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Antineoplastic Activity of *N*-maleamide Homocysteine Thiolactone Amide Encapsulated within Liposomes (42143)

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Abstract. The antineoplastic activity of *N*-maleamide homocysteine thiolactone amide (MHTA) encapsulated within liposomes was studied in mice with transplanted tumors. Tumor weight was decreased by 4–5 biweekly intraperitoneal injections of MHTA in liposomes in DBA/2N females with MTG mammary adenocarcinoma (35% of control value, $P < 0.005$) and in C57Bl/6N males with MUO4 rhabdomyosarcoma (11% of control value, $P < 0.0000001$). Tumor incidence was reduced from 84 to 63% ($P < 0.05$) and from 100 to 32% ($P < 0.001$) in the two systems, respectively. When the compound was administered in dimethyl sulfoxide to A/HeJ females with A10 mammary adenocarcinoma by daily intraperitoneal injection, tumor weight was reduced to 70% of control value ($P < 0.05$), and there was no decrease in tumor incidence (100%). No toxicity was observed at the therapeutic dose utilized, 10 mg/kg/day. *N*-Maleamide homocysteine thiolactone amide is a derivative of the normal biochemical constituents, maleic acid and homocysteine thiolactone. The results show that the N-substituted maleamide derivative of homocysteine thiolactone decreases the growth of murine tumors of two different histological types, when administered encapsulated within liposomes.

Homocysteine thiolactone metabolism is abnormal in several examples of malignant human and animal cells in culture (1). In these malignant cells the free amino groups of proteins are acylated by homocysteine thiolactone, forming a peptide bond and a free sulfhydryl group. In normal cells the sulfur atom of homocysteine thiolactone is converted to sulfate esters of proteoglycans (2). An hypothetical N-substituted derivative of homocysteine thiolactone is believed to prevent thiolation of proteins in normal cells (1). If this formulation is correct, administration of such a derivative to malignant cells may modify tumor growth by preventing abnormal thiolation of amino groups by homocysteine thiolactone. A series of N-substituted derivatives of homocysteine thiolactone were synthesized to test this hypothesis (3). The results of assaying one of these compounds, *N*-maleamide homocysteine thiolactone amide, for antineoplastic activity are presented in this report.

In a previous study two synthetic derivatives of homocysteine thiolactone, pyridoxal homocysteine thiolactone enamine hydrochloride and arachidonoyl homocysteine thiolactone amide, were found to decrease the growth of transplanted murine mammary adenocarcinoma (4). The inhibition of tumor growth was attributed to decreased conversion of methionine to homocysteine thiolactone derivatives and increased conversion of methionine to cystathionine, taurine, and sulfate (4). The hydroperchlorate salt of homocysteine thiolactone was found to increase the amount of necrosis within a transplanted malignant neoplasm when injected with dextrose and water (5). The N-substituted maleamide derivative of homocysteine thiolactone was found to decrease the growth of a transplanted malignant neoplasm moderately when injected with dimethyl sulfoxide (3). In this report we demonstrate prominent antineoplastic activity of *N*-maleamide homocysteine thiolactone amide in two transplanted murine tumor systems, when injected within liposomes.

Materials and Methods. *Reagents.* Homocysteine thiolactone hydroperchlorate was prepared as previously described (5, 6). Homocysteine thiolactone hydrochloride (30 g) was suspended in chloroform–methanol (1 liter, 4:1 by vol). Two equivalents of 70% $HClO_4$ were slowly added with mixing to achieve solution. Crystallization of homocysteine thiolactone hydroperchlorate occurred

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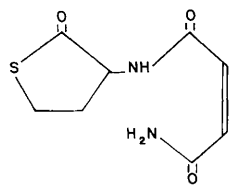
when the solution was concentrated on a rotary evaporator. The crystals were recrystallized from chloroform–methanol (mp 186°C).

N-Maleamide homocysteine thiolactone amide was prepared (3) by suspending 8.7 g of homocysteine thiolactone hydroperchlorate in a solution of maleimide (3.9 g) in toluene (200 ml). Triethylamine (4.0 g) was added gradually, and the mixture was refluxed for 1 hr. Toluene was removed under reduced pressure, and the residue was extracted with cold ethyl ether (100 ml) in several portions. The viscous orange residue was dissolved in hot 95% ethanol (400 ml), filtered, and cooled. The yellow precipitate was recrystallized from 95% ethanol (mp 80–82°C). The chemical structure of *N*-maleamide homocysteine thiolactone amide (3) is presented in Fig. 1.

Animals and tumors. C57Bl/6N male mice (20–25 g) and DBA/2N female mice (20–25 g) were obtained from the National Cancer Institute, Bethesda Md. A/HeJ female mice (20–25 g) were obtained from Jackson Laboratories, Bar Harbor, Maine. The MTG mammary adenocarcinoma and the MUO4 rhabdomyosarcoma were supplied by Dr. A. Bogden, Mason Research Institute, Worcester, Massachusetts. The MUO4 and MTG lines were maintained by monthly transplantation of tumor fragments subcutaneously in C57Bl/6N males and DBA/2N females, respectively. The A10 mammary adenocarcinoma was supplied by Dr. B. Sanford, Jackson Laboratory, and it was maintained by transplantation of ascites cells in A/HeJ females every 7–10 days.

The MUO4 rhabdomyosarcoma arose from a C57Bl/6 male treated with 7,12-dimethylbenzanthracene at Mason Research Institute. The lag phase before tumor growth is evident is 10–20 days, and the average survival time is 65 days after implantation. The subcutaneous tumor does not metastasize. The gross appearance is light gray-tan and moderately firm with slight central hemorrhage and necrosis. Microscopically the tumor cells are arranged in sheets with bundles, whorls, and fascicles in various directions. The cells are spindle shaped with oval, round, and irregular nuclei. Nucleoli are prominent, and nuclear chromatin is condensed along the nuclear membrane. Mitotic figures are frequent, and multinuclear cells are observed. Tests for viral contaminants were negative. The tumor grows in 100% of untreated controls.



N-MALEAMIDE HOMOCYSTEINE THIOLACTONE AMIDE (MHTA)

FIG. 1. Chemical structure of *N*-maleamide homocysteine thiolactone amide.

The MTG adenocarcinoma arose as a spontaneous breast tumor of a DBA/212 female mouse in Dr. A. Goldfeder's laboratory, New York University. The lag phase before tumor growth is evident is 10–14 days, and the average survival time is 30 days after implantation. The subcutaneous tumor does not metastasize. The gross appearance is off white and soft. Microscopically the tumor cells are undifferentiated and epithelial with no acinar structures and a fine connective tissue stroma. The nuclear to cytoplasmic ratio is high, and mitotic figures are numerous. The cells are pleomorphic with oval, round, and irregular vesicular nuclei with prominent nucleoli. Tests for viral contaminants were negative except for LDH agent. Because of extinction of the DBA/212 strain in which the tumor originated, the tumor grows in about 85% of untreated DBA/2 controls.

The A10 adenocarcinoma arose as a spontaneous breast tumor of an A/HeJ female mouse in Dr. B. Sanford's laboratory, Massachusetts General Hospital. The lag phase and survival are similar to that of the MTG adenocarcinoma system. The A10 tumor grows either in the ascites form or as a solid subcutaneous mass, depending upon the site of injection. The subcutaneous tumor does not metastasize. The gross appearance is gray-tan and soft with areas of necrosis. The microscopic appearance is similar to that of the MTG adenocarcinoma. The tumor grows in 100% of untreated controls.

Preparation of liposomes. Liposomes were freshly prepared for each day of use, according to the method of Brassine *et al.* (7). *N*-Maleamide homocysteine thiolactone amide

was dissolved in equal parts of acetonitrile and chloroform–methanol (2:1 by vol). Egg phosphatidyl choline, cholesterol, and stearylamine (Sigma Chemical Company, St. Louis, Mo.) were dissolved in chloroform–methanol at molar ratios of 4:3:1. An equimolar quantity of the solution of lipids was added to the solution of *N*-maleamide homocysteine thiolactone amide to yield a clear yellow solution. The solvents were evaporated under a stream of nitrogen. The residue was suspended in 50 m*M* Tris buffer containing 150 m*M* NaCl, pH 7.4. The ratio of lipids to buffer was 15 mg/ml. The suspension was sonicated at 0°C for 15 min by intermittent pulsation with the microprobe of an Ultrasonics Model W225R sonicator. The uniform milky suspension was kept at 0°C until injected, usually within 2 hr of preparation.

Therapeutic assays. Fragments of the MUO4 and MTG tumors (2 mm^3) were injected subcutaneously by trocar in C57Bl/6N male and DBA/2N female mice, respectively. *N*-Maleamide homocysteine thiolactone amide was given twice weekly by intraperitoneal injection with liposomes. Dosages were calculated on a daily basis. After 12–14 days (4–5 biweekly injections of liposome preparations), the tumors were dissected and weighed. Average body weight for each group was measured weekly.

The A10 ascites tumor cells (10^7) were injected subcutaneously in A/HeJ female mice. *N*-Maleamide homocysteine thiolactone amide dissolved in dimethyl sulfoxide was given daily by intraperitoneal injection. After 11 days of injections, the tumors were dissected and weighed.

Significances of differences of tumor weights from controls were calculated, using Student's *t* test, with correction for unequal group size. Significances of differences in tumor incidence from controls were calculated, using χ^2 test.

Results. *N*-Maleamide homocysteine thiolactone amide encapsulated within liposomes reduced tumor growth in two different transplanted murine tumor systems at a dose of 10 mg/kg/day (Table I). The weight of the MTG mammary adenocarcinoma was reduced to 35% of the control value ($P < 0.005$), and the weight of the MUO4 rhabdomyosarcoma was reduced to 11% of the control value ($P < 0.0000001$). When the compound was given in dimethyl sulfoxide to mice with the A10 mammary adenocarcinoma, the tumor weight was reduced to 70% of the control value ($P < 0.05$).

The data in Table I show that *N*-maleamide homocysteine thiolactone amide encapsulated within liposomes resulted in a significant decrease in the number of animals with tumors. Tumor incidence was reduced from 84 to 63% in the MTG mammary adenocarcinoma system ($P < 0.05$) and from 100 to 32% in the MUO4 rhabdomyosarcoma system ($P < 0.001$). When the compound was given in dimethyl sulfoxide to mice with the A10 adenocarcinoma, there was no effect on tumor incidence (100%).

In the experiments with liposome vehicle the tumor weights were recalculated, using only those animals with tumors (Table I). The weight of the MTG tumors was 0.27 ± 0.32 g for the 15 tumors in the treated group, compared to 0.58 ± 51 g for the 21 tumors in the liposome only group. The weight of the treated group was 47% that of the control group ($P < 0.05$). The weight of the MUO4 tumors was 0.40 ± 0.28 g for the 8 tumors in the treated group, compared to 1.17 ± 0.53 g for the 25 tumors in the liposome only group. The weight of the treated group was 34% of the control group ($P < 0.0005$).

No toxic effects of *N*-maleamide homocysteine thiolactone amide were observed when injected at a dose of 10 mg/kg/day. There was no effect of the compound on survival when injected in liposomes or in dimethyl sulfoxide (Table I). There was no effect of the compound on body weight in the animals with the MTG or MUO4 tumors (Table I). Autopsy revealed no pathological abnormalities other than the transplanted tumors.

Discussion. Encapsulation of *N*-maleamide homocysteine thiolactone amide within liposomes resulted in prominent antineoplastic activity against transplanted murine mammary adenocarcinoma and rhabdomyosarcoma. Not only did the compound reduce the weight of these tumors, it also reduced significantly the number of animals with tumors, compared to controls. Moreover, the weight of the tumors was reduced in those animals with tumors, compared to controls, showing a correlation between reduced tumor size and decreased tumor incidence.

TABLE I. EFFECT OF *N*-MALEAMIDE HOMOCYSTEINE THIOLACTONE AMIDE ON TUMOR GROWTH, TUMOR INCIDENCE, SURVIVAL, AND BODY WEIGHT

Dose of MHTA (mg/kg/d)	Vehicle	Days treated[a]	Strain	Tumor[b]	Survivors/ total	Tumor weight g ± SD	Inhibition of tumor growth (%)	Tumor incidence	Average body weight (g)	
									Start	Finish
—	Liposomes	12	DBA/2N	MTG	25/25	0.49 ± 0.51	—	21/25	24.4	23.4
10	Liposomes	12	DBA/2N	MTG	24/25	0.17 ± 0.29[d]	65	15/24[c]	24.4	24.3
—	Liposomes	14	C57Bl/6N	MUO4	25/25	1.17 ± 0.53	—	25/25	20.4	22.7
10	Liposomes	14	C57Bl/6N	MUO4	25/25	0.13 ± 0.24[f]	89	8/25[e]	19.9	23.8
—	DMSO	11	A/HeJ	A10	19/19	0.60 ± 0.16	—	19/19	—	—
10	DMSO	11	A/HeJ	A10	24/24	0.52 ± 0.16[c]	30	24/24	—	—

[a] Animals were treated twice weekly by intraperitoneal injection of MHTA in liposomes or by daily intraperitoneal injection of MHTA in DMSO.
[b] MTG and A10 are mammary adenocarcinomas, and MUO4 is a rhabdomyosarcoma.
[c] $p < 0.05$.
[d] $p < 0.005$.
[e] $p < 0.001$.
[f] $p < 0.0000001$.

Less prominent antineoplastic activity against transplanted mammary adenocarcinoma was observed when the compound was injected in dimethyl sulfoxide. With this solvent, there was smaller inhibition of tumor growth and no reduction in the number of animals with tumors. Apparently enhanced biological activity is associated with binding of the compound to the lipids of liposomes.

The results show that the compound has antineoplastic activity against both carcinomatous and sarcomatous types of transplanted tumors. In order to characterize further the range of antineoplastic activity of the compound, it would be desirable to study its effect on malignant cells in culture and in animals with transplanted leukemia and tumors of other histological types. Moreover, the present results are preliminary in nature, and further study is needed to optimize the antineoplastic activity and to characterize the toxicity of *N*-maleamide homocysteine thiolactone amide.

N-substituted derivatives of homocysteine thiolactone may control or prevent neoplastic growth by preventing the thiolation of cellular proteins by homocysteine thiolactone (1). The conjugated double bond system of the carbonyl, ethylene, and amide groups of the *N*-maleamide substituent of homocysteine thiolactone is probably necessary for biological activity. The *N*-succinylamide derivative of homocysteine thiolactone lacks this conjugated double bond system and is biologically inactive when injected in dimethyl sulfoxide (4). The *N*-arachidonoyl amide and pyridoxal enamine derivatives of homocysteine thiolactone have antineoplastic activity, possibly because of their extensive conjugated double bond systems (4). Further study is required to determine whether these antineoplastic derivatives of homocysteine thiolactone modify or diminish endogenous thiolation of cellular protein by homocysteine thiolactone within transplanted malignant neoplasms.

Homocysteine thiolactone arises from metabolism of methionine and is bound to the lipids of liver (6). Homocysteine thiolactone is subsequently converted to homocysteic acid, phosphoadenosine phosphosulfate, and sulfate esters of proteoglycans (2). Homocysteic acid promotes growth of hypophysecto-

mized rats, and the increased growth is mediated by the formation of somatomedin (8). Homocysteine is atherogenic in children with homocystinuria (9) and in experimental animals (10, 11). A prominent feature of the vascular lesions in homocysteinemia is hyperplasia of smooth muscle cells within the intima. The apparent enhancement of biological activity of *N*-maleamide homocysteine thiolactone amide by liposome encapsulation suggests that lipoproteins may increase the atherogenic potential of homocysteine derivatives. Cell cultures of many malignant tumors require methionine for growth and fail to grow or grow slowly when supplied with homocystine and a methyl donor (12).

In a study of response of monocytes to chemoattractants, transmethylation reactions were found to be required (13). The synthetic derivative, erythro-9-(2-hydroxy-3-nonyl)adenine, and homocysteine thiolactone depressed adenosyl methionine transmethylation, inhibited chemotaxis, and prevented release of [^{3}H]arachidonic acid from monocytes. The effects of *N*-maleamide homocysteine thiolactone amide on monocytes and the immune system are unknown. Enhancement or inhibition of immune function by antineoplastic homocysteine thiolactone derivatives may be of importance to their effects on tumor growth.

N-Malemide homocysteine thiolactone amide is an example of a class of antineoplastic compounds composed of normal biochemical constituents (4). Maleic acid occurs as maleylacetoacetic acid in the oxidative catabolism of homogentisic acid by homogentisic acid oxidase (14). Maleylacetoacetic acid is converted to fumarylacetoacetic acid by an isomerase requiring glutathione (15). Homocysteine thiolactone and *N*-maleamide homocysteine thiolactone amide are intermediates or potential intermediates in metabolic pathways important in controlling normal (8) and neoplastic (1) growth. The results of the present study show that the presumed alteration of these metabolic pathways by an exogenous homocysteine thiolactone derivative offers a promising approach to the control of neoplastic growth.

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