

Enhancement of Antitumor Activity of Ascorbate against Ehrlich Ascites Tumor Cells by the Copper:Glycylglycylhistidine Complex¹

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ABSTRACT

Ascorbate in an aqueous solution is easily oxidized by molecular oxygen in the presence of cupric ion, thus producing reactive oxygen species and exhibiting cytotoxicity.

In order to increase the antitumor activity of ascorbate, we used the innocuous form of cupric ion complexed with glycylglycylhistidine, a tripeptide designed to mimic the specific Cu(II) transport site of albumin molecule. Although this square planar copper:glycylglycylhistidine complex did not significantly oxidize ascorbate at pH 7.4, it killed Ehrlich ascites tumor cells *in vitro* in a high concentration of ascorbate. The injections of large doses of ascorbate together with copper:glycylglycylhistidine prolonged the life span of mice inoculated i.p. with Ehrlich tumor cells. The target specificity against tumor cells was primarily attributable to their high peptide-cleaving activity.

INTRODUCTION

Ascorbate in an aqueous solution is easily oxidized by oxygen in the presence of transition metal ions and produces reactive oxygen species (5). The reaction path for the metal-catalyzed oxidation of ascorbate by oxygen was proposed by Taqui Khan and Martell (22) to involve molecular oxygen bound to the metal:ascorbate complex causing an electron transfer within the complex from ascorbate anion to metal ion and generating the partially reduced oxygen species.

Malignant cells of several different types have been shown to have lowered levels of reactive oxygen-scavenging enzymes which should be primary factors in the biological defense against oxidative stress (3, 12, 15). High-energy irradiation and some cytotoxic antitumor drugs (13, 20), which cause the formation of reactive oxygen species in biological systems, have injurious effects on the viability of malignant cells. Therefore, ascorbate in certain circumstances should be considered to exhibit potent cytotoxicity against malignant cells.

Several investigators have already pointed out the cytotoxicity of a large intake of ascorbate, in the presence of cupric ion, against tumor cells. Yamafuji *et al.* (23) disclosed that ascorbate, in cooperation with cupric ion, had a strong inhibitory potency against the growth of Sarcoma 180 implanted i.p. in mice. Stich *et al.* (19) pointed out the enhancement of chromosome-damaging action of ascorbate by transition metal ions. Bram *et al.* (4) reported that ascorbate possessed a preferential toxicity against malignant melanoma cells due to their elevated copper concentration.

Cupric ion is, however, protein avid. An excess of copper has toxic effects in a wide variety of animals and in humans (17). In the presence of ascorbate, naked cupric ion may exert damaging action to malignant cells as well as normal cells.

Serum albumin is a physiologically important Cu(II) transport protein. GGH⁴ is a tripeptide designed to mimic the specific Cu(II) transport site of the albumin molecule (8). Accordingly, the copper:GGH complex may be considered as an innocuous form of cupric ion.

The present paper deals with the *in vitro* and *in vivo* antitumor activity of ascorbate and copper:GGH complex against Ehrlich ascites tumor cells and with the mechanism of its target specificity against malignant cells.

MATERIALS AND METHODS

Chemical Reagents. GGH was synthesized according to the method of Lau *et al.* (8). The purity was checked by thin-layer chromatography. Catalase (2400 units/mg) from bovine liver was purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of analytical reagent grade.

The 1:1 mixture (molar ratio) of cupric chloride and GGH was dissolved in Krebs-Ringer phosphate-buffered saline and adjusted to pH 7.4 by adding a few drops of concentrated sodium carbonate solution. The absorption maximum and extinction coefficient of the complex was 526 nm and 88 M⁻¹ cm⁻¹, respectively.

ESR Studies. ESR spectra were obtained using a JEL-FE 1X spectrometer, JEOL, Ltd., Tokyo, Japan. The spectra of the copper complexes were measured at liquid nitrogen temperature (-196°), and those of ascorbate radicals and spin-trapped radicals were measured at room temperature. ESR signals were obtained as the first derivative of the absorption. The g-values and hyperfine splitting constant were determined by comparison with the standard of Mn(II) in MgO.

Oxidation of Ascorbate and Epinephrine and the Spin-trapping of Short-lived Radical Intermediates. The rate of oxidation of ascorbate in the presence of cupric ion or copper:GGH complex was measured by the decrease in UV absorption at 265 nm.

Hydrogen peroxide was determined using leuco crystal violet and horseradish peroxidase as catalyst, according to the method of Mottola *et al.* (10). The formation of oxygen-centered radicals, superoxide radical and hydroxyl radical, was observed by the spin-trapping technique using BPN as a spin adduct, as adapted for the Fe(II):bleomycin:O₂ system (20). The oxidation of epinephrine in the presence of cupric ion or copper:GGH was examined in terms of the production of adrenochrome (9), which had an absorption maximum at 480 nm.

Cytotoxicity Test *in Vitro*. Ascites of ddY mice, 10 to 12 days after i.p. inoculation with Ehrlich tumor cells, was used for the cytotoxicity test. Tumor cells (10⁶ cells/ml) in Krebs-Ringer medium at pH 7.4 were incubated at 37° with varied concentrations of ascorbate and cupric ion or copper:GGH for varying periods of time and assayed for their

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⁴ The abbreviations used are: GGH, glycylglycylhistidine; ESR, electron spin resonance; BPN, *N-tert-butyl- α -phenylnitron*.

viability by trypan blue dye exclusion test. The inhibitory effect of catalase upon the cytotoxicity was investigated.

Spleen cell suspension of ddY mouse was prepared by passing the minced spleen suspension in cold Eagle's minimal essential medium through a 100 mesh stainless sieve. It was used for the cytotoxicity test as a reference.

Analysis of the Change of the Copper:GGH Complex After Incubation in Tumor Cell Suspension. Tumor cell suspension in Krebs-Ringer medium (10^8 cells/ml) in the presence or absence of 10^{-4} M ascorbate was added with copper:GGH at the final concentration, 10^{-4} M, and incubated at 37° for 30 min. It was deproteinized with picric acid, passed through the Dowex 1-X8 column, and analyzed for ninhydrin-positive compounds with an amino acid analyzer. ESR spectra of copper:GGH (10^{-4} M) were measured after incubation in tumor cell suspension in the presence of 10^{-4} M ascorbate.

Antitumor Activity *in Vivo*. Two-tenths ml of ascites fluid containing 5×10^5 Ehrlich tumor cells was inoculated i.p. into ddY mice weighing about 25 g. Forty-eight hr after this inoculation, 0.2 ml of sodium ascorbate and/or copper:GGH was injected i.p. each day for 12 days. Increase in life span calculated on the basis of the mean survival time of the treated relative to the control animals and the number of 60-day survivors were used for the expression of antitumor activity of compounds.

RESULTS

The ESR spectrum of the copper:GGH complex at -196° (Chart 1) is quite similar in shape and in g -values to that of the copper:macrocytic dioxotetraamine complex possessing a quadridentate coordination (6). It has an intense signal at g -value lower than the perpendicular component (g_\perp) due to an overshoot phenomenon which occurs from the particular angular dependence. The g_\parallel value and calculated hyperfine cou-

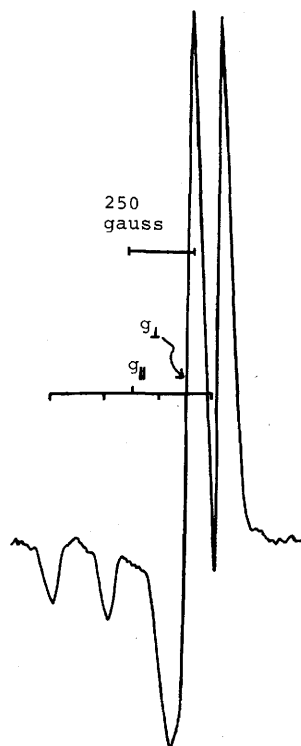


Chart 1. ESR spectrum of the copper:GGH complex at -196° . It is measured at 9.15 GHz with 100 kHz modulation and at 8 milliwatts microwave power. The concentration of copper:GGH is 10^{-4} M. g_\parallel and g_\perp are parallel and perpendicular components, respectively.

pling constant $|A_\parallel|$ are plotted on a scheme g_\parallel versus $|A_\parallel|$ for CuS_4 , CuN_4 , and CuO_4 complexes (Chart 2), as reported by Yokoi and Addison (24). The magnetic parameter of the copper:GGH complex places it at the low g_\parallel and high $|A_\parallel|$ extreme on the CuN_4 line, indicating the highest degree of square planar conformation and electron-donating ability among CuN_4 complexes. The stability of this complex at pH 7.4, as shown by Sakurai and Nakahara (16), is as high as those of macrocyclic tetraamine complexes. Accordingly, GGH may coordinate around cupric ion (Chart 3) through the α -amino nitrogen of NH_2 -terminal glycine, 2 intervening peptide nitrogens, and an imidazole nitrogen of histidine.

The time course of ascorbate oxidation in the presence of cupric ion or copper:GGH complex is shown in Chart 4. Although cupric ion greatly accelerates the ascorbate oxidation, copper:GGH accelerates it only slightly.

After 10 min of incubation of the mixture of 2.5×10^{-4} M ascorbate and 2.5×10^{-6} M cupric ion at 37° , $34 \mu\text{g}$ of H_2O_2 per 100 ml is detectable. The ESR signal of spin-trapped BPN (Chart 5) after an addition of BPN is demonstrable, beside the doublet signal of ascorbate radical (7). This indicates the occurrence of oxygen-centered radicals in this reaction mixture, as found in the $\text{Fe(II):bleomycin:O}_2$ system (20). Instead, copper:GGH, even at 2.5×10^{-5} M, produces neither a detectable amount of H_2O_2 nor an ESR signal of spin-trapped BPN at any time during 30-min incubation.

The Cu(II) catalysis of epinephrine oxidation is also strongly inhibited by the complex formation with GGH as with EDTA.

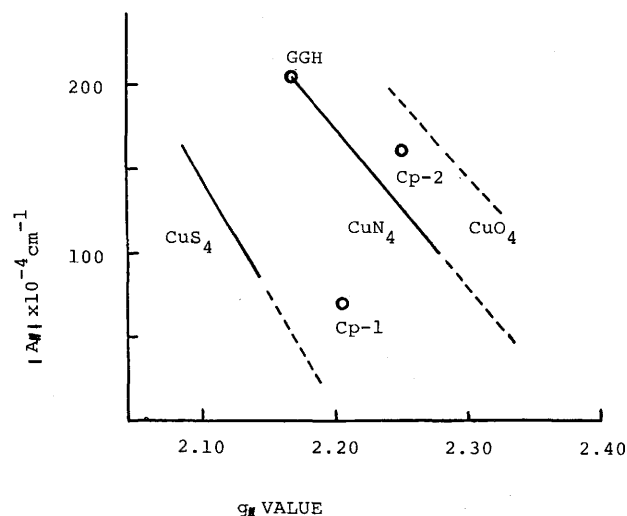


Chart 2. Relationship between g_\parallel and $|A_\parallel|$ for copper complexes. Type 1 and 2 copper centers of ceruloplasmin (Cp) are plotted as references. Their ESR parameters are compatible to CuS_2N_2 and CuN_2O_2 , respectively (24).

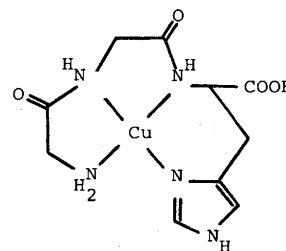


Chart 3. Structure of the copper:GGH complex. Nitrogens are deprotonated when the coordination occurs.

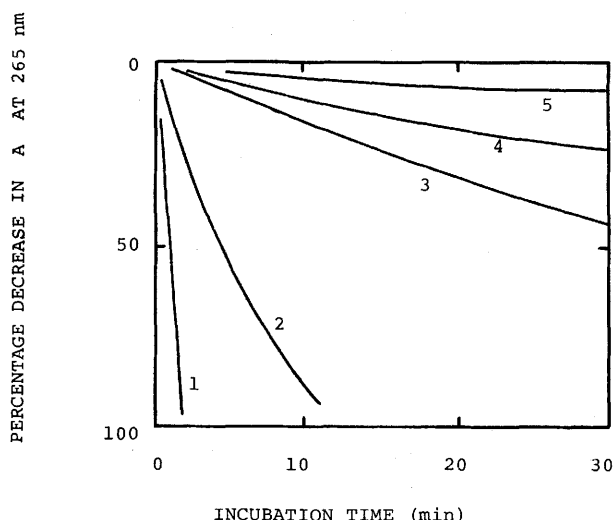


Chart 4. Oxidation of ascorbate (decrease in absorbance) in the presence of cupric ion or copper:GGH complex. Ascorbate, 10^{-3} M, in Krebs-Ringer medium of pH 7.4. 1, cupric ion, 10^{-4} M; 2, cupric ion, 10^{-5} M; 3, cupric ion, 10^{-6} M; 4, copper:GGH, 10^{-5} M; 5, without cupric ion or copper:GGH.

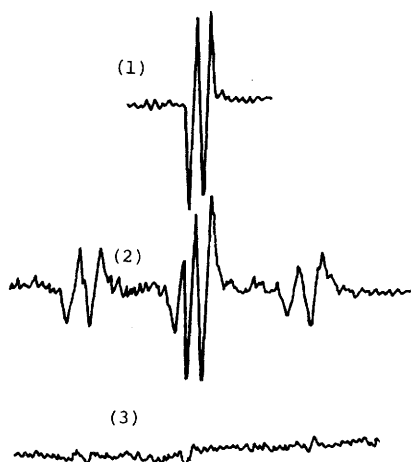


Chart 5. Spin-trapping of oxygen-centered radicals by BPN. It is measured at 9.43 GHz with 100 kHz modulation and at 25 milliwatts microwave power. All of the reagents are dissolved in Krebs-Ringer medium at pH 7.4. 1, Mixture of 0.1 ml of 10^{-5} M cupric ion, 0.1 ml of 10^{-3} M ascorbate, and 0.2 ml of Krebs-Ringer medium; 2, mixture of 0.1 ml of 10^{-5} M cupric ion, 0.1 ml of ascorbate, and 0.2 ml of 0.08 M BPN; 3, mixture of 0.2 ml of 0.08 M BPN and 0.2 ml of Krebs-Ringer medium.

Cupric ion, possessing a relatively high oxidation-reduction potential and coordinating to ascorbate anion or epinephrine, is easily reduced by these reducing agents, whereas the mononuclear planar copper:GGH complex is not. As mentioned by Taqui Khan and Martell (22), the complex formation with increased stability and lowered oxidation-reduction potential decreases the metal-catalyzed oxidation of these biological reducing agents.

Amino acid analysis (Chart 6) indicates that, after 30 min incubation at 37° of Ehrlich tumor cell suspension plus copper:GGH, the peak of GGH disappears with an increase of NH_3 but without change in histidine peak. Tumor cells possessing a high peptidase activity, as reported by Sylvén and Bois-Svensson (21), cause a hydrolytic cleavage of this tripeptide. If the released histidine remains intact, its peak should become

much higher. It may be utilized by tumor cells and decomposed at least in part to NH_3 . With or without 10^{-4} M ascorbate, a similar change in the amino acid elution pattern is demonstrable. Thus, in contact with tumor cells, the copper:GGH complex undergoes cleavage to release free cupric ion. ESR analysis indicates that, after incubation with 10^{-4} M ascorbate, the ESR signal of copper:GGH disappears almost completely. Without tumor cells, however, such a remarkable decrease in ESR signal does not occur. After incubation in a suspension of spleen cells or RBC, this complex undergoes no significant change.

The results of cytotoxicity of ascorbate and cupric ion or copper:GGH against Ehrlich tumor cells *in vitro* are shown in Chart 7. Cupric ion, in the presence of ascorbate, possesses a strong killing effect. At higher concentrations of both ascorbate and cupric ion, the percentage of dead (stained) cell increases. The killing effect of copper:GGH is much less com-

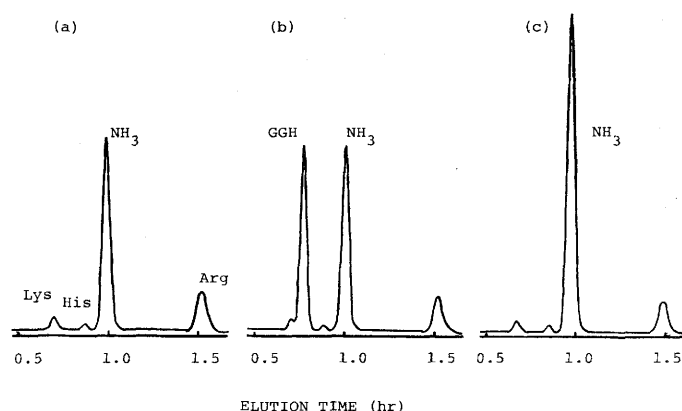


Chart 6. Elution pattern of amino acids and added GGH in Ehrlich tumor cell suspension. Elution patterns of basic amino acids and GGH are indicated. a, Tumor cell suspension; b, immediately after an addition of copper:GGH at 10^{-4} M; c, after 30 min incubation at 37° of copper:GGH-added tumor cell suspension.

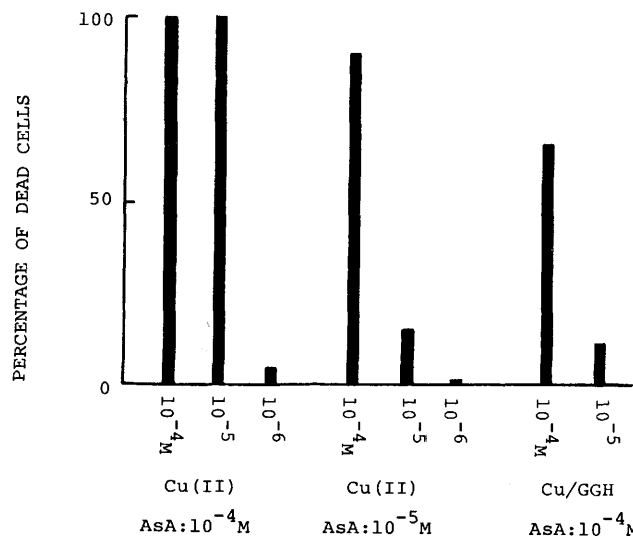


Chart 7. Killing effects of ascorbate and cupric ion or copper:GGH against Ehrlich tumor cells. Tumor cell suspension (10^6 cells/ml) in Krebs-Ringer medium are used for all of the experiments. Final concentrations of ascorbate, cupric ion, and copper:GGH are shown. Dead (stained) cells are counted after 2 hr of incubation at 37° . At 10^{-4} M concentrations each of ascorbate (AsA) and Cu(II), all of tumor cells are killed within 1 hr incubation.

pared with free cupric ion. In the presence of 10^{-4} M ascorbate (1.76 mg/100 ml, corresponding to the blood plasma level of a human adult after an administration of several g of ascorbate, approximately to the kidney threshold value), copper:GGH exhibits the cytotoxicity to an appreciable extent. However, in a lower concentration, 10^{-5} M, of ascorbate (0.176 mg/100 ml, corresponding to plasma level without supplemental ascorbate), copper:GGH does not show a significant tumor-killing effect. Although Benade *et al.* (1) reported that ascorbate at an extremely high concentration is lethal to Ehrlich tumor cells, ascorbate by itself does not show any significant cytotoxicity at the level of kidney threshold value.

The tumor cell-killing action of ascorbate and copper:GGH is completely inhibited by addition of catalase (600 units/ml). It appears to be due to the formation of H_2O_2 and the subsequent appearance of higher reactive radicals, as mentioned by Stich *et al.* (19), on the mitosis-inhibiting and chromosome-damaging action of ascorbate:transition metal against Chinese hamster ovary cells.

Microscopic observations reveal that ascorbate and copper:GGH cause blebbing of tumor cells before the appearance of stained cells while leaving the nuclei apparently intact. The bleb formation is much more remarkable in the presence of free cupric ion in place of copper:GGH. Although cupric ion together with ascorbate causes marked damage to spleen cells and to RBC, copper:GGH does not.

In vivo antitumor activity of ascorbate and copper-GGH is shown in Chart 8. Copper:GGH alone does not show an antitumor activity. Ascorbate by itself shows only weak activity. The administration of both ascorbate and copper:GGH exhibits much greater activity. Increase in life span (%) is 0 for the copper:GGH group, a little more than 24 for the ascorbate group, and much more than 64 for the ascorbate:(copper:GGH) group. The rate of 60-day survivors is 0 of 10 for the control and copper:GGH groups, only 1 of 10 for the ascorbate group, and 4 of 10 for the ascorbate:(copper:GGH) group. The 60-day-surviving mice reject the tumor growth even after *i.p.* inoculation with additional Ehrlich tumor cells (10^6 cells) and

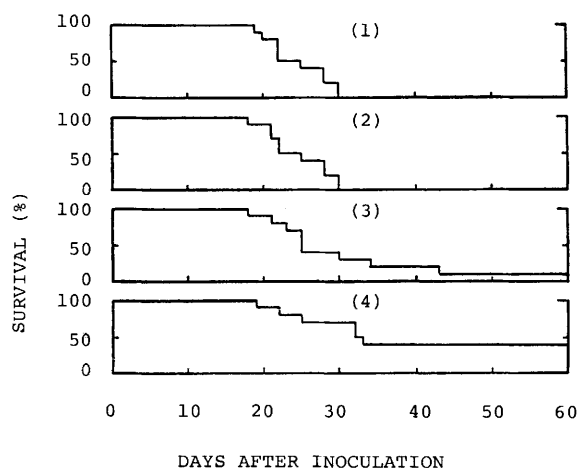


Chart 8. Life span of mice after inoculation with Ehrlich tumor cells. Two-tenths ml of Ehrlich ascites tumor cells (5×10^5 cells) is inoculated *i.p.* into ddY mice weighing about 25 g on Day 0. Ascorbate (0.2 ml, 4%) and/or copper:GGH (0.2 ml, 10^{-3} M) are injected *i.p.* 48 hr after the tumor inoculation, and this injection is repeated each day for 12 days. For the control group, the same amount of 0.9% NaCl solution is injected. 1, Control; 2, Cu/GGH; 3, ascorbate; 4, ascorbate-Cu/GGH.

survive for a long period without an accumulation of ascites.

Injection *i.p.* with 3 or 5 times larger amounts of copper:GGH does not show better results, indicating that the amount (0.2 ml of 10^{-3} M copper:GGH; equal to $12.7 \mu\text{g}$ as cupric ion) is close to that for maximum antitumor activity.

Free cupric ion instead of copper:GGH, injected *i.p.* together with ascorbate, causes trembling and often sudden death of tumor-inoculated mice. The innocuous copper:GGH complex is much more securely applied to the living body.

DISCUSSION

It is well known that naked cupric ion increases greatly the cytotoxicity of ascorbate. In order to apply the innocuous copper *in vivo*, we intended to construct "metal buffer," [the terminology was introduced by G. Schwarzenbach (2)] which consists of a complex that can release free metal ion little by little and react preferentially against malignant cells. Moreover, the ligand was preferred to be a physiological compound.

GGH, mimicking the specific copper transport site of albumin, possesses the requisite planar geometry needed for Cu(II) binding. As found by ESR studies, the copper:GGH complex possesses the highest degree of square planar conformation and electron-donating ability among CuN_4 complexes. A square planar CuN_4 complex, as mentioned by Oishi *et al.* (14), does not give rise to the deteriorative oxidation of physiological reducing agents such as ascorbate and epinephrine.

The physicochemically stable copper:GGH complex is easily decomposed, in contact with tumor cells, due to their high peptide-cleaving activity. In such circumstances, even such an inert copper complex liberates free copper ion in the medium surrounding tumor cells and exerts a synergistic killing of tumor cells by ascorbate.

Microscopic observations *in vitro* reveal that the copper:GGH complex in the presence of high concentrations of ascorbate causes the blebbing and killing of tumor cells while leaving the nuclei apparently intact. DNA-damaging or mutagenic properties of ascorbate and cupric ion have been demonstrated on bacteriophage (11) and even on eukaryotic cells (18, 19). Although the reaction of copper:GGH is mild, it may have some DNA-damaging effects, as seen in any H_2O_2 -producing compounds.

The *i.p.* administration of both ascorbate and the copper:GGH complex increases significantly the life span of mice after an inoculation of Ehrlich ascites tumor cells. However, ascorbate or copper:GGH alone has only weak effects. Ascorbate, which undergoes an enhancement of tumor-killing activity by copper:GGH, may be expected to behave similarly *in vivo*.

Ascorbate and the copper:GGH system exerts mild carcinostatic effects. Maximum effect corresponds to a survival of about one-half of tumor-inoculated mice. Such host-nontoxic compounds, although exerting a mild cytotoxicity to malignant cells, may be promising for cancer chemotherapy.

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