378**, 260 (1975).

(9) A. C. Sartorelli, *Biochem. Biophys. Res. Commun.*, **27**, 26 (1967).

(10) J. L. Toohey, *Proc. Natl. Acad. Sci. USA*, **72**, 73 (1975).

(11) W. C. Hymer and E. L. Kuff, *J. Histochem. Cytochem.*, **12**, 359 (1964).

(12) H. Sawada, K. Tatsumi, M. Sasada, S. Shirakawa, T. Nakumura,

and G. Wakisaka, *Cancer Res.*, **34**, 3341 (1974).
(13) K. M. Anderson, I. S. Mendelson, and G. Guzik, *Biochim. Biophys. Acta*, **383**, 56 (1975).
(14) I. H. Hall, G. L. Carlson, G. S. Abernethy, and C. Piantadosi, *J. Med. Chem.*, **17**, 1253 (1974).
(15) F. Maley and S. Ochoa, *J. Biol. Chem.*, **233**, 1538 (1958).
(16) A. Kampf, R. L. Barfknecht, P. J. Schaffer, S. Osaki, and M. P. Mertes, *J. Med. Chem.*, **19**, 903 (1976).
(17) M. K. Ho, T. Hakalo, and S. F. Zakrzwski, *Cancer Res.*, **32**, 1023 (1972).
(18) R. T. Borchardt, J. A. Huber, and Y. S. Wu, *J. Med. Chem.*, **19**, 1094 (1976).
(19) E. C. Moore and R. B. Hurlbert, *J. Biol. Chem.*, **241**, 4802 (1966).
(20) K. Randerath, *Biochim. Biophys. Acta*, **76**, 622 (1963).
(21) M. K. Spassova, G. C. Russev, and E. V. Golovinsky, *Biochem. Pharmacol.*, **25**, 923 (1976).
(22) J. B. Wyngaarden and D. M. Ashton, *J. Biol. Chem.*, **234**, 1492 (1959).
(23) S. M. Kalman, P. H. Duffield, and T. Brzozowski, *Am. Biol. Chem.*, **24**, 1871 (1966).
(24) R. M. Archibald, *J. Biol. Chem.*, **156**, 121 (1944).
(25) S. B. Koritz and P. P. Cohen, *ibid.*, **209**, 145 (1954).
(26) A. Raineri, R. C. Simsiman, and R. K. Boutwell, *Cancer Res.*, **33**, 134 (1973).
(27) Y. M. Kish and L. J. Kleinsmith, *Methods Enzymol.*, **40**, 201 (1975).
(28) A. C. Gilman, *Proc. Natl. Acad. Sci., USA*, **67**, 305 (1970).
(29) I. H. Hall, K. S. Ishaq, and C. Piantadosi, *J. Pharm. Sci.*, **63**, 625 (1974).
(30) Y. S. Cho-Chung and P. M. Gullino, *J. Biol. Chem.*, **248**, 4743 (1973).
(31) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *ibid.*, **193**, 265 (1951).
(32) G. W. Snedecor, "Statistical Methods," Iowa State College Press, Ames, Iowa, 1956, sec. 2.
(33) T. Posternak, *Annu. Rev. Pharmacol.*, **14**, 23 (1974).
(34) Y. S. Cho-Chung and P. M. Gullino, *Science*, **183**, 87 (1974).
(35) C. S. Rubin and O. M. Roseau, *Annu. Rev. Biochem.*, **44**, 81 (1975).

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Antitumor Agents XXXV: Effects of Brusatol, Bruceoside A, and Bruceantin on P-388 Lymphocytic Leukemia Cell Respiration

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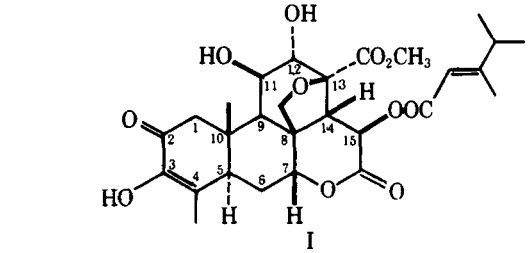
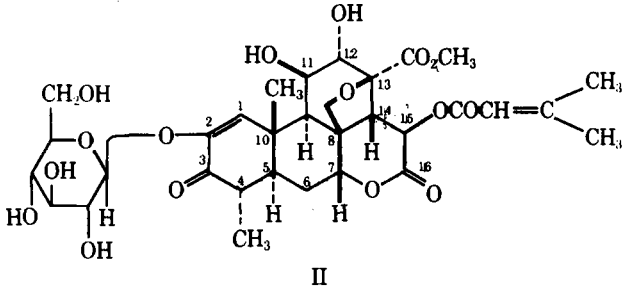
Abstract □ Brusatol, a quassinoid with potent antineoplastic activity against P-388 lymphocytic leukemia cell proliferation, significantly inhibited P-388 cell hexokinase, phosphofructokinase, malic dehydrogenase, and succinic dehydrogenase. Mitochondrial oxidative phosphorylation, basal, and adenosine diphosphate-stimulated respiration, utilizing succinate and α-ketoglutarate as the substrate, was suppressed significantly by *in vivo* treatment with brusatol. However, brusatol treatment had no effect on liver oxidative phosphorylation. Brusatol greatly increased P-388 cyclic AMP levels but had no effect on liver cyclic nucleotides. Similar inhibitory effects on P-388 cell oxidative phosphorylation were found *in vitro* with brusatol, bruceoside A, and bruceantin. Brusatol had no effect on adenosine triphosphatase activity or on uncoupling of oxidative phosphorylation. Rather, brusatol appeared to increase the concentration of reduced mitochondrial electron-transport cofactors, thereby blocking aerobic respiration. A proposed mechanism of action is discussed.

Keyphrases □ Antineoplastic agents—brusatol, bruceoside A, bruceantin, effect on cellular respiration of P-388 leukemia cells, *in vivo, in vitro* □ Quassinoids—antineoplastic activity, effect on cellular respiration of P-388 leukemia cells, *in vivo, in vitro* □ Leukemia, P-388—effect of quassinoids on cellular respiration, *in vivo, in vitro* □ Cellular respiration—P-388 leukemia cells, effect of various quassinoids

Numerous quassinoids have been isolated recently, *e.g.*, bruceantin (I) from *Brucea antidysenterica* (1), bruceoside A (II) from *Brucea javanica* (2), and its subsequent hydrolysis product brusatol (III). All three agents are active in the P-388 lymphocytic leukemia survival system. Bruceantin has a T/C = 197 at 1 mg/kg/day, bruceoside A has a T/C = 156 at 6 mg/kg/day, and brusatol has a T/C = 158 at 125 μg/kg/day (3). At a concentration of 0.015 m*M*,



I



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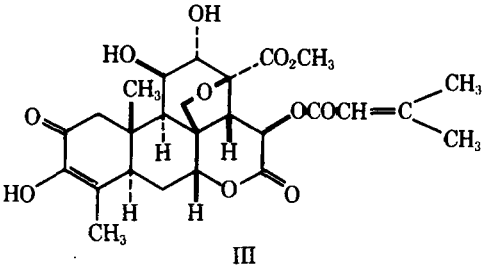

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