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γ-Glutamyl Transpeptidase-Dependent Mutagenicity and Cytotoxicity of γ-Glutamyl Derivatives: A Model for Biochemical Targeting of Chemotherapeutic Agents

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Many carcinomas in humans are rich in γ-glutamyl transpeptidase (GGT), a plasma membrane enzyme that reacts with extracellular substrates. Thus, biochemical targeting of chemotherapeutic agents may be achieved by converting anticancer drugs into their γ-glutamyl derivatives. Chemical conversion of phenylhydrazine (PH) and biochemical modification of daunomycin (DM) into their γ-glutamyl derivatives γ-glutamyl phenylhydrazine (GGPH) and γ-glutamyl DM (GGDM) resulted in the abolishment of their mutagenicity and cytotoxicity, as judged by decreased viability and increased mutant yields in cultures of several Salmonella Ames strains. Commercial γ-glutamyl-*p*-nitroanilide (GGPNA) was not toxic or mutagenic. Mutagenicity and/or cytotoxicity of these γ-glutamyl derivatives were restored upon reaction with GGT, with concomitant release of PH, and *p*-nitroaniline (PNA). The GGT-dependent release of DM from GGDM was demonstrated by thin layer chromatography (TLC), spectral analysis, and specific mutagenicity. Mutagenicity and/or cytotoxicity of γ-glutamyl derivatives increased in the presence of glycylglycine, a GGT activator, and decreased in the presence of serine-borate, a GGT inhibitor. GGDM retained considerable DNA binding capacity. Its inability to kill and mutagenize was due to altered transport properties. The results are compatible with the notion that γ-glutamylation is a feasible method for biochemical targeting of drugs containing a primary amino group to GGT-rich tumors. Environ. Mol. Mutagen. 32:377–386, 1998

Key words: γ-glutamyl transpeptidase; phenylhydrazine; daunomycin; mutagenicity; cytotoxicity; *Salmonella typhimurium;* drug targeting

INTRODUCTION

Biochemical targeting is an interesting approach to increase the specificity of drugs for tumor cells. Biochemically targeted drugs are substrates for enzymes that exist at high level/activity in tumor cells as compared to normal ones, and are specifically converted into active forms upon reaction with the enzymes.

Example 1: Conversion of a Prodrug Into a Chemically Reactive Agent

γ-Glutaminyl-*p*-hydroxybenzene (GHB) is a tyrosine analog and a natural product of *Agaricus bisporus.* Tyrosinase oxidizes GHB to γ-glutaminyl-3,4-benzoquinone; the latter converts nonenzymatically to the corresponding 2-hydroxy-4-iminoquinone, which ultimately inhibits many enzymes involved in normal energy metabolism, leading to cell death. Most melanoma cells are rich in tyrosinase, whereas other cells are deficient in tyrosinase: GHB is a tyrosinase-targeted specific antimelanoma agent [Vogel et al., 1977, 1979; Burger et al., 1979; Chen et al., 1979; Boekelheide et al., 1980].

Example 2: Release of a Chemically Reactive Agent From an Adduct

L-γ-glutamyl-4-(hydroxymethyl) phenylhydrazine (agaritine) is another metabolite of *A. bisporus.* Removal of the γ-glutamyl moiety forms the cytotoxic 4-(hydroxymethyl)-phenylhydrazine. The latter is converted to the reactive diazonium ion by microsomal enzymes [Hiramoto et al., 1995a,b; Walton et al., 1997]. The first step of activation is catalyzed by γ-glutamyl transpeptidase (GGT), a plasma membrane enzyme that is induced to high levels at early stages of experimental hepatocarcinogenesis in rodents.

Abbreviations: DM, daunomycin; GGDM, putative γ-glutamyl-daunomycin; GGT, γ-glutamyl transpeptidase; GGPNA, γ-glutamyl-*p*-nitroanilide; GGPH, γ-glutamyl phenylhydrazine; GHB, γ-glutaminyl-*p*-hyrdoxybenzene; GSH, glutathione; PH, phenylhydrazine; PNA, *p*-nitroaniline; TLC, thin layer chromatography.

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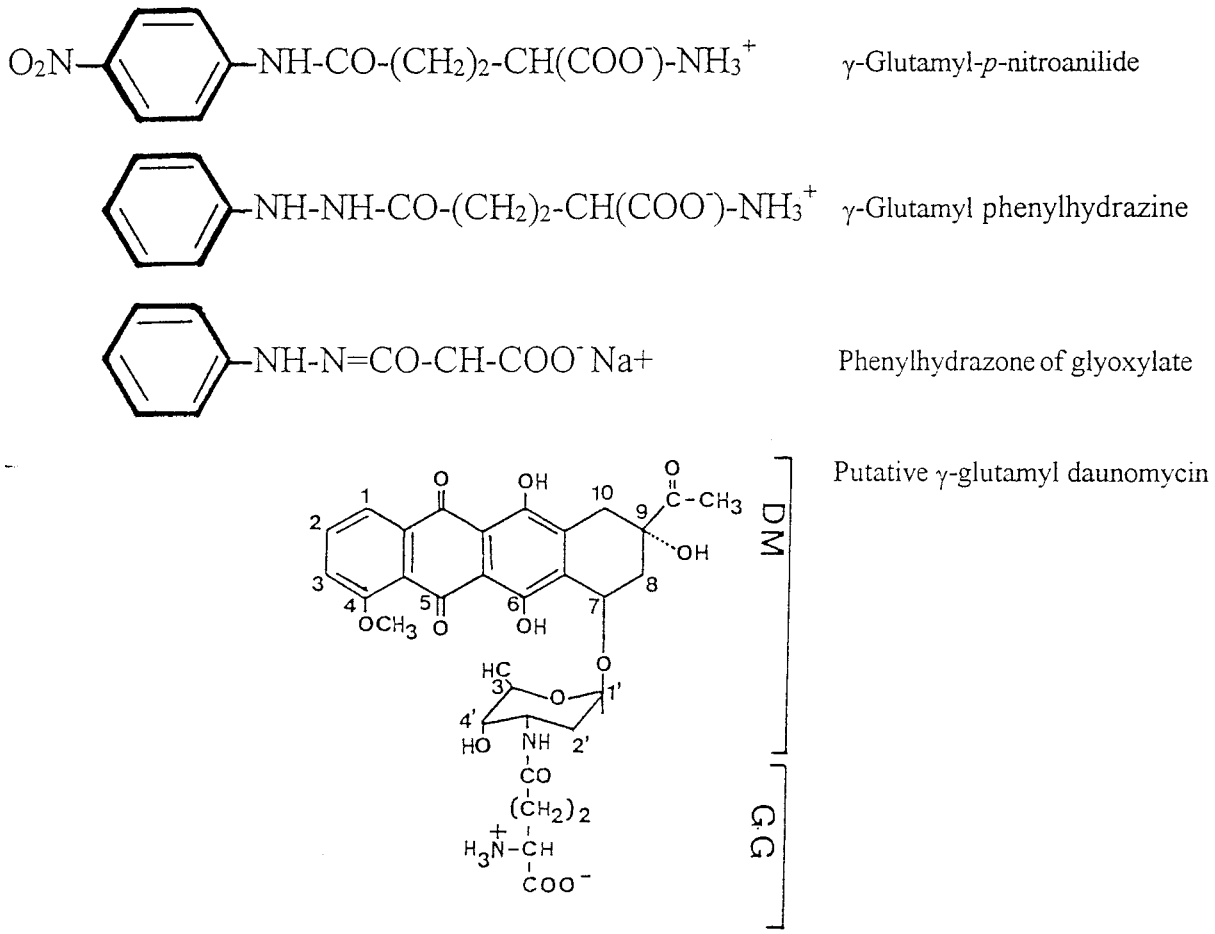

Fig. 1. Structures of GGPNA, GGPH, PH, and GGDM.

High GGT levels persist in carcinomas in later stages. Increased GGT levels are also found in many human hepatic carcinomas, and in other human and animal carcinomas [Gerber and Thung, 1980; Selvaraj et al., 1981; Hanigan and Pitot, 1985; Farber, 1986; Bannasch, 1986; Sarma et al., 1986; Pitot, 1990; Stark, 1991]. Thus, γ-glutamyl derivatives are potentially GGT-targeted prodrugs.

The use of GGT for drug targeting is appealing in that the active site of membranal GGT is exclusively directed towards the extracellular space [Tsao and Curthoys, 1980]. The action of GGT on a γ-glutamyl derivative of a nonpolar drug may release the nonpolar drug in the vicinity of the GGT-rich tumor cell, thus increasing the effective concentration (and uptake) of the drug at the tumor site. In order to test this possibility, we synthesized and partially characterized a γ-glutamyl derivative of daunomycin (Fig. 1) (GGDM), and compared its mutagenic and cytotoxic properties with those of daunomycin (DM). The results indicate that mutagenicity and cytotoxicity of GGDM depend on GGT activity and that γ-glutamylation of DM alters mainly its uptake properties.

MATERIALS AND METHODS

Materials

GSH, glycylglycine, γ-glutamyl-*p*-nitroanilide (GGPNA), amino acids, phenylhydrazine, pyroglutamic acid, bovine GGT (25 U/mg protein), GGPNA, NADP, glucose-6-phosphate, G6PD, and calf thymus DNA were from Sigma (Milwaukee, WI). Kieselgel-60 was from Merck (Darmstadt, Germany). Sodium glyoxylate was from ICN (Costa-Mesa, CA). Silica gel-60 TLC plates were from Riedel-De Häen (Hannover, Germany). Daunomycin was from Rhône-Poulenc (Courbevoie, France). *Salmonella typhimurium* strains TA1538, TA98, and TA100 were kindly provided by B.N. Ames, Division of Biochemistry and Molecular Biology, University of California, Berkeley.

Methods

Preparation of γ-Glutamyl Derivatives

γ-Glutamyl phenylhydrazine (GGPH) (Fig. 1) was synthesized from pyroglutamic acid and PH, and was purified as described previously [Levenberg, 1970]. GGDM was prepared enzymatically in a reaction mixture containing 250 mM HEPES buffer, pH 8.5, 3 U/ml GGT, 13 mM DM, and 130 mM GSH (adjusted to pH 7) in a final volume of 1.5 ml. Mixtures for small-scale preparations contained 250 mM

HEPES, pH 8.5, 4.3 mM DM, 50 mM GSH, and 3 U/ml GGT. The mixture was incubated up to 25 hr at 25°C in the dark and was extracted four times with an equal volume of chloroform. Phase separation and precipitation of denatured proteins was by centrifugation (10,000*g*, 25°C, 5 min). The aqueous phase was loaded onto 20 × 20 cm, 5 mm-thick silica gel-60 plates (750 µl/plate) and chromatographed with chloroform:methanol:water (13:6:1) [Levin and Sela, 1979] until the solvent front reached 15 cm. The gel area containing the polar product (orange to deep red) was scraped, extracted 3–5 times with methanol:water (5:1) and was centrifuged as above. Methanol was evaporated under reduced pressure at 40°C, the remaining aqueous phase was frozen and lyophilized, and the final product (red threads) was stored desiccated at −20°C. Preparation, handling and experiments with GGDM were carried out under yellow (>550 nm) light in order to prevent photodegradation. Prior to the experiments, DM or the aglycone resulting from dissociation or degradation during storage were removed by dissolving GGDM in 50 mM HEPES, pH 8, and extracting 4–5 times with chloroform as above. Purity of GGDM was tested on TLC plates developed as above. The extinction coefficients of DM in ethanol and in HEPES buffer pH 8.5 are 11,500 M^{-1} cm^{-1} and 7,419 M^{-1} cm^{-1} at 480 nm, respectively. Since GGDM is insoluble in ethanol, its concentration was determined in HEPES buffer using the coefficient of DM in HEPES. IR spectra were carried out with DM and GGDM in KBr pellets.

Determination of GGT Activity With Various γ-Glutamyl Derivatives

Hydrolysis of L-γ-glutamyl-*p*-nitroanilide was essentially as described previously [Stark et al., 1993] in reaction mixtures containing 100 mM Tris HCl, pH 8.2, 20 mM glycylglycine, 0.1–2 mM GGPNA, and 100 mU/ml GGT, and the rate of increase in A_{412} was followed (ε = 8,800 M^{-1} cm^{-1}). *p*-Nitroaniline was used as a standard. Hydrolysis of GGPH was determined in 50 mM phosphate buffer pH 8.5 containing 0.3–5 mM GGPH, 300 mU/ml GGT, and 5 mM glyoxylate. Continuous monitoring of A_{320} (phenylhydrazone-α-keto acid adduct, ε = 14,000 M^{-1} cm^{-1}) was used to determine reaction rates. Commercial phenylhydrazine was used as a standard.

Rat kidney and rat liver microsomes were prepared by homogenization of kidney cortex or liver tissue in 100 mM Tris buffer, pH 8.0, and centrifugation at 10,000*g* for 30 min. The supernatant was centrifuged at 130,000*g* for 60 min and the pellet was suspended in the same buffer, aliquoted, and stored at −80°C. GGT activity was 10 U/mg protein and 5 mU/mg protein in rat kidney and rat liver microsomes, respectively.

Cytotoxicity Assays

The toxicity of GGPH, phenylhydrazine, and GGPNA was assayed in Salmonella strain TA1538 [Malca-Mor and Stark, 1982]. Cells were grown in minimal medium [Maron and Ames, 1983]. Exponentially growing cells were diluted to 0.8 − 1 × 10^8/ml in 60 mM HEPES, pH 8.35, 40 mM $MgCl_2$, 27.5 mM KCl, 27 mM glucose, 5 µM biotin, 100 µM histidine, and, when appropriate, 3 U/ml GGT, 40 mM glycylglycine, and the test compound. Cultures were shaken at 37°C and samples were withdrawn with time for viable counts (triplicates) on LB agar. The release of *p*-nitroaniline from GGPNA was assayed by withdrawing 5 µl samples at various time points (as indicated in the figures), diluting in 150 mM NaCl and determination of *p*-nitroaniline at A_{412}. The release of phenylhydrazine was assayed by withdrawing 50 µl samples into 1.2 ml of 5 mM Na-glyoxylate in 150 mM NaCl, centrifugation, and determination of A_{320} of the supernatant. Cytotoxicity of DM and GGDM in strain TA98 was assayed on exponential cultures in LB medium at 4 × 10^8 cells/ml. Cultures were diluted to 4 × 10^7 cells/ml in LB, pH 8.5, containing, when appropriate, 40 mM glycylglycine, 5–20 U/ml GGT, and test compounds. Cultures were shaken at 37°C and viability was determined as above. The release of biologically active compounds from their γ-glutamyl derivatives was linear with time. Thus, the cells were exposed to linearly accumulating concentrations, and actual exposure at a certain time point was half of that of the released material at that time point. Specific toxicity was defined as the accumulated concentration of a test compound that caused one lethal hit.

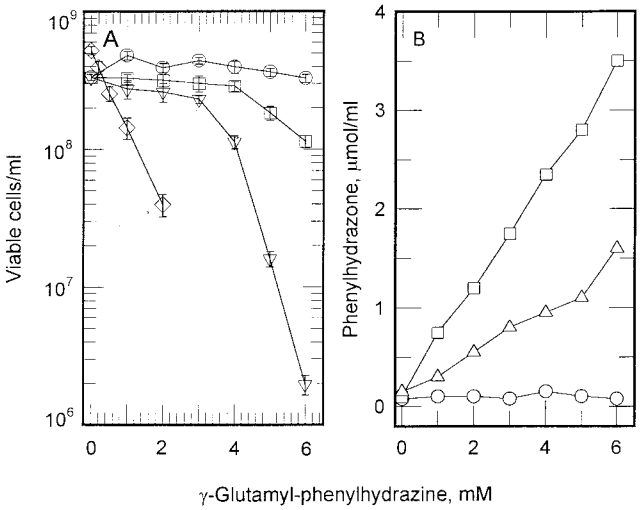

Fig. 2. GGT-dependent cytotoxicity of GGPH in strain TA1538. (**A**) Reaction mixtures (as described in Materials and Methods) contained 3.5 × 10^8 cells/ml, 40 mM glycylglycine, and the indicated concentrations of phenylhydrazine (◇); GGPH (○); GGPH and 3 U/ml GGT (▽) or (□) GGPH, 3 U/ml GGT and 5mM serine-10 mM borate. Reactions were shaken at 37°C for 6 hr. Viability was determined as described in Materials and Methods. Presented are means of triplicates. (**B**) Release of PH from GGPH. Samples of reactions containing GGPH were centrifuged and the concentration of PH was determined by reaction with glyoxylate as described in Materials and Methods. (○) GGPH; (□) GGPH and 3 U/ml GGT; (△) GGPH, 3 U/ml GGT and 5 mM serine-10 mM borate. Presented are means of triplicates.

Mutagenicity Assays

Bacterial cultures, growth media, and the procedure for the standard plate incorporation mutagenicity test were essentially as described by Maron and Ames [1983] except that rat kidney microsomes replaced S9. The NADPH generation system was not included because neither DM nor PH require oxidative metabolism for their activation. The microsomes were used solely as a GGT source. Reaction mixtures with liver microsomes included the NADPH generation system. Reaction mixtures contained 10^8 TA98 or TA100 cells, 83 mM HEPES, pH 8.0, 12 mM $MgCl_2$, 27.5 mM KCl, 10 mM glucose, and, when appropriate, 40 mM glycylglycine and rat kidney microsomes at 7 mg protein/ml and test compound in a final volume of 0.5 ml. The pH of the top agar was adjusted to 8.0. Triplicate plates were poured for each concentration point. Colonies were counted manually.

Binding of DM and GGDM to DNA was determined by fluorescence quenching [Levin and Sela, 1979]. The fluorescence of reaction mixtures containing 10 mM Tris.HCl, 100 mM NaCl, 0.5 mM EDTA, pH 7, and 5 µM DM (λex 485 nm, λem at 580 nm) or 10 µM GGDM (λex 503 nm, λem 590 nm) was determined in the absence of DNA, and after incremental addition of calf thymus DNA up to 90 µg/ml. Scatchard

plots were used to determine frequency and affinity constants of binding [Levin and Sela, 1979].

RESULTS AND DISCUSSION

GGT catalyzes the cleavage of a γ-glutamyl moiety of a γ-glutamyl-containing compound (hydrolysis of a donor) and transfers it to an acceptor molecule containing a primary amino group (transpeptidation) (Equations 1, 2, below). Its specificity is low in that numerous compounds can serve as donors or acceptors, and the kinetics of the reactions depend on the nature of the donor and the acceptor. A serine-borate complex is a transition state analog, and thus a competitive inhibitor of GGT, whereas the acceptor glycylglycine increases the cleavage of the γ-glutamyl bond by 6–10-fold. We utilized the broad specificity of GGT to test whether it can release mutagenic/cytotoxic compounds from their respective γ-glutamyl analogs.

The kinetic parameters of the reaction of GGT with GGPH were determined at 0.312–3.75 mM GGPH, 20 mM glycylglycine, and 1 U/ml GGT. Vmax was 3 nmol/min/40 μg protein (75 nmol/min/mg protein), and Km was 0.8 mM. Reaction rate was linear with time at every GGPH concentration. At 10 mM serine/20 mM boric acid, Vmax was unchanged and Km was 2.2 mM, indicative of competitive inhibition (data not shown). Cleavage of GGPH by GGT is approximately 170-fold slower than the reaction with GGPNA (Vmax = 50 nmol/min/4 μg protein (12.5 μmol/min/mg protein) at 100 mU GGT/ml, 1 mM GGPNA, 20 mM glycylglycine).

$$\gamma\text{-Glutamyl-NH-R} + \text{GGT} \rightarrow \gamma\text{-glutamyl-GGT} + \text{R-NH}_2 \quad \text{Hydrolysis} \quad (1)$$

$$\gamma\text{-Glutamyl-GGT} + \text{R}'\text{-NH}_2 \rightarrow \gamma\text{-glutamyl-NH-R}' + \text{GGT} \quad \text{Transpeptidation} \quad (2)$$

GGT-Dependent Cytotoxicity of GGPH and GGPNA

Synthetic GGPH and commercial GGPNA (Fig. 1) served as model compounds. The synthesis of GGPH from pyroglutamic acid yields a racemic mixture of L- and D-GGPH; however, GGT cleaves L- and D-γ-glutamyl moieties equally well. Salmonella cells were exposed to PH, or to GGPH with and without GGT, for 6 hr. Figure 2A shows that PH was toxic in a concentration-dependent manner. GGPH without GGT was not toxic, whereas marked toxicity was obtained in the presence of GGT at 4, 5, and 6 mM GGPH. Addition of serine-borate complex (a competitive inhibitor of GGT) resulted in lower toxicity. The amounts of released PH (Fig. 2B) under each condition indicate that toxicity depended on

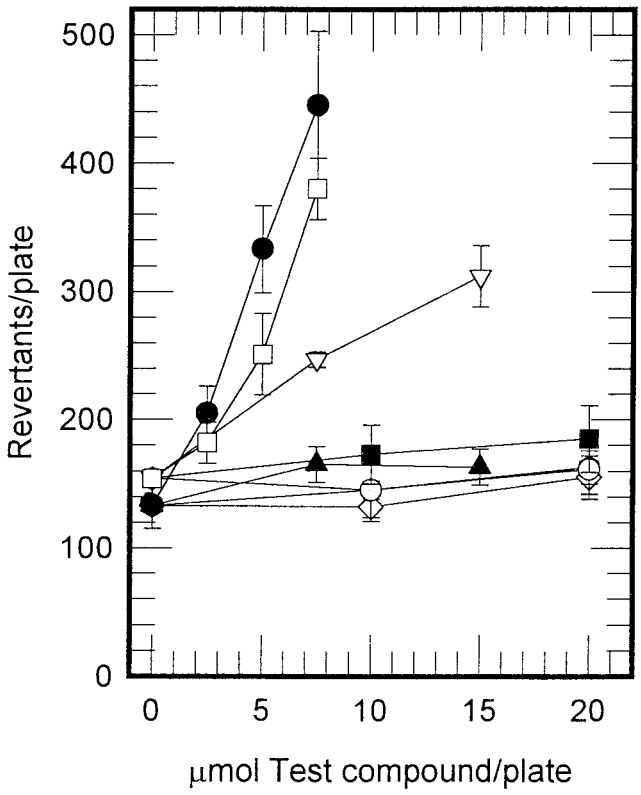

Fig. 3. GGT-dependent mutagenicity of GGPH and GGPNA in strain TA100, plate incorporation assay. Reaction mixtures (as described in Materials and Methods) contained 1×10^8 cells and the indicated amounts of test compounds: (●) PH; (□) PH with kidney microsomes; (▲) GGPH or GGPH with liver microsomes; (▽) GGPH with kidney microsomes; (◇) PNA or PNA with liver or kidney microsomes; (○) GGPNA; (■) GGPNA with kidney or liver microsomes. Kidney and liver microsomes were at 7 mg protein/ml. Presented are means ± SD of triplicate plates.

the cleavage of GGPH by GGT. The killing obtained at constant (2 mM) concentration of commercial PH for 6 hr (1.22 logs) was comparable to that obtained at 5 mM GGPH with GGT (1.2 logs), where free PH accumulated to 3 mM within the same time. Since PH accumulation was linear with time, the effective concentration of the released PH (integral of zero to 6 hr) was 1.5 mM. The addition of serine-borate at 6 mM GGPH resulted in release of 1.6 mM PH (effective concentration 0.8 mM) and in 0.5 logs killing, correlated with 0.6 logs killing at 1 mM commercial PH. The toxicity of GGPNA was GGT-dependent, was partially inhibited by serine borate, and correlated with the amounts of PNA released from GGPNA: 3.5 and 0.7 lethal hits were obtained at 20 mM GGPNA, 1U/ml GGT for 5 hr without and with serine-borate, respectively. Under these conditions, PNA accumulated to 16 and 8 mM with and without serine-borate, respectively (data not shown). The specific toxicity of the released PH (0.72 mM) was 2.9-fold higher than that of

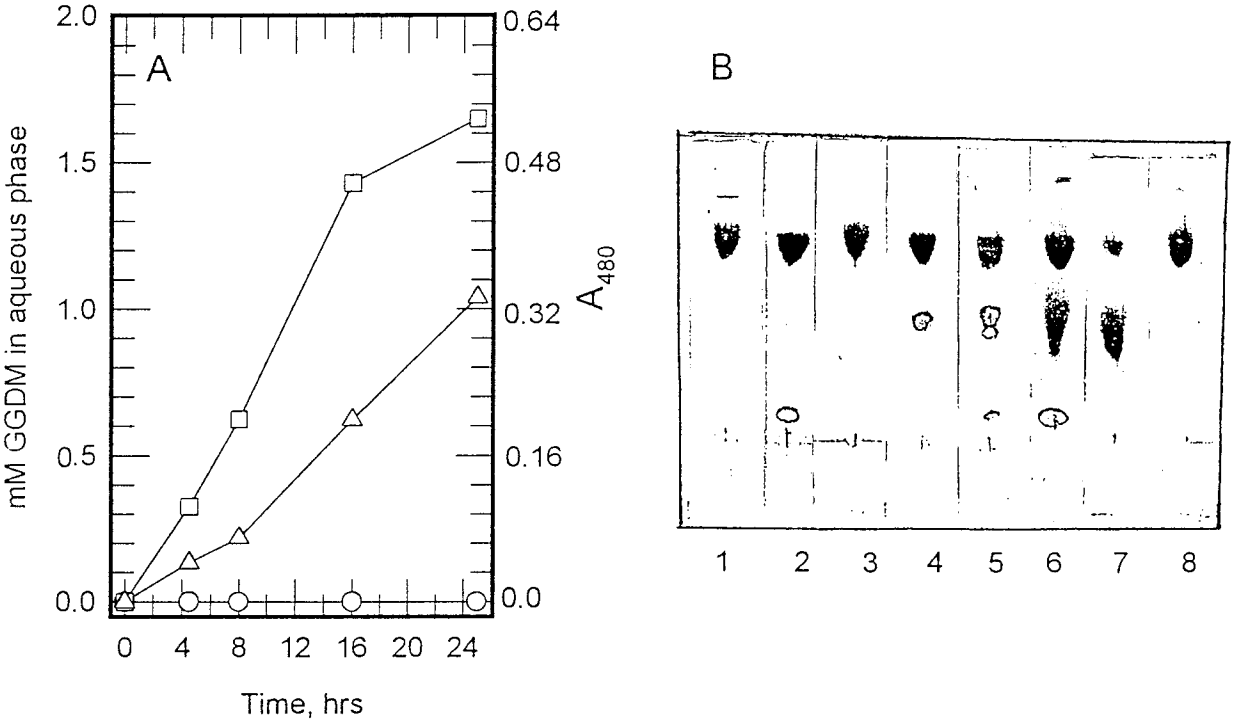

Fig. 4. Synthesis of GGDM. The procedure was essentially as described in Materials and Methods. Reaction mixtures contained 4.3 mM DM and as indicated, 50 mM GSH, 3 U/ml GGT, 12.5 mM serine, and 25 mM boric acid in a final volume of 500 µl. At the indicated time points, 30 µl samples were withdrawn into 500 µl 200 mM HEPES pH 8.5 and the mixture was extracted with chloroform. The volume of the aqueous phase was adjusted to 700 µl with HEPES buffer (to allow measurement in a microcuvette) and the concentration of GGDM was determined at 480 nm. (**A**) (□) DM, GSH, and GGT; (△) DM, GSH, and serine-borate; (○) DM and GSH, DM and GGT, or DM and serine-borate. (**B**) TLC of 2 µl samples of reaction mixtures shown in **A,** lanes 1 and 8, DM standard; lane 2, DM and GSH, 25 hr time point; lanes 3–7, complete reaction, 0, 4, 8.5, 16, and 25 hr time points, respectively. Circled are fainter spots. The aglycone of DM appears as a thin red line at the top (apolar) position in lanes 1 and 6.

the released PNA (2.07 mM), very similar to the ratio obtained at pH 7.4 [Malca-Mor and Stark, 1982]. Thus, the effective cell death obtained with GGPNA (a precursor for the less toxic PNA) as compared to that of GGPH (a precursor for the more toxic PH) was due to the fact that GGPNA is a good substrate for GGT, whereas GGPH is a poor one.

GGT-Dependent Mutagenicity of GGPH and GGPNA

The plate incorporation assay was used to determine whether GGT can activate GGPH and GGPNA to mutagens. Here, rat kidney or rat liver microsomes replaced soluble GGT in order to test whether metabolism by enzymes other than GGT converts γ-glutamyl derivatives into mutagens. Figure 3 shows that GGPH and GGPNA were not mutagenic without kidney microsomes. With kidney microsomes, significant dose-dependent mutagenicity was obtained only with GGPH. A very weak response was obtained with GGPNA and kidney microsomes, similar to the weak mutagenicity of PNA in the liquid preincubation assay [Malca-Mor and Stark, 1982]. No mutagenicity of GGPH and GGPNA was observed with liver microsomes, which lack GGT. Mutagenicity of PH did not require metabolic activation, and inclusion of liver or kidney microsomes slightly decreased its mutagenic activity.

The above results indicate that cytotoxicity/mutagenicity of a chemically reactive compound is abolished upon conjugation with a γ-glutamyl residue, and that cleavage of the γ-glutamyl residue by GGT restores cytotoxicity and/or mutagenicity. PH and PNA are not used as anticancer drugs. We therefore tested whether a known mutagenic/cytotoxic chemotherapeutic agent can be converted into its γ-glutamyl derivative and whether the biological activities of the adduct depend on GGT activities.

GGT-Dependent Synthesis and Partial Characterization of a Putative GGDM Adduct

Daunomycin (Fig. 1) seemed a good candidate, in that it contains a single primary 3′-amino group in the daunosamine (amino sugar) moiety. Attempts to attach a γ-

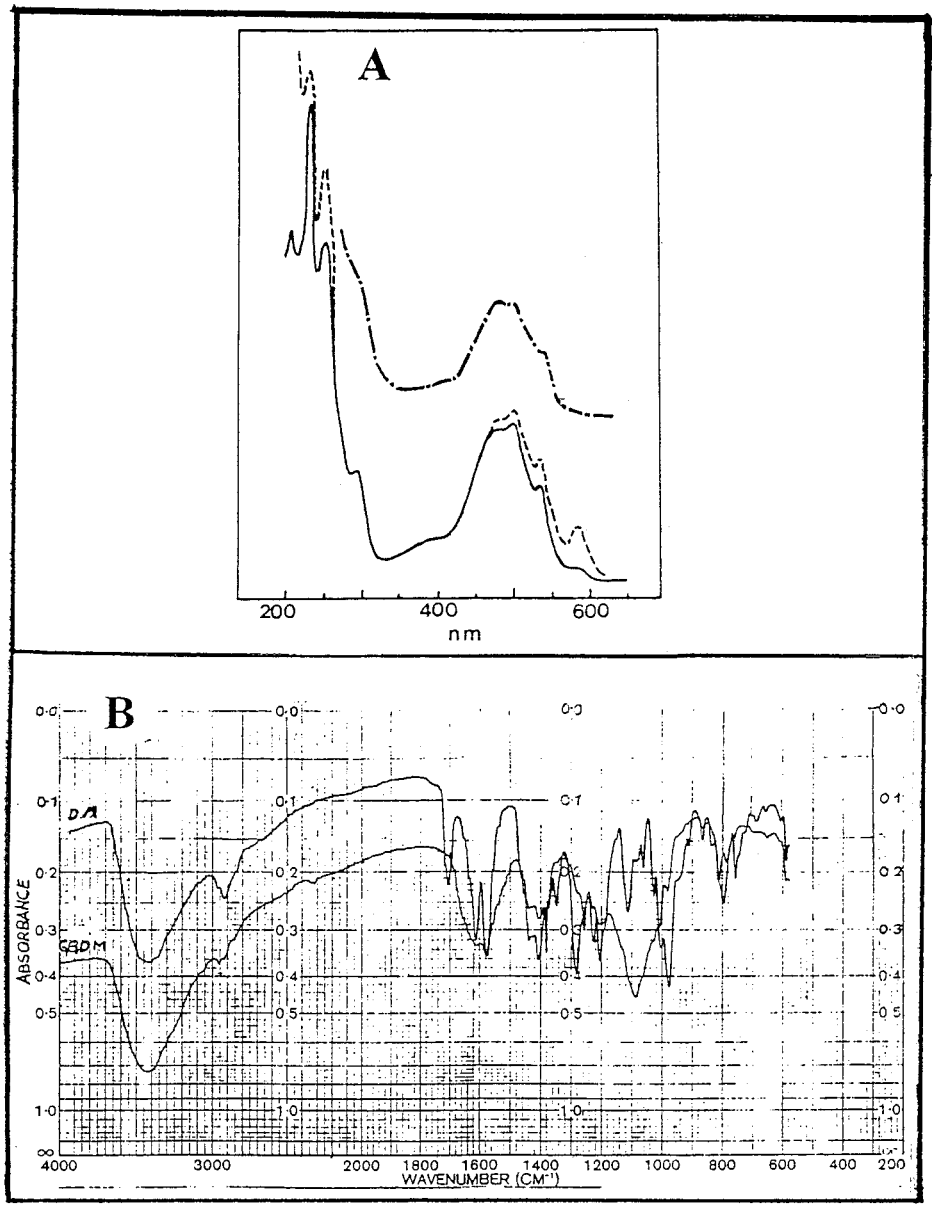

Fig. 5. (**A**) Activity spectrum of (bottom to top): DM, GGDM, and DM released from the adduct by GGT. (**B**) IR spectrum of DM (top line) and GGDM (bottom line).

glutamyl residue to DM by reacting it with pyroglutamic acid were unsuccessful: no polar products were formed, and most of the DM remained unchanged save some cleavage of the daunosamine and appearance of the aglycone, as judged by TLC (data not shown). We attempted, therefore, to use this 3′-amino group as an acceptor and GSH as a γ-glutamyl donor in a transpeptidation reaction catalyzed by GGT. Such a reaction resulted in the time-dependent appearance of a polar product in TLC of mixtures that contained GGT (Fig. 4B, lanes 4–7), but not without GGT (lane 2) or in a complete mixture at time zero (lane 3). Determination of the concentration of the extracted polar product (Fig. 4A) revealed that its accumulation was slow, linear with time, and that addition of serine-borate partially inhibited its accumulation. The above indicate that the formation of the product depended on the activity of GGT, and thus it may be the putative GGDM adduct. Considerable amounts of DM were apparent at early time points, and a small amount was still visible at 25 hr. Since GGT was present, it was unclear whether it was due to incomplete conversion, GGT-dependent or nonenzymatic cleavage of GGDM.

The activity spectrum of GGDM was very similar to that of DM (Fig. 5A). Comparison of the IR spectra of DM and GGDM (Fig. 5B) revealed a broad peak appearing at 1,630–1,680 cm^{-1} [Williams and Fleming, 1995], consistent with an amide bond. We could not determine the molecular mass of GGDM due to the instability of the glycosidic bond, and the amounts of GGDM were too low to allow NMR and CMR analyses, but other evidence (see below) indicates that GGDM is a γ-glutamyl derivative of DM.

Binding of DM and GGDM to DNA

Binding of DM to DNA occurs by intercalation of the aglycone between the bases, and the daunosamine moiety lies in the minor groove [Gao et al., 1996] without bonding to DNA [Quigley et al., 1980]. However, modifications of the 3′-amino group in the daunosamine moiety alter the DNA-binding properties of DM more drastically than modifications of the aglycone [Jolles et al., 1996; Chaires et al., 1996]. Further, N-alkylations of the 3′-amino group by a small moiety (e.g., N,N-dimethyldaunomycin) abolish or greatly reduce their mutagenicity in bacterial and mammalian cells [Westendorf et al., 1984]. We therefore compared the DNA binding properties of GGDM and DM as judged by fluorescence quenching. The binding parameters, calculated from Scatchard plots of DM (Fig. 6A) indicate that at saturating DM concentrations, the minimal target size was 7–9 basepairs (n = 0.11–0.146), and the apparent association constant (Kapp) was $2.1–2.82 \times 10^6\ M^{-1}$. The affinity for a basepair ($2n \times Kapp$) was $6.3 \times 10^5\ M^{-1}$. The values calculated for saturating GGDM (Fig. 6B) were target size of 32–40 basepairs (n = 0.025–0.0316), Kapp = $2.27–3.14 \times 10^6\ M^{-1}$ and $2n \times Kapp = 1.53 \times 10^4\ M^{-1}$. DNA binding of GGDM was not due to nonenzymatic release of DM (see below) in that the latter process is very slow, whereas its reaction with DNA is instantaneous.

Cytotoxicity of DM and GGDM

In view of the fact that GGDM had a significant capability to bind DNA, we tested whether the molecule was cytotoxic as compared to DM. TA98 cells were exposed to various concentrations of GGDM or DM without GGT, and viability was followed with time. Figure 7 shows that GGDM at concentrations up to 200 μM was not toxic, but decreased growth rates in a concentration-dependent manner. DM at 0.9–3.5 μM similarly decreased growth rate and was cytotoxic at 7.2 μM. The ability of GGDM to decrease growth rate was approximately 60-fold less than that of DM. This was unexpected, in that the concentrations of GGDM exceeded by far those required to saturate DNA. The half-life time of GGDM without GGT was

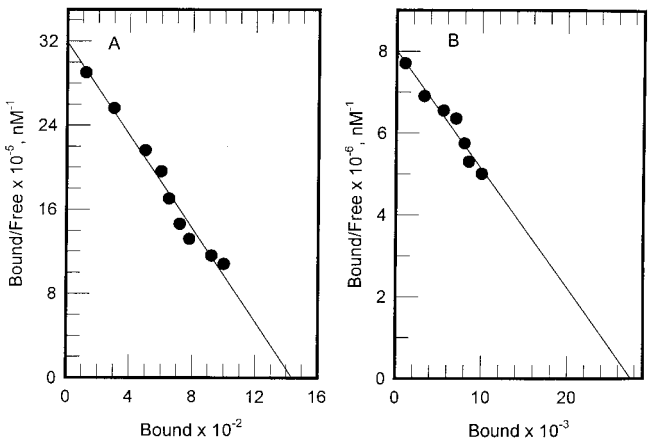

Fig. 6. Scatchard plotting of DNA binding data. (**A**) DNA was at 5 μg/ml in the presence of 0.2–2.4 μM DM. (**B**) DNA was at 10 μg/ml in the presence of 0.2–2.2 μM GGDM. DNA-bound DM or GGDM do not fluoresce. One μg DNA contains 3,080 pmols deoxyribonucleotide phosphate. The bound fraction (*B*) was calculated from the difference between the fluorescence values in the absence and the presence of DNA. The minimal target size (1/*n*, or *B max*) and the apparent association constant (Kapp) were calculated according to the relationship

$$\frac{B}{Free} = \frac{B\ max - B}{Kapp}$$

where the slope = $-1/Kapp$ and intercept = *B max*.

102 hr (see below); thus, the effective DM concentrations which accumulated within 6 hr at 20, 60, 120, and 200 μM GGDM were 0.4, 1.2, 2.4, and 4 μM, respectively. This indicates that the toxicity without GGT was mainly due to release of DM, which readily penetrates the cells, rather than due to the penetration of the intact GGDM molecule. GGDM was highly toxic to TA98 cells in the presence of GGT, where cell death depended on GGDM concentration at a constant concentration of GGT (Fig. 8A) or on GGT concentration at constant concentration of GGDM (Fig. 8B). The cytotoxic agent is suggested to be DM released from GGDM by GGT, in that TLC of reaction mixtures revealed that the appearance of spots with Rf corresponding to DM was GGT- and time-dependent (data not shown), and the activity spectrum of the chloroform-soluble material was identical to that of DM (Fig. 5A, top line). Prolonged incubation of GGDM alone also resulted in limited, nonenzymatic release of DM.

GGT-Dependent Mutagenicity of GGDM

Determination of mutagenicity is a more sensitive assay than cytotoxicity for the detection of the biological activity of potent mutagens such as DM. Since the plate incor-

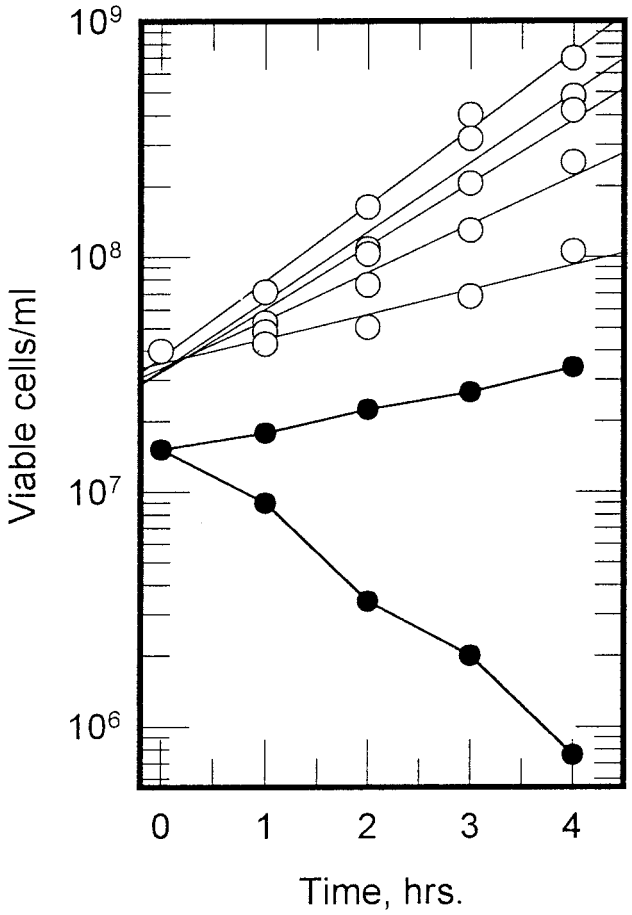

Fig. 7. Cytotoxicity of DM and GGDM in strain TA98 without activation. Exponentially growing TA98 cells were diluted into fresh LB medium (adjusted to pH 8.5) containing GGDM or DM. Cultures were shaken at 37°C and samples were withdrawn for viable counts, as described in Material and Methods. GGDM (○) was at (top to bottom) 0, 20, 60, 120, and 200 μM. DM (●) was at (top to bottom) 3.5 and 7.2 μM. Presented are means of triplicates.

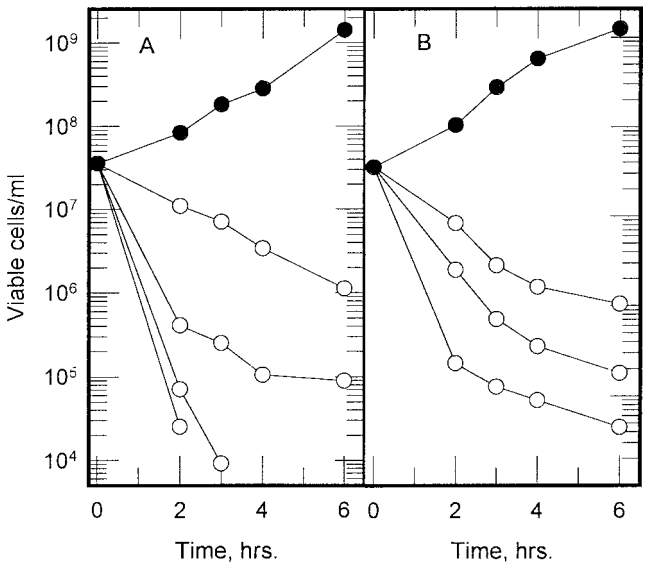

Fig. 8. GGT-dependent cytotoxicity of GGDM in strain TA98. Exponentially growing TA98 cells were diluted into fresh LB medium containing 40 mM glycylglycine and GGDM in one of the following combinations, shaken at 37°C, and samples were withdrawn for viable counts as described in Material and Methods. (**A**) Response to GGDM concentration. Cultures contained (●) 80 μM GGDM; (○) (top to bottom) 20 U/ml GGT and 10, 20, 40, and 80 μM GGDM. (**B**) Response to GGT concentrations. Cultures contained (●) 20 μM GGDM without GGT and (○) 20 μM GGDM in the presence of 5, 10, and 20 U/ml GGT. Presented are means of triplicates.

poration assay involves prolonged exposure which would lead to significant nonenzymatic release of DM, the enzymatic reactions of GGDM with GGT and the mutagenicity assay were separated. Samples from the synthesis mixture were withdrawn with time, the released DM was extracted with chloroform, dried, dissolved in ethanol, and the concentration of DM was determined spectrophotometrically. The solution was diluted 1:1 in water and mutagenicity was determined in TA98 cells by the plate incorporation assay. Table I shows that a slow release of DM from GGDM occurred without GGT. The rate of release was higher with GGT and further increased in the presence of glycylglycine. The half-life of GGDM (calculated from the remaining GGDM vs. time) was 102, 70, and 32 hr, respectively. Likewise, mutagenicity of extracts from reactions with GGT was higher than from those without GGT, and highest in extracts from reactions with GGT and glycylglycine, whereas GGDM without GGT was not mutagenic (Table I). The identity of the activity spectra of authentic DM and the chloroform-soluble material released from GGDM nonenzymatically or by GGT (Fig. 5A), and the specific mutagenicity of the released material which was indistinguishable from that of DM (Table I) indicate that the released material was DM.

Although transport experiments with labeled GGDM were not performed, the results presented here suggest that the low toxicity/mutagenicity of GGDM was mainly due to its transport. The less polar DM readily enters the cells and binds DNA, resulting in cell death and mutagenesis. The more polar GGDM, although capable of DNA binding, is not readily transported. The possibility of release of DM from GGDM by the bacterial, periplasmic GGT is remote, in that coliforms contain negligible (less than 0.1 mU/10^9 cells) GGT activity [Suzuki et al., 1986]. The transport properties of GGDM into mammalian cells are, however, unknown. The results are also consistent with the idea that in addition to the conversion of a prodrug into a biologically active compound, γ-glutamylation may increase the specificity of a chemotherapeutic agent by biochemical targeting of their transport. The slow enzymatic release of DM from its γ-glutamyl derivative may

TABLE I. Mutagenicity of Daunomycin Released From γ-Glutamyl-daunomycin by GGT

Rxn no.	GGT	Gly-gly	4 μl/plate nmol/plate[a]	4 μl/plate Rev/plate	4 μl/plate Net Rev/nmol	8 μl/plate nmol/plate[a]	8 μl/plate Rev/plate	8 μl/plate Net Rev/nmol	16 μl/plate nmol/plate[a]	16 μl/plate Rev/plate	16 μl/plate Net Rev/nmol
1	(−)	(−)	0.19	129 ± 32	426	0.38	205 ± 29	413	0.76	367 ± 26	420
2	+	(−)	0.26	222 ± 12	669	0.53	401 ± 36	666	1.06	750 ± 84	662
3	+	+	0.38	266 ± 20	574	0.76	523 ± 57	625	1.53	998 ± 96	621
4	(−)	(−)	0.28	190 ± 36	507	0.57	518 ± 30	824	1.14	738 ± 41	605
5	+	(−)	0.36	287 ± 41	664	0.72	579 ± 34	738	1.44	952 ± 49	628
6	+	+	0.53	532 ± 34	913	1.06	931 ± 64	833	2.12	1458 ± 277	665
7[b]	(−)	+	0.32[b]	50 ± 10	(−)	0.64[b]	58 ± 17	(−)	1.28[b]	51 ± 15	(−)

Reaction mixtures contained 50 mM HEPES buffer pH 8.5, 400 μM GGDM (Nos. 1, 4) and 20 U/ml GGT without (Nos. 2, 5) or with (Nos. 3, 6) 40 mM glycylglycine in a final volume of 1.5 ml. Samples (500 μl) were withdrawn after 48 hr (Nos. 1–3) and 96 hr (Nos. 4–6) incubation at 37°C. Samples were extracted four times with chloroform; the organic phases were pooled, evaporated, and dissolved in 200 μl ethanol. The activity spectrum (220–650 nm) of each sample was identical to that shown in Figure 5. Concentrations of DM in extracted samples were 235, 330, 478, 356, 444, and 661 μM in reactions 1–6, respectively. Extracts were diluted 5-fold in ethanol:water (1:1) and 4, 8, and 16 μl aliquots were mixed with 1.6×10^8 TA98 cells in top agar and plated onto minimal plates. Further operations were as described in Materials and Methods. The spontaneous mutant yield was 48 ± 7 revertants/plate. Authentic DM at 1 and 2 nmol/plate yielded 702 ± 54 and 1.256 ± 131 revertants/plate, respectively (mean 629 ± 35 revertants/nmol).

[a]The amount of DM was calculated from the concentrations of the extracted material.

[b]Aliquots of an aqueous solution of 80 μM GGDM were tested for mutagenicity as above.

be an advantage in that it may serve as a ''time-release'' capsule in chemotherapy.

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