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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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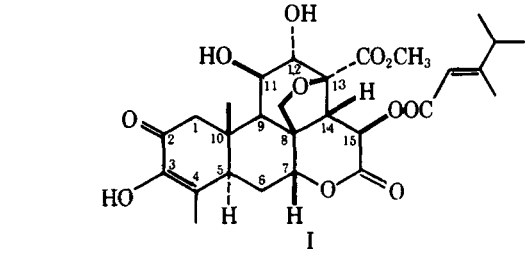
Received October 28, 1978, from the *Division of Medicinal Chemistry, School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514.* Accepted for publication January 9, 1979.

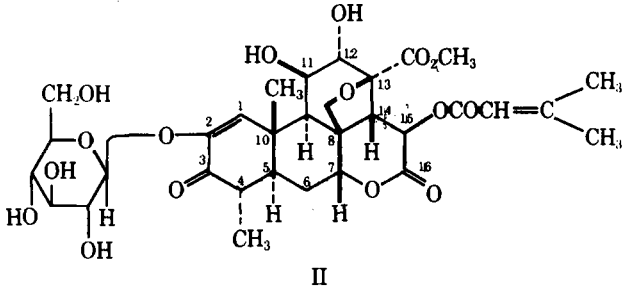
Abstract □ Brusatol, a quassinoid with potent antineoplastic activity against P-388 lymphocytic leukemia cell proliferation, significantly inhibited P-388 cell hexokinase, phosphofructokinase, malic dehydrogenase, and succinic dehydrogenase. Mitochondrial oxidative phosphorylation, basal, and adenosine 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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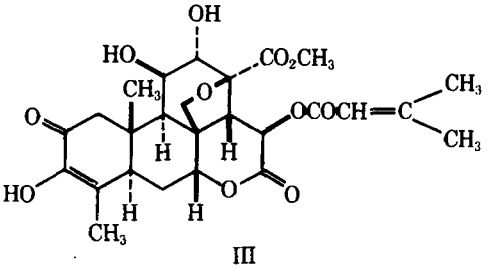

III

Table I—Effects of Brusatol on Aerobic and Anaerobic Processes of DBA/2 Mice

Process	Control	Brusatol, 100 μg/kg/day on Days 7, 8, and 9, % of control
	P-388 Lymphocytic Leukemia Cells (n = 6)	
Hexokinase activity	100 ± 21	62 ± 17[a]
Phosphofructokinase activity	100 ± 16	54 ± 11[b]
Lactic dehydrogenase activity	100 ± 9	97 ± 13
Malic dehydrogenase activity	100 ± 16	43 ± 15[b]
Succinic dehydrogenase activity	100 ± 15	25 ± 3[b]
Adenosine triphosphatase activity	100 ± 6	107 ± 11
Cyclic AMP	100 ± 8	1269 ± 60[b]
Oxidative phosphorylation		
With succinate: state 4	100 ± 13	22 ± 6[b]
With succinate: state 3	100 ± 7	18 ± 5[b]
With α-ketoglutarate: state 4	100 ± 6	26 ± 5
With α-ketoglutarate: state 3	100 ± 11	29 ± 13
	Liver from Nontumor-Bearing Animals (n = 5)	
Cyclic AMP levels	100 ± 22	111 ± 14
Oxidative phosphorylation		
With succinate: state 4	100 ± 12	96 ± 5
With succinate: state 3	100 ± 8	102 ± 8
With α-ketoglutarate: state 4	100 ± 11	97 ± 10
With α-ketoglutarate: state 3	100 ± 13	93 ± 15

[a] p = 0.025. [b] p = 0.001.

all of these quassinoids significantly inhibited DNA, RNA, and protein syntheses in P-388 cells.

A positive correlation between aerobic respiration inhibition and antitumor–antimitotic activity has been observed for a number of nonrelated compounds, *e.g.*, a podophyllotoxin derivative, ellipticine, 4,6-diaminotriazines, methotrexate, naphthoquinone derivatives, tritylthioalanine, carminomycin, piperazinedione (4), 5-fluorinated pyrimidine-6-carboxyaldehydes (5), benzamalecene, triparanol (6), and sesquiterpene lactones (7). Sesquiterpene lactone antineoplastic agents also have been observed to suppress tumor cell glycolytic and Krebs cycle dehydrogenase activity enzymes (8). This paper reports the effect of quassinoids on P-388 lymphocytic leukemia aerobic and anaerobic cell metabolism.

EXPERIMENTAL

Materials—Bruceantin (I) was obtained in small quantities from the National Cancer Institute. Bruceoside A (II) was extracted from *B. javanica* according to the literature technique (2), and brusatol (III) was obtained by treating II with 3 *N* sulfuric acid–methanol (1:1) to hydrolyze the glycosidic linkage (2).

P-388 Lymphocytic Leukemia Cell Metabolism Studies—DBA/2 male mice (~25 g) were inoculated with 10^6 P-388 lymphocytic leukemia cells intraperitoneally on Day 0. For the *in vitro* studies, P-388 lymphocytic leukemia cells were harvested from the peritoneal cavity on Day 10. For the *in vivo* studies, the mice were treated on Days 7, 8, and 9 with 100 μg/kg/day ip of brusatol or 5 mg/kg/day ip of bruceoside A. Drugs were prepared by homogenization in 0.05% polysorbate 80 and water.

The number of tumor cells per milliliter and the 0.4% trypan blue uptake were determined with a hemocytometer (9). Phosphofructokinase activity of 10-day cells was determined by a literature method (10) based on the absorbance change of 1.125 μmoles of reduced nadide (nicotinamide adenine dinucleotide) at 340 nm. Hexokinase activity was assayed by a literature method (11) based on the absorbance change of 500 μmoles of nadide phosphate at 340 nm. Lactic dehydrogenase activity was determined by a literature method (12) based on absorbance changes of 0.12 μmole of reduced nadide at 340 nm.

Succinic dehydrogenase activity was determined by a literature method (13) based on the reduction of potassium ferric cyanide to the ferrous ion at 455 nm. Malic dehydrogenase activity was measured by a literature technique (14) based on absorption changes of 15 μmoles of reduced nadide. Protein concentrations were determined by the method of Lowry *et al.* (15).

In vitro respiration studies were carried out on 10-day P-388 lymphocytic leukemia cells. Oxygen consumption was measured with an electrode[1] connected to an oxygraph[2]. The reaction vessel typically 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 the substrate, 22 μmoles of adenosine triphosphate, and 0.015 or 0.005 μmole of test drugs in a total volume of 1.8 ml at 37°. After the basal metabolic level (state 4) was obtained, 0.257 μmole of adenosine diphosphate was added to obtain the adenosine diphosphate-stimulated respiration rate (state 3) (7).

In vivo studies were conducted on 10-day cells after treatment of the DBA/2 mice. The method of Lindahl and Oberg (16) was used to determine if oxidative phosphorylation was uncoupled with 0.2 μmole of 2,4-dinitrophenol as an internal standard. The reaction medium contained, in 2 ml, 50 μmoles of phosphate buffer, 10 μmoles of magnesium chloride, 2 μmoles of diphosphate, 80 μmoles of glucose, excess hexokinase, 60 μmoles of pyruvate or succinate, and P-388 isolated mitochondria. Studies were carried out using the oxygraph. Measurement of individual electron-transport system components was carried out by a literature method (17) by recording differences in spectral absorption after incubating the P-388 lymphocytic leukemia mitochondrial suspensions (9000×*g* × 10 min) at pH 7.4 with 50 m*M* tromethamine hydrochloride, 50 m*M* potassium chloride, 50 m*M* sucrose, 10 m*M* inorganic phosphate, and either 4.2 m*M* glutarate plus 4.2 m*M* malate or 1.3 m*M* succinate as the substrate.

Nadide was measured at 340 and 374 nm, flavin adenine dinucleotide was measured at 455 and 500 nm, cytochrome b was measured at 410 and 430 nm, cytochrome c and c_1 was measured at 540 and 550 nm, and cytochrome oxidase was measured at 603 and 605 nm. The difference was obtained by calculating the difference between the reduced and oxidized cofactor forms, which was expressed as percent of the control. *In vitro* spectral studies were carried out by incubating 3.16 μmoles of nadide with 0.005 μmole of brusatol in phosphate buffer, pH 7.2, and measuring the appearance of the reduced form at 340 nm. Cytochrome c at 0.1 μmole was also incubated with brusatol, and the appearance of the reduced form was measured at 550 nm over 48 hr. Reduced nadide (6.5 μmoles) and brusatol (10 μmoles) were incubated at room temperature in 1 ml of 0.067 *M* phosphate buffer at pH 7.2 for 72 hr. The reaction medium was subjected to preparative TLC separation on silica gel plates eluted with chloroform–methanol (10:1). Spots were identified by UV absorption. Individual spots were scraped off the plate. An unknown spot was separated from the silica gel by column chromatography, which was eluted with acetone. Silica gel TLC showed a single spot at R_f 0.55 as compared to brusatol at R_f 0.64 whereas the cofactor remained at the origin.

Adenosine triphosphatase activity was measured in P-388 leukemia cells by the method of Suolinna *et al.* (18), and inorganic phosphate released was measured by the method of Chen *et al.* (19). Cyclic AMP levels were measured by a literature radioimmunoassay (20), using ^{3}H(G)-cyclic

[1] Clark.
[2] Gilson.

Table II—*In Vitro* Effects of Quassinoids on Oxidative Phosphorylation Process of P-388 Lymphocytic Leukemia Cells and DBA/2 Mouse Liver Homogenates

Substrate	0.05% Polysorbate 80,%	Percent Control: Bruceantin (0.015 μmole)	Bruceoside A (0.015 μmole)	Brusatol (0.005 μmole)	2,4-Dinitrophenol (0.2 μmole)
		P-388 Oxidative Phosphorylation Processes (n = 6)			
Succinate					
State 4	100 ± 13	50 ± 7[a]	54 ± 7[a]	50 ± 9[a]	—
State 3	100 ± 7	57 ± 10[a]	55 ± 10[a]	50 ± 7[a]	—
α-Ketoglutarate					
State 4	100 ± 6	60 ± 7[a]	58 ± 10[a]	59 ± 9[a]	—
State 3	100 ± 11	52 ± 6[a]	66 ± 5[a]	61 ± 11[a]	—
		Liver Oxidative Phosphorylation Processes (n = 5)			
Succinate					
State 4	100 ± 12	102 ± 14	105 ± 20	105 ± 19	—
State 3	100 ± 12	95 ± 14	86 ± 9	98 ± 17	—
α-Ketoglutarate					
State 4	100 ± 13	74 ± 13[b]	89 ± 19	101 ± 8	—
State 3	100 ± 21	70 ± 3[b]	105 ± 30	95 ± 28	—
		Isolated Mitochondria from P-388 Cells			
Pyruvate: state 3	100 ± 8	—	—	71 ± 7[a]	21 ± 5[a]
Succinate: state 3	100 ± 8	—	—	51 ± 6[a]	15 ± 4[a]

[a] p = 0.001. [b] p = 0.025.

AMP (39.8 Ci/mmole). Liver oxidative phosphorylation studies were carried out on nontumor-bearing DBA/2 male mice. The liver was excised, and a 10% homogenate in 0.25 *M* sucrose and 0.001 *M* edetic acid was prepared (21). Respiration was carried out on 0.1 ml of the homogenate in the reaction vessel. Cyclic AMP levels also were measured on the 10% homogenate.

RESULTS

Brusatol treatment of P-388 lymphocytic leukemia-bearing mice significantly reduced the activity of the glycolytic pathway regulatory enzymes of P-388 cells (Table I). Ten-day P-388 cells demonstrated a hexokinase activity as an absorbance change of 0.300 optical density (O.D.) unit/min/mg of protein, which brusatol reduced 38%. The phosphofructokinase activity for 10-day P-388 cells was 0.270 O.D. unit/min/mg of protein, which was reduced 46% by brusatol treatment. Krebs cycle dehydrogenase enzymes were also suppressed by brusatol. Malic dehydrogenase activity for control P-388 cells was 135.7 O.D. units/min/mg of protein, which brusatol reduced 57%. Succinic dehydrogenase activity for 10-day P-388 cells was 0.145 O.D. unit/min/mg of protein. Brusatol inhibited succinic dehydrogenase activity 75%.

In vitro oxidative phosphorylation studies showed that 0.015 μmole of bruceantin, bruceoside A, or brusatol at 0.015 and 0.005 μmole reduced states 3 and 4 respiration significantly (Table II). Basal respiration (state 4) with succinate as the substrate resulted in 11.44 μl of oxygen consumed/min/mg of protein. Adenosine diphosphate-stimulated respiration (state 3) with succinate was 20.76 μl of oxygen consumed/min/mg of protein. Basal respiration using α-ketoglutarate as the substrate resulted in 10.01 μl of oxygen consumed/min/mg of protein. State 3 consumption resulted in 19.17 μl of oxygen consumed/min/mg of protein.

Brusatol at 0.005 μmole *in vitro* caused a 50% inhibition of state 4 and state 3 with succinate as the substrate. Brusatol with α-ketoglutarate as the substrate caused a 41% inhibition of state 4 and a 39% inhibition of state 3. Both concentrations of brusatol, *i.e.*, 0.005 and 0.015 μmole, caused the same degree of suppression. Bruceoside A with succinate caused a 46% inhibition of state 4 and a 45% of inhibition of state 3. With α-ketoglutarate, bruceoside A caused a 42% inhibition of state 4 and a 34% inhibition of state 3. Bruceantin caused a 50% inhibition of state 4 and a 43% inhibition of state 3 with succinate and a 40% inhibition of state 4 and a 48% inhibition of state 3 with α-ketoglutarate.

At 100 μg/kg/day, brusatol suppressed *in vivo* state 4 basal respiration 78% and adenosine diphosphate respiration (state 3) 82% in the presence of succinate (Table I). α-Ketoglutarate basal respiration was suppressed 74% by brusatol treatment, and state 4 respiration was inhibited 71%. Bruceoside A treatment *in vivo* at 5 mg/kg/day caused a 7% inhibition of state 4 respiration and a 31% inhibition of state 3 with succinate. State 4 with α-ketoglutarate was inhibited 18% and state 3 was inhibited 25% by bruceoside A.

In vitro and *in vivo* studies with liver homogenates of nontumor-bearing animals demonstrated no changes in states 4 and 3 with either succinate or α-ketoglutarate as the substrate. *In vitro* state 3 studies with P-388 lymphocytic leukemia mitochondria with 0.005 μmole of brusatol showed that respiration was inhibited 29% with pyruvate and 49% with succinate as the substrate, respectively (Table II). 2,4-Dinitrophenol addition further suppressed state 3 respiration by 79% with pyruvate and 85% with succinate, indicating that oxidative phosphorylation was not uncoupled by brusatol. Adenosine triphosphatase activity after brusatol treatment was within normal limits (Table I).

Examination of the different spectra of cofactors of the electron-transport system showed that *in vivo* administration of brusatol increased the concentration of all reduced forms of the chain cofactors. Incubation with malate showed a 24% increase in reduced nadide, a 7% increase in reduced flavin adenine dinucleotide, a 13% increase in reduced cytochrome b, a 110% increase in reduced cytochrome c_1 and c, and a 100% increase in reduced cytochrome $\alpha_1 + \alpha_3$. Incubation with succinate resulted in a 198% increase in reduced flavin adenine dinucleotide, a 49% increase in reduced cytochrome b, a 216% increase in reduced cytochromes c_1 and c, and a 100% increase in reduced cytochrome $\alpha_1 + \alpha_3$.

In vitro studies showed the same trends but not as markedly. When brusatol was incubated with isolated nadide for 24 hr, there was a 26% increase in the reduced form; there was a 35% increase in reduced nadide after 40 hr, indicating that brusatol chemically reacted with the cofactor. When cytochrome c was incubated with brusatol, there was a 21% increase in reduced cytochrome c after 24 hr; after 48 hr, there was a 13% increase in reduced cytochrome c. Incubation of brusatol with reduced nadide demonstrated that the two agents chemically interacted to give a product that did not have the same R_f value as the two starting materials, suggesting the formation of an adduct between brusatol and the cofactor.

DISCUSSION

Brusatol treatment of P-388 lymphocytic leukemia-bearing mice significantly reduced the activities of enzymes of the Embden-Meyerhoff cycle and of the Krebs cycle. The key regulatory enzymes, hexokinase and phosphofructokinase, were significantly depressed by brusatol treatment. Krebs cycle dehydrogenase activities of malate and succinate were also significantly reduced. Succinate, which is a flavin adenine dinucleotide-linked dehydrogenase, was more severely depressed than malate dehydrogenase, which is a nadide-linked dehydrogenase. Oxidative phosphorylation processes of P-388 lymphocytic leukemia cells were significantly reduced by the quassinoids tested. With succinate as the substrate, both basal respiration (state 4) and adenosine diphosphate-stimulated respiration (state 3) were suppressed slightly more than with α-ketoglutarate as the substrate. Brusatol *in vivo* was more effective in reducing states 4 and 3 respiration with succinate or α-ketoglutarate.

Brusatol *in vivo*, however, did not affect normal liver respiration processes, nor did any of the quassinoids affect *in vitro* liver respiration at 0.015 μmole. The mitochondria studies showed that brusatol did not uncouple oxidative phosphorylation like 2,4-dinitrophenol does. Nor did brusatol treatment stimulate mitochondrial adenosine triphosphatase activity, which would facilitate the uncoupling of mitochondrial oxidative phosphorylation. Rather, brusatol appeared to act on the mitochondrial

electron-transport chain. *In vivo* studies showed drastic increases in the reduced forms of chain cofactors with either malate or succinate as the substrate. *In vivo* brusatol effects were more striking than *in vitro* effects on reducing cofactors, which may explain the observed increased effects of brusatol on *in vivo* states 4 and 3 respiration after 3 days of treatment as compared to *in vitro* effects. UV *in vitro* studies showed that brusatol chemically interacted with nadide increasing the reduced form absorbance at 340 nm. Cytochrome c, a heme, also was reduced in the presence of brusatol to the ferrous form with an increase in absorbance at 550 nm.

Although separation of the brusatol adduct and reduced nadide has not been achieved to date, incubation of the two agents showed that a possible reaction product existed. Nucleophilic attack of reduced nadide on the diosphenol ring A of brusatol may be postulated. If such an attack occurs, then complexation or alkylation of cofactor or functional groups of enzymes could account for the inhibitory effects of brusatol on P-388 lymphocytic leukemia cell metabolism with respect to the suppression of tumor cell anaerobic and aerobic respiration.

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^{13}C-NMR Spectra of α-Adrenergic Blocking Agents

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Received October 13, 1978, from the *Departments of *Physiology and ‡Chemistry, University of North Dakota, Grand Forks, ND 58202.* Accepted for publication January 17, 1979.

Abstract □ The natural abundance ^{13}C-NMR spectra of five α-adrenergic blocking agents, tolazoline, dibenamine, azapetine, phenoxybenzamine, and phentolamine, are reported. The chemical shifts of various carbon resonances were assigned on the basis of chemical shift theory, multiplicities observed in single-frequency off-resonance-decoupled spectra, relaxation times, and comparisons with the chemical shifts of model compounds.

Keyphrases □ NMR spectroscopy—analysis, tolazoline, dibenamine, azapetine, phenoxybenzamine, phentolamine □ α-Adrenergic blocking agents—analysis, NMR spectroscopy

Earlier studies reporting the ^{13}C-NMR spectra of antimalarials (1, 2), anti-inflammatory agents (3, 4), antipyretic analgesics (5), and central nervous system acting agents (6–10) prompted the assignments of the natural abundance ^{13}C-NMR chemical shifts of α-adrenergic blocking agents, 2-benzyl-2-imidazolone (I) (tolazoline), *N,N*-dibenzyl-β-chloroethylamine (II) (dibenamine), 6,7-dihydro-6-(2-propenyl)-5*H*-dibenz[*c,e*]azepine (III) (azapetine), *N*-(2-chloroethyl)-*N*-(1-methyl-2-phenoxyethyl)benzenemethanamine (IV) (phenoxybenzamine), and 3-[[(4,5-dihydro-1*H*-imidazol-2-yl)methyl](4-methylphenyl)amino]phenol (V) (phentolamine). The ^{13}C-NMR spectra of these therapeutic agents are of theoretical as well as of biological interest.

Both the proton noise-decoupled and single-frequency off-resonance-decoupled (SFORD) spectra of I–V were recorded using the Fourier transform technique. The proton noise-decoupled spectra of these compounds gave the chemical shifts of various carbon resonances, while the SFORD spectra provided the distinction of methyl, methylene, methine, and nonprotonated carbons. Furthermore, the relaxation time measurements differentiated nonprotonated carbons from protonated carbons.

The assignments of various carbon-13 signals were made on the basis of chemical shift theory (11), multiplicities observed in the SFORD spectra, percent integration of the signals in the proton noise-decoupled spectra, relaxation times, and chemical shifts of the corresponding carbons of model compounds (12).

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