Antitumor Agents XXVII: Effects of Helenalin on Anaerobic and Aerobic Metabolism of Ehrlich Ascites Cells

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Abstract □ Evidence is presented that the antitumor agent helenalin, a sesquiterpene lactone, suppresses anaerobic glycolytic enzymes of tumor cells at a number of sites and not exclusively at glycogen synthetase and phosphofructokinase, previously proposed sites for inhibition by α-methylene-γ-lactones. Of the enzymes tested, the sulfhydryl-containing enzyme hexokinase was inhibited the maximum, *i.e.*, 83%, by helenalin treatment, whereas phosphofructokinase and glycogen synthetase were suppressed approximately 45%. Another sulfhydryl-bearing enzyme, aldolase, was decreased approximately 43%. Phosphorylase *a* was inhibited 65%, glucose-6-phosphatase was inhibited 46%, and succinic dehydrogenase was inhibited 59% by helenalin treatment. Mitochondrial oxidative phosphorylation processes were also significantly depressed in the presence of helenalin *in vitro* with either succinate or α-ketoglutarate as substrates. Thus, a number of enzymes of anaerobic and aerobic carbohydrate metabolism of Ehrlich ascites cells appear to be inhibited by helenalin, which supposedly can alkylate functional groups, *e.g.*, sulfhydryl groups of these enzymes, by a rapid Michael-type addition.

Keyphrases □ Helenalin—effects on anaerobic and aerobic metabolism of Ehrlich ascites cells □ Metabolism, anaerobic and aerobic—Ehrlich ascites cells, effects of helenalin □ Enzyme activity—effects of helenalin on anaerobic and aerobic metabolism of Ehrlich ascites cells □ Antitumor agents—helenalin, effects on anaerobic and aerobic metabolism of Ehrlich ascites cells

Helenalin (1), a sesquiterpene lactone, recently was shown to possess significant antitumor activity against Walker 256 ascites carcinosarcoma (Sprague–Dawley rats at 2.5 mg/kg/day, T/C = 316%) and Ehrlich ascites (CF_1 mice at 33.3 mg/kg/day, 99% inhibition) and to be marginally active against P-388 lymphocytic leukemia (DBA/2 mice at 25 mg/kg/day, T/C = 127%) (1) and Lewis lung carcinoma (C57BL 6 mice at 25 mg/kg/day, T/C = 142%)[1]. Further studies demonstrated that helenalin inhibited deoxyribonucleic acid (1) and protein synthesis but had little effect on ribonucleic acid synthesis of Ehrlich tumor cells (2). Deoxyribonucleic acid polymerase activity and phosphorylation of nuclear histones and nonhistone proteins were inhibited, whereas the level of 3′,5′-cyclic adenosine monophosphate was tripled by drug treatment of CF_1 male Ehrlich ascites tumor-bearing mice (1).

Kupchan and coworkers (3–5) demonstrated that the α-methylene-γ-lactone system can act as the alkylating center for cytotoxic antitumor lactone, *e.g.*, elephantopin, vernolepin, and euparotin acetate. A Michael-like reaction between biological nucleophiles, such as L-cysteine (3), or the sulfhydryl-containing enzymes, *e.g.*, phosphofructokinase (4) and glycogen synthetase (5), was proposed as the mechanism of these agents. *In vitro* helenalin, which contains both the α-methylene-γ-lactone and cyclopentenone moieties, alkylates sulfhydryl groups of cysteine and reduced glutathione (2). The effects of helenalin on the aerobic and anaerobic respiration of Ehrlich ascites tumor-bearing mice are now reported.

[1] Unpublished results. Lewis lung screening was carried out by the procedure outlined by R. I. Geran, N. H. Greenberg, M. M. MacDonald, A. M. Schumacher, and B. J. Abbott, *Cancer Chemother. Rep.*, **3**, 13 (1972), and references cited therein.

EXPERIMENTAL

Male CF_1 mice (~30 g) were implanted intraperitoneally on Day 0 with 10^6 Ehrlich ascites tumor cells. On Days 5, 6, and 7, animals were treated with 0.125 mg ip of helenalin suspended by homogenization in 0.05% polysorbate 80–water. Animals were sacrificed on Day 8, and the ascites fluid was collected from the peritoneal cavity and homogenized.

The following enzymatic activities were determined for both control and treated cells: glucose-6-phosphate dehydrogenase (6), glucose-6-phosphatase (7), hexokinase (8), glycogen synthetase (9), phosphofructokinase (10), aldolase (11), glyceraldehyde-3-phosphate dehydrogenase (12), lactic dehydrogenase (13), malic dehydrogenase (14), succinic dehydrogenase (15), total cellular adenosine triphosphatase (16), mitochondrial adenosine triphosphatase (17), and monoamine oxidase (18).

Phosphorylase *a* activity was determined as the disappearance of inorganic phosphate from the medium used for the phosphorylation of glucose when released from glycogen. The reaction medium contained 1 μmole of sodium phosphate, 2% glycogen, 0.3 μmole of 3′,5′-cyclic adenosine monophosphate, 2.0 μmoles of sodium fluoride, and 10 μmoles of tris(hydroxymethyl)aminomethane at pH 6.1 in a total volume of 0.1 ml. After incubation for 60 min at 37°, the inorganic phosphate was determined.

Fructose-1,6-diphosphatase was determined utilizing a reaction medium containing 60 μmoles of pH 9.4 glycine buffer, 1 μmole of fructose-1,6-diphosphate, and 1 μmole of manganese chloride (19). After incubation at 37° for 15 min, the inorganic phosphate was determined by the method of Chen *et al.* (20). The protein content for all assays was determined by the method of Lowry *et al.* (21).

In vitro effects of helenalin on Ehrlich ascites tumor oxidative phosphorylation in the presence and absence of adenosine diphosphate, utilizing succinate (flavin adenine dinucleotide-linked dehydrogenase) or α-ketoglutarate (nicotinamide adenine dinucleotide phosphate-linked dehydrogenase) as substrates, were measured. Oxygen consumption was measured with a Clark oxygen electrode connected to a Gilson oxygraph.

The reaction vessel contained 55 μmoles of sucrose, 22 μmoles of potassium chloride, 22 μmoles of dibasic potassium phosphate, 90 μmoles of succinate or 60 μmoles of α-ketoglutarate, 2 μmoles of adenosine triphosphate, and helenalin (0.2 mg in 0.05% polysorbate 80–water) in a total volume of 1.8 ml. After the basal metabolic (state 4) level was obtained, 0.257 μmole of adenosine diphosphate was added to obtain the adenosine diphosphate-stimulated respiration rate (state 3) (22).

RESULTS

Helenalin significantly reduced the enzymatic activity of glycolytic and Krebs cycle enzymes (Table I). Glucose-6-phosphate dehydrogenase activity for 8-day Ehrlich ascites cells was 0.5158 O.D. (optical density) unit/min/mg of protein based on the presence of 0.0056 μmole of nicotinamide adenine dinucleotide phosphate. Helenalin caused a 30% reduction in the activity of this enzyme. Glucose-6-phosphatase activity for the control was 7.594 μg of inorganic phosphate/mg of protein released in 15 min. Helenalin caused a 46% reduction in enzymatic activity. Hexokinase activity for the control was 0.169 O.D. unit/min/mg of protein based on the presence of 500 μmoles of nicotinamide adenine dinucleotide phosphate. Helenalin reduced the activity by 83%.

Glycogen synthetase activity for the control was 5036 dpm/mg of

Table I—Effects of Helenalin on Ehrlich Ascites Metabolism of 8-day CF_1 Male Mice (n = 6)

Compound	Control, 0.05% Polysorbate 80, $\bar{x} \pm SD$[a]	Helenalin, 0.125 mg/day, $\bar{x} \pm SD$[a]
In Vivo Enzymatic Assay		
Glucose-6-phosphate dehydrogenase	100 ± 27	70 ± 26
Glucose-6-phosphatase	100 ± 23	54 ± 25[b]
Hexokinase	100 ± 32	17 ± 2[b]
Glycogen synthetase	100 ± 24	56 ± 24[b]
Phosphorylase *a*	100 ± 19	35 ± 12[b]
Phosphofructokinase	100 ± 39	55 ± 15[b]
Fructose-1,6-diphosphatase	100 ± 26	70 ± 14
Aldolase	100 ± 22	57 ± 15[b]
Glyceraldehyde-3-phosphate dehydrogenase	100 ± 20	115 ± 8
Lactic dehydrogenase	100 ± 15	100 ± 22
Malic dehydrogenase	100 ± 18	92 ± 14
Succinic dehydrogenase	100 ± 22	41 ± 22[b]
Total cellular adenosine triphosphatase	100 ± 18	93 ± 29
Mitochondrial adenosine triphosphatase	100 ± 24	74 ± 20
Monoamine oxidase	100 ± 2	111 ± 4
In Vitro Oxidative Phosphorylation (0.2 mg of Helenalin)		
Succinate		
State 3	100 ± 4	60 ± 8[b]
State 4	100 ± 3	72 ± 8[b]
α-Ketogluturate		
State 3	100 ± 11	67 ± 10[b]
State 4	100 ± 5	48 ± 15[b]

[a] Mean of the percent of the control value and the standard deviation. The probable significance level (p) was determined by the Student t test. [b] p = 0.001.

protein; helenalin reduced the activity by 44%. Phosphorylase *a* activity for the control was 4.43 μg of inorganic phosphate taken up from the medium/hr/mg of protein; helenalin reduced this activity by 65%. Phosphofructokinase activity for the control was 0.252 O.D. unit/min/mg of protein based on 1.125 μmoles of reduced nicotinamide adenine dinucleotide; helenalin treatment reduced the activity by 45%. Fructose-1,6-diphosphatase activity of the control was 10.12 μg of inorganic phosphate released/mg of protein in 15 min; helenalin reduced the activity by 30%.

Aldolase activity for the control was 282 mU/mg of protein, which was reduced 43% by helenalin treatment. Glyceraldehyde-3-phosphate dehydrogenase activity for the control was 2.405 O.D. units/min/mg of protein based on 20.3 μmoles of nicotinamide adenine dinucleotide; helenalin treatment had no effect on this enzyme. Lactic dehydrogenase activity for the control was 5.696 O.D. units/min/mg of protein based on 0.12 μmole of reduced nicotinamide adenine dinucleotide and was not affected by helenalin treatment.

Malic dehydrogenase activity for the control was 0.7975 O.D. unit/min/mg of protein based on 15 μmoles of reduced nicotinamide adenine dinucleotide; helenalin had very little effect on this enzyme. Succinic dehydrogenase activity for the control was 0.0669 O.D. unit/min/mg of protein based on 15 μmoles of potassium ferricyanide; helenalin treatment caused a 59% suppression of enzymatic activity. Total cellular adenosine triphosphatase for the control was 9.873 μg of phosphate hydrolyzed/10 min/mg of protein. Helenalin treatment resulted in no change in activity.

Mitochondrial adenosine triphosphatase activity for the control was 1.139 μg of phosphate released/10 min/mg of protein. Helenalin treatment caused a 26% suppression of enzymatic activity, but this amount was not significant. The enzyme monoamine oxidase was very low in activity in Ehrlich ascites cells; no alteration was observed with helenalin treatment.

The respiration of Ehrlich ascites tumor cells in the presence of sodium succinate as a substrate in the absence of adenosine diphosphate (basal state 4 respiration) was 5.506 μl of oxygen/hr/mg of protein; in the presence of adenosine diphosphate-coupled oxidative phosphorylation (state 3), it was 6.487 μl of oxygen/hr/mg of protein. Sodium α-ketoglutarate resulted in a state 4 respiration of 2.525 μl of oxygen/hr/mg of protein, and state 3 respiration was 4.049 μl of oxygen/hr/mg of protein. Helenalin treatment caused a 40% reduction in state 3 and a 28% reduction in state 4 with succinate (flavin adenine dinucleotide-linked enzyme) and a 33% reduction in state 3 and a 52% reduction in state 4 with α-ketoglutarate (a nicotinamide adenine dinucleotide-linked enzyme). The vehicle polysorbate 80–water itself did not alter enzymatic activities of Ehrlich ascites cells, with the exception of state 3 respiration, which was depressed 12% in the presence of this vehicle.

DISCUSSION

Several studies have indicated that cancer cells are dependent on energy from the anaerobic glycolytic cycle and that the mitochondria of these cells are altered; *i.e.*, the mitochondria are tightly coupled (23), vary in number and morphology, and have modified swelling properties (24–26), depending on the type of cells and stage of differentiation, generally with a reduction in number and a more heterogeneous size and distribution (27). Reduction of adenosine diphosphate stimulated respiration; *i.e.*, coupled oxidative phosphorylation (state 3), as well as accelerated uncoupled electron transport respiration (state 4), has been observed with mitochondria from cancer cells (24). Supposedly, the tumor cell is dependent on the Embden–Meyerhof cycle as an energy source (25), which is critical for the cell's survival in view of the energy required for rapid nucleic and protein synthesis in proliferating cells.

Since the observation had previously been made that compounds containing the α-methylene-γ-lactone moiety inhibited sulfhydryl-bearing enzymes, a number of enzymes of the Embden–Meyerhof and Krebs cycle were examined as possible sites of inhibition by helenalin in Ehrlich ascites cells. Enzymes that regulate the availability of glucose for glycolysis, *e.g.*, hexokinase and phosphorylase *a*, were drastically inhibited by helenalin treatment. Other sulfhydryl-containing enzymes, such as the regulatory enzyme phosphofructokinase and aldolase, were also inhibited but to a lesser degree by helenalin. Enzymes later in the cycle, *e.g.*, glyceraldehyde-3-phosphate dehydrogenase and lactic dehydrogenase, were unaffected by helenalin treatment. Enzymes required for the reversal of glycolysis, *e.g.*, glucose-6-phosphatase, fructose-1,6-diphosphatase, as well as glycogen synthetase, were inhibited significantly by helenalin treatment.

The regulatory enzyme of the hexose monophosphate shunt, glucose-6-phosphate dehydrogenase, was reduced, but not significantly, by helenalin treatment. The mitochondrial enzyme succinic dehydrogenase was significantly reduced by helenalin treatment. This enzyme is reported to contain an iron–sulfur protein as an integral component. Malic dehydrogenase and monoamine oxidase activities were not affected by helenalin treatment.

Gosálvez *et al.* (28) attempted to correlate antitumor activity of compounds with the suppression of tumor respiration, *e.g.*, 4,6-diaminotriazine derivatives, naphthoquinone analogs, tritylthioalanine, carminomycin, piperazinedione, and methotrexate. Helenalin *in vitro* reduced basal oxygen consumption (electron transport) with both nicotinamide adenine dinucleotide and flavin adenine dinucleotide-linked enzymes of Ehrlich ascites cells. Oxidative phosphorylation (coupled respiration) also was inhibited in the presence of helenalin. The reduction in these processes did not appear to be due to the suppression of mitochondrial adenosine triphosphatase activity since the activity of this enzyme was not significantly different from the control activity, as was the total cellular adenosine triphosphatase activity. Helenalin had little effect on normal mouse liver oxidative phosphorylation processes.

CONCLUSION

Helenalin has the ability to inhibit both aerobic and anaerobic energy processes of tumor cells. The thesis that the α-methylene-γ-lactone component or the cyclopentenone ring (1) of the molecule has the ability to alkylate functional groups of these enzymes by a Michael-type addition is attractive. Whereas the inhibition of glycolytic metabolism is probably not the major mechanism of action of helenalin as an antitumor agent (*i.e.*, the mechanism is most likely inhibition of nucleic acid and protein synthesis), the fact that the energy available to cells for synthetic processes is seriously hampered and rate limiting in the presence of helenalin has an additive effect on the drug's actions on cellular metabolism and tumor inhibition.

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Intrapatient Variability of Serial Steady-State Plasma Tricyclic Antidepressant Concentrations

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Abstract □ Nine or 10 serial steady-state plasma measurements of amitriptyline, desipramine, desmethyldoxepin, doxepin, imipramine, nortriptyline, or protriptyline were made in 23 depressed patients. Each patient was monitored for compliance by pill counts, and sampling time was controlled carefully to determine intrapatient variability of steady-state tricyclic levels on a day-to-day basis. The coefficients of variation during serial sampling of the various ingested drugs were: amitriptyline, 21%; desipramine, 26%; doxepin, 21%; imipramine, 14%; nortriptyline, 13%; and protriptyline, 17%. The therapeutic ranges for the tricyclic antidepressants are relatively wide, so coefficients of variation of these magnitudes indicate that the position of an individual patient in relation to the optimal therapeutic range can be reliably determined on a clinical basis.

Keyphrases □ Plasma drug levels—various tricyclic antidepressants, intrapatient variability at steady state □ Antidepressants, various—plasma drug levels, intrapatient variability at steady state

Although it has been over 10 years since Hammer *et al.* (1) first demonstrated a 36-fold difference between the lowest and highest steady-state levels of desipramine in patients ingesting equal amounts of the same drug, the use of plasma tricyclic antidepressant levels as a practical way to improve patient management is only slowly being accepted. Since the evidence that plasma drug levels more closely correspond to therapeutic response than oral dosage, there has been marked interest in the routine determination of plasma tricyclic antidepressant levels as a method of improving clinical response (2–8) and predicting toxicity (9).

Although therapeutic ranges have been suggested for most of the marketed tricyclic antidepressants, the interpretation of the results of plasma tricyclic determinations is often difficult. Differences in optimal plasma therapeutic ranges have been caused by varying purity of the standard drugs used, assay and blood collection methods, and the patient populations studied. In addition, day-to-day variation occurs in the steady-state level in individual patients. This variation is influenced not only by the reliability of the drug assay techniques but by biological variation within the individual.

The purpose of this study was to determine the variation for the entire procedure, controlling for such factors as is practical in a clinical setting.

EXPERIMENTAL

Plasma tricyclic antidepressant levels were assayed by GLC–mass fragmentography by a method described previously (10). This method is specific for each drug monitored and does not assay undescribed metabolites. Differences between duplicate samples are less than 10%.

To examine the biological and laboratory variability of steady-state plasma levels, outpatients undergoing treatment for depression on level doses of drug for at least 2 weeks were subjected to nine or 10 serial samplings over 3 weeks. Patient compliance was monitored by pill counts.