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MECHANISM(S) REGULATING INHIBITION OF THYMIDYLATE SYNTHASE AND GROWTH BY γ-L-GLUTAMINYL-4-HYDROXY-3-IODOBENZENE, A NOVEL MELANIN PRECURSOR, IN MELANOGENIC MELANOMA CELLS

JOSEPH A. PREZIOSO,* KALYANI M. DAMODARAN, NU WANG and WILLIAM D. BLOOMER

Department of Radiation Oncology, University of Pittsburgh, Pittsburgh Cancer Institute and Joint Radiation Oncology Center, Pittsburgh, PA 15213, U.S.A.

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Abstract—A proposed mechanism for the melanoma specific activity of phenolic amines is based upon the ability of the enzyme tyrosinase to oxidize these prodrugs to toxic intermediates. In this study, we synthesized an iodinated analog of γ-L-glutaminyl-4-hydroxybenzene (GHB) with increased antimelanoma activity in both human and murine melanoma cell lines. GHB and γ-L-glutaminyl-4-hydroxy-3-iodobenzene (I-GHB) were shown to be substrates for both mammalian and mushroom tyrosinase. Glutathione, a cellular antioxidant, inhibited tyrosinase mediated formation of γ-L-glutaminyl-3,4-benzoquinone (GBQ) from GHB, inhibited melanin production, and blocked the inhibition of the enzyme thymidylate synthase by oxidized GHB. Buthionine sulfoximine (BSO) depletion of cellular glutathione enhanced the growth inhibitory activity and the inhibition of *in situ* thymidylate synthase by phenolic amines in melanoma cells. GHB and I-GHB were shown to be approximately 5- and 10-fold more cytotoxic, respectively, in highly metastatic B16-BL6 cells than in weakly metastatic B16-F1 cells with approximately equal tyrosinase activity. B16-BL6 cells had approximately 20-fold higher γ-glutamyltranspeptidase (γ-GTPase) activity than B16-F1 cells which suggested the possible involvement of this enzyme in the activation of the cytotoxicity of the phenolic amines. 4-Aminophenol, a product of γ-GTPase reaction with GHB, was a substrate for tyrosinase and a potent inhibitor of *in situ* thymidylate synthase activity in melanogenic cells. In pigmented melanoma cells containing the enzyme tyrosinase, the quinone mediated mechanism of phenolic amine cytotoxicity may be uniquely important and the cellular antioxidant glutathione essential in the detoxification of these quinone-generated intermediates.

Malignant melanoma is a cancer with increasing world-wide incidence [1]. The effectiveness of existing therapy against disseminated melanoma is inadequate and radiation therapy has been shown to be ineffective. Most of the drugs currently used against melanoma induce responses of less than 25% [2, 3]. Dacarbazine (DTIC), an anticancer agent commonly used in melanoma, has a response rate of less than 20% [4]. Better chemotherapeutic agents for the treatment of patients with disseminated malignant melanoma are needed desperately.

Melanoma-targeting strategies based on specific cell markers [5, 6] and on melanin-targeted radiotherapy [7–9] are currently under investigation. The most characteristic feature of malignant melanoma is its pigmentation. Tyrosinase, a unique enzyme to the melanocyte and melanoma cell, has several functions in the process of pigmentation, including hydroxylation of tyrosine and oxidation of L-dopa to dopaquinone. The process of melanogenesis has been shown to be potentially lethal to cells because it is associated with the generation of toxic intermediates and free radicals [for review see Ref. 10].

Melanospecificity and antimelanoma effects have been observed with a variety of phenolic amine compounds [11–14]. The naturally occurring γ-L-glutaminyl-4-hydroxybenzene (GHB)†, isolated from the mushroom *Agaricus bisporus*, has been shown to have *in vivo* antimelanoma activity in B16 and Kent melanoma [15, 16]. The antimelanoma activity was reported to be dependent upon the intracellular activation of GHB by tyrosinase [16]. The quinone resulting from tyrosinase-catalyzed oxidation of GHB strongly inhibits DNA synthesis, whereas protein and RNA syntheses are largely unaffected [15]. Oxidized GHB has been shown to inhibit DNA polymerase in cell free extracts from L1210 leukemia cells [17]. These observations suggest that the melanocytotoxicity of phenolic amines is due to the strong affinity of tyrosinase-generated quinones for sulfhydryl-dependent enzymes involved in DNA synthesis.

* Corresponding author: Joseph A. Prezioso, Ph.D., Pittsburgh Cancer Institute, W944 Biomedical Science Tower, DeSoto at O'Hara Streets, Pittsburgh, PA 15213. Tel. (412) 624-0342; FAX (412) 624-0295.

† Abbreviations: GHB, γ-L-glutaminyl-4-hydroxybenzene; I-GHB, γ-L-glutaminyl-4-hydroxy-3-iodobenzene; GBQ, γ-L-glutaminyl-3,4-benzoquinone; HIQ, 2-hydroxy-4-iminoquinone; γ-GTPase, γ-glutamyltranspeptidase; BSO, buthionine sulfoximine; and MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

Thymidylate synthase (5,10-methylenetetrahydrofolate: dUMP C-methyltransferase, EC 2.1.1.45) is an enzyme that catalyzes the reductive methylation of deoxyuridylate (dUMP) to deoxythymidylate (dTMP), an essential precursor for DNA synthesis. Inhibition of thymidylate synthase significantly contributes to the cytotoxicity of many antitumor agents, e.g. 5-fluoropyrimidines and antifolates [18]. Inhibition of dTMP synthesis leads to a complex situation in which DNA synthesis is impaired but protein and RNA syntheses are not, a phenomenon termed "thymineless death" [19]. In this study an intact cell assay, which measures thymidylate synthase activity [20], was utilized to investigate the mechanism of cytotoxicity of GHB and γ-L-glutaminyl-4-hydroxy-3-iodobenzene (I-GHB). This assay permits direct and rapid assessment of enzyme activity and inhibition by cytotoxic agents. Its advantage over cell-free systems is that the cell membrane, multi-enzyme complexes, and network of metabolic pathways and their control within the cell remain unperturbed.

We have synthesized a novel iodinated derivative of GHB and examined it and GHB for antimelanoma activity. We also examined the mechanism of activation of these compounds in melanoma cells and the role of the cellular antioxidant glutathione in the detoxification of these oxidized compounds and its protection of the sulfhydryl-dependent enzyme thymidylate synthase.

MATERIALS AND METHODS

Chemicals. Tyrosinase, buthionine sulfoximine (BSO), L-γ-glutamyl-*p*-nitroanilide, glycylglycine, L-cysteine, glutathione (reduced form), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), melanin, tetrahydrofolic acid, crude porcine kidney γ-glutamyltranspeptidase (γ-GTPase), L-dopa and L-tyrosine were purchased from the Sigma Chemical Co., St. Louis, MO. 4-Aminophenol and L-glutamic acid were purchased from the Aldrich Chemical Co., Milwaukee, WI. The γ-*N*-hydroxysuccinimidyl ester of *N*-Boc-L-glutamic acid α-t-butyl ester was purchased from Bachem, Inc., Philadelphia, PA. L-[3,5-^{3}H]Tyrosine (sp. act. 40–60 Ci/mmol) and [5-^{3}H]deoxycytidine (sp. act. 24.2 Ci/mmol) were purchased from New England Nuclear, Boston, MA. [5-^{3}H]Deoxyuridine monophosphate (sp. act. 20 Ci/mmol) was obtained from Amersham, Arlington Heights, IL.

Synthesis of GHB and I-GHB. GHB was synthesized according to the method of Vogel *et al.* [16]. I-GHB was synthesized by the following sequence of reactions: 2-Iodo-4-nitrophenol was reduced with titanium(III) chloride to yield 4-amino-2-iodophenol which was coupled *in situ* with the γ-*N*-hydroxysuccinimidyl ester of *N*-Boc-L-glutamic acid α-t-butyl ester to give the amino and carboxyl protected I-GHB. Treatment of the protected I-GHB with trifluoroacetic acid followed by purification yielded I-GHB.

α-t-Butyl-N-Boc-γ-L-glutaminyl-4-hydroxy-3-iodobenzene. A solution of 2-iodo-4-nitrophenol (230 mg) [21] in an acetic acid–water mixture (1:1) (1 mL) was added to titanium(III) chloride (1.23 g) in an atmosphere of argon and the mixture stirred vigorously at room temperature for 15 min. Water (3 mL) was added and the reaction mixture was neutralized carefully by the addition of solid sodium bicarbonate under argon. The mixture was diluted further with water and extracted with ethyl acetate. The extract was dried over anhydrous magnesium sulfate and filtered into a reaction flask under argon. Dimethylformamide (DMF; 2 mL) and γ-*N*-succinimidyl-*N*-Boc-α-t-butyl-L-glutamate (200 mg) were added to the solution. The ethyl acetate was evaporated *in vacuo* under a stream of argon at room temperature leaving the DMF solution in the flask. The reaction mixture was stirred at room temperature for 48 hr and then diluted with water and extracted with ethyl acetate. The extract was washed with water and dried over anhydrous sodium sulfate. Evaporation of the solvent *in vacuo* gave an oily material which was purified by preparative layer chromatography using an ethyl acetate–hexane (1:1) solvent system to give a colorless glassy material (68 mg, 15%) characterized as α-t-butyl-*N*-Boc-γ-L-glutaminyl-4-hydroxy-3-iodobenzene; TLC on silica gel: ethyl acetate–hexane (1:1) solvent system, R_f, 0.25, and chloroform–methanol (9:1) solvent system, R_f, 0.7. NMR: (300 MHz, acetone-d_6) δ 1.39 (s, 9H, t-Bu), 1.44 (s, 9H, t-Bu), 2.45 (t, 2H, —NH—CO—*CH*$_2$—CH$_2$—), 4.05 (m, 1H, —CH$_2$—*CH*—NH—CO—OtBu), 6.85 (d, 1H, aromatic C-5H), 7.42 (d of d, 1 H, aromatic C-6H) and 8.13 (d, 1H, aromatic C-2H). MS: (EI) *m/z* 520 (M^+, 3%). MS_{HR}: found, M^+, 520.1070; $C_{30}H_{29}IN_2O_6$ requires, M^+, 520.1070.

γ-L-Glutaminyl-4-hydroxy-3-iodobenzene. A solution of α-t-butyl-*N*-Boc-γ-L-glutaminyl-hydroxy-3-iodobenzene (55 mg) in trifluoroacetic acid (0.2 mL) was stirred at room temperature in an atmosphere of argon for 16 hr. The trifluoroacetic acid was evaporated under a stream of argon. Ethyl acetate was added to the residue and evaporated, and the procedure was repeated. Addition of ethyl acetate (1 mL) followed by water (1 drop) and trituration gave an off-white crystalline powder which was filtered and dried (27 mg, 70%); m.p. 196–198° (dec.). TLC on silica gel: *n*-butanol–acetic acid–water (4:1:1) solvent system, R_f, 0.5. NMR: (300 MHz, D_2O, pH 10) δ 1.98 (m, 2H, —NH—CO—CH$_2$—*CH*$_2$—CH—), 2.42 (t, 2H, —NH—CO—*CH*$_2$—CH$_2$—), 3.43 (t, 1H, —NH—CO—CH$_2$—CH$_2$—*CH*—COOH), 6.60 (d, 1H, aromatic C-5H), 7.03 (d of d, 1H, aromatic C-6H) and 7.64 (d, 1H, aromatic C-2H). Elemental analysis (in percent): found, C, 36.33, H, 3.70, N, 7.50 and I, 34.60; required for $C_{11}H_{13}IN_2O_4$: C, 36.28; H, 3.60; N, 7.70 and I, 34.85.

Cell lines. The human SK-MEL-28 melanoma cell line was purchased from the American Type Culture Collection, Rockville, MD. The murine B16-F1 cell line [22] and the highly invasive and metastatic B16-BL6 cell line isolated by Dr. Ian Hart [23] were obtained from Dr. Isiah J. Fidler, MD Anderson Cancer Center, Houston, TX. The human SK-MEL-30 melanoma cell line was a gift from Dr. Michael Wick, Dana Farber Cancer Institute, Boston, MA. Cell lines were maintained as monolayers in 75 cm^2 Falcon plastic flasks containing RPMI-1640 medium

supplemented with 10% fetal bovine serum or 10% calf serum, 2 mM glutamine, 0.1 mM MEM non-essential amino acids, 1 mM sodium pyruvate, 50 μg insulin/mL, 100 μg streptomycin/mL, 100 U of penicillin/mL, and 2.5 μg gentamicin/mL. All cultures were maintained in a 5% CO_2 humidified air incubator at 37°.

Preparation of cell-free extracts. Cell-free extracts were prepared from melanoma and K562 leukemia cells. Melanoma cells ($\sim 2 \times 10^7$) were trypsinized from 75 cm^2 culture flasks, harvested by centrifugation and washed twice with Hanks' Balanced Salt Solution (HBSS). K562 leukemia cells were harvested by centrifugation and washed with HBSS. Cells were resuspended in 1.5 vol. of 20 mM Tris buffer containing 1 mM dithioerythritol (pH 7.4). The resuspended cells were sonicated using a Sonics and Materials Sonifier (model Vibra cell; Danbury, CT) at a 70 setting with 5 pulses of 20-sec durations. The suspension was centrifuged at 15,000 rpm for 30 min and the pellet discarded. The supernatant was decanted, aliquoted and stored at −135° until assayed for enzyme activity.

Tyrosinase assay. In situ tyrosinase activity was determined by a modification of the procedure of Halaban and Lerner [24]. Culture medium was removed from 24-well microtiter plates containing cells in log phase growth and replaced with 0.5 mL HBSS containing 0.5 μCi L-[3,5-^{3}H]tyrosine which had been reduced previously to dryness under a stream of nitrogen gas. Following incubation of intact cells for 60 min at 37°, the accumulation of ^3H_2O in 0.5 mL of medium was measured. The buffered salt solution was removed from the wells and added to 1.5-mL microfuge tubes. To each tube was added 0.5 mL of charcoal suspension [300 mg charcoal in 10% trichloroacetic acid (TCA)] and the mixture was shaken periodically for 30 min before centrifugation at 10,000 rpm for 6 min. Aliquots of 0.5 mL were pipetted off and added to 6 mL of CytoScint ES scintillation fluid (ICN Biomedical Inc., Irvine, CA). Radioactivity was measured in a Packard Tri-Carb Liquid scintillation counter (model 1900CA).

Inhibition of mammalian tyrosinase by GHB and I-GHB was examined using a modification of the radiometric assay described by Halaban *et al.* [25]. GHB or I-GHB was incubated with 20 μL B16-BL6 cell-free extract, 50 μM L-tyrosine, 50 μM L-dopa and 1 μCi [^{3}H]tyrosine at 37° for 2 hr. The reaction was terminated and radioactivity measured by the charcoal absorption method [25].

Melanin formation. GHB or I-GHB (2 mM) was incubated with mushroom tyrosinase (1 mg/mL) at 37°. After a 48-hr incubation period, melanin formation was determined by adding an equal volume of 5% TCA and the mixture centrifuged at 12,000 rpm for 20 min. The pellet was washed with ethyl alcohol–ethyl ether (3:1) and centrifuged at 12,000 rpm for 20 min. The melanin was resuspended in 600 μL of 0.85 N NaOH by heating at 100° for 15 min. After cooling, the absorbance at 400 nm was determined. The amount of melanin formed was calculated from a synthetic melanin standard curve.

MTT viability assay. To assay growth inhibitory activity, cell lines were seeded at 3.5×10^4 cells/well in 24-well flat bottom microtiter plates in complete RPMI-1640 medium containing 10% fetal bovine serum or 10% calf serum. Cell lines were incubated with selected concentrations of GHB or I-GHB (2 mM–20 μM) in complete medium containing 10% serum at 37° in a 5% CO_2 humidified atmosphere 24 hr after initial plating. The IC_{50} values (drug concentration causing a 50% reduction in cell viability) were determined by the MTT viability assay after a 48 hr incubation period. The MTT viability assay uses a tetrazolium dye which is reduced to a blue formazan by the mitochondria of living but not dead cells or cell debris. The amount of formazan dye reduced is directly proportional to the number of remaining viable target cells [26]. The absorbance of each well was measured on a Fisher Biotech Series 2000 automated microplate reader at 420 nm.

Determination of tyrosinase generated oxidized products from GHB and 4-aminophenol. Inhibition of γ-L-glutaminyl-3,4-benzoquinone (GBQ) formation from GHB by glutathione and L-cysteine was determined using a spectrophotometric assay. GHB (2.5 mM) was incubated with mushroom tyrosinase (1 mg/mL) in the presence or absence of 4 mM glutathione or L-cysteine at 37°. The amount of GBQ formed was determined at 15-min intervals by measuring the increase in absorbance at 440 nm (Abs_{max} of GBQ) in a Beckman model 34 spectrophotometer [12]. A molar extinction coefficient of 1.38 $mM^{-1}\,cm^{-1}$ was used for absorbance conversions [27].

Tyrosinase-dependent formation of 2-hydroxy-4-iminoquinone (HIQ) from 4-aminophenol was assayed as follows. 4-Aminophenol (1.25 mM) was incubated with mushroom tyrosinase (1 mg/mL) in the presence or absence of 4 mM glutathione at room temperature. The amount of HIQ formed was determined at 1-min intervals by spectrophotometrically measuring the increase in absorbance at 490 nm (Abs_{max} of HIQ) [12]. A molar extinction coefficient of 10.0 $mM^{-1}\,cm^{-1}$ was used for absorbance conversions [17].

γ-GTPase assay. γ-GTPase activity was measured in cell extracts. Cell extract was added to a reaction mixture containing 2 mM L-γ-glutamyl-*p*-nitroanilide, 20 mM glycylglycine, 75 mM NaCl and 200 μM Trizma-HCl buffer, pH 7.5. The reaction mixture was incubated at room temperature and the absorbance measured every 5 min for 1 hr spectrophotometrically at 410 nm [28]. In the presence of enzyme, L-γ-glutamyl-*p*-nitroanilide donates a glutamyl group to glycylglycine forming the conversion products *p*-nitroaniline and glutamylglycylglycine. The activity was expressed as nanomoles of *p*-nitroaniline released/10^6 cells. A molar extinction coefficient of 10.4 $mM^{-1}\,cm^{-1}$ was used for absorbance conversions.

GHB and I-GHB were tested for reactivity with crude porcine kidney γ-GTPase as described by Boekelheide *et al.* [12]. Formation of 4-aminophenol and 4-amino-2-iodophenol from GHB and I-GHB was measured spectrophotometrically at Abs_{294} and Abs_{344}, respectively.

Assay of thymidylate synthase activity in cell extracts. Thymidylate synthase activity was assayed

as described by Roberts [29]. This sensitive procedure measures the release of tritium (as tritiated water) from the 5-position of [5-^{3}H]deoxyuridine monophosphate ([5-^{3}H]dUMP) during the formation of dTMP [30]. Briefly, 20 μL of cell extract was mixed with 180 μL of reaction mixture containing (final concentration): 0.15 M Trizma (pH 7.4), 60 mM NaF, 0.052% formaldehyde, 10 mM tetrahydrofolate, and 0.667 μCi of [5-^{3}H]dUMP. Radiolabeled dUMP had been reduced previously to dryness under a stream of nitrogen gas. The reaction mixture was incubated at 37° for 45 min and the reaction was terminated by adding 0.5 mL of an activated charcoal suspension (300 mg charcoal in 10% TCA) which removed unreacted [^{3}H]dUMP. The reaction mixtures were vortexed periodically for 30 min and then centrifuged for 10 min at 15,000 rpm. Aliquots of 0.5 mL were pipetted off, added to 6 mL of CytoScint ES scintillation fluid, and the radioactivity was measured.

In situ *thymidylate synthase assay.* *In situ* thymidylate synthase activity was assayed by a modification of the procedure described by Yalowich and Kalman [20] which measures ^3H_2O generated from [5-^{3}H]deoxycytidine ([^{3}H]dCyd). To perform this assay, cell lines were seeded at 7.5×10^4 cells/well and incubated for 24 hr in Falcon 24-well flat bottom microtiter plates in complete RPMI-1640 medium containing 10% fetal bovine or calf serum. Cells were then incubated for 2 hr with selected concentrations of GHB or I-GHB (2 mM–100 μM) in complete medium containing 10% serum at 37° in a 5% CO_2 humidified atmosphere; 1 μCi [^{3}H]-dCyd which had been reduced previously to dryness under nitrogen gas was then added and the cells were incubated with GHB or I-GHB for an additional 90 min. The reaction was terminated by transferring 0.5-mL aliquots into 1.5-mL microfuge tubes containing 0.5 mL of an activated charcoal suspension. The mixtures were vortexed periodically for 30 min to remove unreacted [^{3}H]dCyd and then centrifuged for 6 min at 15,000 rpm. Aliquots of 0.5 mL were pipetted off and the radioactivity was determined.

RESULTS

I-GHB as a novel melanin precursor. Both I-GHB and GHB competed with L-[3,5-^{3}H]tyrosine for tyrosine hydroxylase in cell free extracts of B16-BL6 melanoma cells (Fig. 1). In the presence of 1 mM GHB or I-GHB, 5,360 ± 160 or 17,680 ± 810 cpm/hr, respectively, were released from [^{3}H]tyrosine as ^3H_2O after a 2 hr incubation period. In the absence of drug, 23,620 ± 830 cpm/hr were released. GHB and I-GHB significantly ($P < 0.005$) inhibited ^3H_2O formation from [^{3}H]tyrosine with B16-BL6 cell-free extract by 77 and 25%, respectively.

Both I-GHB and GHB generated melanin in the presence of tyrosinase. After a 48-hr incubation period at 37° in the presence of 2 mM GHB or I-GHB, 70 or 44 μg melanin was formed, respectively (results not shown). GHB generated 59% more melanin than I-GHB under identical conditions. In the absence of tyrosinase, no detectable level of melanin was formed with either compound.

Growth inhibitory activity of phenolic amines with melanoma cell lines. I-GHB was approximately 3-fold more growth inhibitory in human SK-MEL-28 melanoma cells and heavily pigmented murine B16-BL6 cells than GHB (Table 1). In the heavily pigmented human SK-MEL-30 cell line, GHB had a 2.4-fold higher IC_{50} value than I-GHB. In the pigmented B16-F1 cell line, both GHB and I-GHB were relatively non-toxic with IC_{50} values of 1900 ± 213 and 1333 ± 53 μM, respectively.

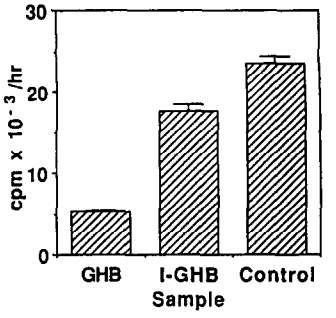

Fig. 1. Inhibition of tyrosine hydroxylase activity in B16-BL6 cell free extracts by GHB and I-GHB. Phenolic amines (1 mM) were incubated with 20 μL extract, 50 μM L-tyrosine, 50 μM L-dopa and 1 μCi [3,5-^{3}H]tyrosine at 37°. Tyrosinase activity in the absence of phenolic amine was determined in parallel experiments. ^3H_2O formation was measured 2 hr after the addition of [3,5-^{3}H]tyrosine. Tyrosinase activity is reported as cpm released per hr. Values are means ± SD, N = 3.

BSO potentiation of GHB and I-GHB cytotoxic activity in melanoma cells. BSO, an inhibitor of the synthesis of the cellular antioxidant glutathione, enhanced growth inhibitory activity of phenolic amines in human and murine melanoma cells (Fig. 2). The IC_{50} values for GHB in the human SK-MEL-28 cell line was 1333 ± 53 μM in the presence of 100 μM BSO and > 2000 μM in its absence. BSO treatment in the heavily pigmented B16-BL6 cell line significantly decreased ($P < 0.005$) the IC_{50} value of GHB by more than 74%. In the presence of BSO the IC_{50} value was < 100 μM and in its absence 382 ± 12 μM (Fig. 2).

The IC_{50} value for I-GHB in the SK-MEL-28 cell line was 656 ± 33 μM and in the presence of 100 μM BSO was 218 ± 13 μM, a significant ($P < 0.005$) 67% decrease (Fig. 2). The IC_{50} value for I-GHB in the pigmented B16-BL6 cell line was 130 ± 8 μM, and in the presence of BSO was < 20 μM. At a 20 μM I-GHB concentration in the presence of BSO, 100% of cells were non-viable as determined by the MTT viability assay, whereas in the absence of BSO 100% of the cells were viable. BSO alone at a 100 μM concentration after a 3-day exposure decreased the cell viability by < 10%. BSO significantly enhanced the growth inhibitory activity of both phenolic amines in human and murine melanoma cells. BSO enhancement of phenolic amine cytotoxicity was not due to increased *in situ* tyrosinase activity since B16-BL6 melanoma cells exposed to 100 μM BSO for 24 hr had a 19% decrease in *in situ* tyrosinase activity (data not shown).

Table 1. Comparison of growth inhibitory activity of GHB and I-GHB with *in situ* tyrosinase and γ-glutamyltranspeptidase activity of various melanoma cell lines

Cell line	Tyrosinase activity* (cpm/5 × 10^5 cells/hr)	γ-GTPase†	GHB IC_{50}‡ (μM)	I-GHB IC_{50}‡ (μM)
Human melanoma				
SK-MEL-30	4390 ± 390	2.0 ± 0.3	1737 ± 213	722 ± 29
SK-MEL-28	440 ± 120	1.1 ± 0.2	> 2000	656 ± 33
Murine melanoma				
B16-BL6	8440 ± 370	124.0 ± 6.5	382 ± 12	130 ± 8
B16-F1	7090 ± 1660	6.2 ± 1.0	1900 ± 213	1333 ± 53

* Tyrosinase activity was determined 24 hr after plating, using the *in situ* assay described in Materials and Methods. Values are means ± SD, N = 3.

† γ-GTPase activity is reported as nmol *p*-nitroaniline released/10^6 cells/hr. Values are means ± SD, N = 4.

‡ Cells were incubated with selected concentrations of GHB or I-GHB for 48 hr. The IC_{50} values represent the concentration of drug causing a 50% reduction in growth as determined by the MTT viability assay. Values are means ± SD, N = 3.

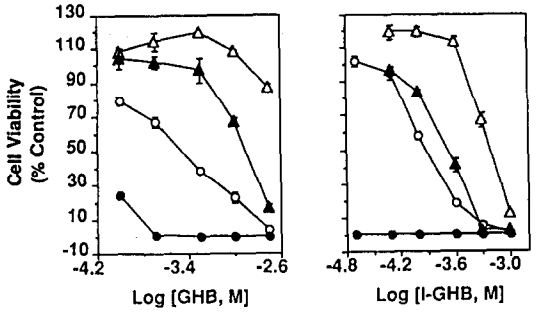

Fig. 2. BSO enhancement of GHB and I-GHB cytotoxicity in human SK-MEL-28 and murine B16-BL6 melanoma cells. Cells were preincubated for 24 hr with 100 μM BSO prior to the addition of GHB or I-GHB. After a 48 hr exposure, in SK-MEL-28 cells, to drug alone (△) or drug + BSO (▲) or in B16-BL6 cells to drug alone (○) or drug + BSO (●), percent growth inhibition was determined. Values are means ± SD, N = 3.

Glutathione and L-*cysteine inhibition of GBQ formation from GHB.* GHB is hydroxylated to γ-L-glutaminyl-3,4-dihydroxybenzene which is further oxidized to GBQ, both reactions being catalyzed by tyrosinase. GBQ has a visible absorption maximum of 440 nm [12]. Figure 3A demonstrates that glutathione and cysteine prevented the production of GBQ from GHB. After a 2-hr incubation period at 37° with tyrosinase, 1.41 μmol of GBQ was generated from GHB. In the presence of 4 mM glutathione or cysteine, only 0.007 or 0.09 μmol, respectively, was produced. Glutathione and cysteine prevented the formation of the sulfhydryl reactive compound GBQ from GHB by 99 and 94%, respectively.

Correlation of γ-GTPase activity with growth inhibition by phenolic amines. The IC_{50} value of GHB was 382 μM with pigmented B16-BL6 melanoma cells but 1900 μM for the equally pigmented variant B16-F1 cell line (Table 1). I-GHB had IC_{50} values of 130 or 1333 μM in B16-BL6 or B16-F1 cells, respectively, even though both cell lines had similar tyrosinase activities (Table 1) and glutathione levels (4.8 and 5.6 nmol/10^6 cells for BL6 and F1 cells, respectively).

GHB and γ-L-glutaminyldihydroxybenzene are both substrates for the enzyme γ-glutamyltranspeptidase [12]. In our study, I-GHB was also shown to be a substrate for γ-GTPase forming 4-amino-2-iodophenol with crude porcine kidney γ-GTPase. γ-GTPase cleaves the glutamyl group of GHB forming 4-aminophenol. 4-Aminophenol has shown *in vivo* antimelanocytic activity in C57BL/6J mice [12]. In this study, 4-aminophenol was shown to inhibit *in situ* thymidylate synthase activity in BL6 melanoma cells with an IC_{50} value of 282 ± 32 μM. These observations prompted us to test the BL6 and F1 variant B16 melanoma cell lines for their γ-GTPase activities. The B16-BL6 cell line had 20 fold higher γ-glutamyltranspeptidase activity than the B16-F1 cell line (Fig. 3B), suggesting that high levels of the enzyme γ-GTPase increase the susceptibility of pigmented melanoma cells to the cytotoxic activity of both GHB and I-GHB. γ-GTPase may activate these prodrugs by catalyzing the formation of 4-aminophenols, which upon oxidation in a melanogenic environment, generate cytotoxic products.

Glutathione inhibition of the formation of 2-hydroxy-4-iminoquinone from 4-aminophenol. Cellular γ-GTPase may activate the prodrugs GHB and I-GHB by generating 4-aminophenols which may then serve as substrates for the enzyme tyrosinase. If 4-aminophenol is a substrate of tyrosinase, it would be initially hydroxylated to aminocatechol and then further oxidized to 2-hydroxy-4-iminoquinone, which has a visible absorption maximum of 490 nm [12]. Figure 3C demonstrates that 4-aminophenol was a substrate for tyrosinase and that glutathione prevented the production of HIQ from 4-aminophenol. After a 20 min incubation with tyrosinase, 96 nmol of HIQ was generated from 4-aminophenol. In the presence

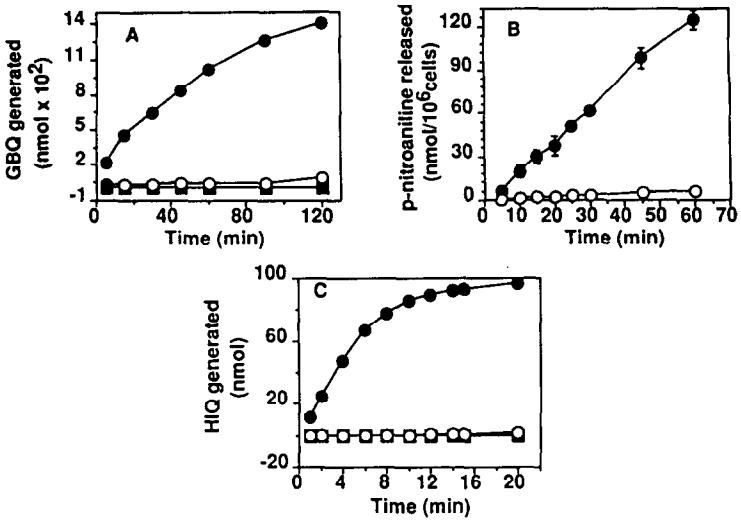

Fig. 3. (A) L-Glutathione and L-cysteine inhibition of γ-L-glutaminyl-3,4-benzoquinone formation from GHB. GHB was incubated with tyrosinase (1 mg/mL) in the absence (●) or presence of 4 mM glutathione (■) or cysteine (○) at 37°. At 15-min intervals the absorbance was determined at a wavelength of 440 nm in a Beckman spectrophotometer. (B) γ-Glutamyltranspeptidase activity in B16-BL6 (●) and B16-F1 (○) cell extracts. Cell extract was added to a reaction mixture containing 2 mM L-γ-glutamyl-*p*-nitroanilide, 20 mM glycylglycine, 75 mM NaCl and 200 mM Trizma-HCl, pH 7.5. The reaction mixture was incubated at room temperature and the absorbance was measured every 5 min for 1 hr spectrophotometrically at 410 nm. Values are means ± SD, N = 3. (C) Glutathione inhibition of 2-hydroxy-4-iminoquinone formation from 4-aminophenol. 4-Aminophenol was incubated with tyrosinase (1 mg/mL) in the presence (■) or absence (●) of 4 mM glutathione at room temperature. At specific time intervals the absorbance was determined spectrophotometrically at a wavelength of 490 nm. Control was 4-aminophenol in the absence of tyrosinase (○).

of 4 mM glutathione, HIQ formation was not detected. In the absence of both tyrosinase and glutathione, 1.3 nmol was generated.

Glutathione inhibition of oxidized GHB inhibitory activity with thymidylate synthase. GHB inhibition of the sulfhydryl-dependent enzyme, thymidylate synthase, in cell-free extracts required oxidation of the drug. In the absence of tyrosinase, 1 mM GHB inhibited thymidylate synthase activity by less than 10% (data not shown). Figure 4 shows that the sulfhydryl containing antioxidant glutathione blocked the inhibition of thymidylate synthase by oxidized GHB. Oxidized GHB was incubated for 30 min with glutathione before the addition of cell extract containing thymidylate synthase. The IC_{50} values for inhibition of thymidylate synthase by oxidized GHB were 21 ± 1 μM vs 100 ± 4 μM in the presence of 5 mM glutathione, a significant ($P < 0.005$) 4.8-fold increase.

BSO potentiation of GHB and I-GHB inhibition of in situ *thymidylate synthase in pigmented melanoma cells.* BSO, an inhibitor of glutathione synthesis, enhanced the inhibition of *in situ* thymidylate synthase by GHB and I-GHB in pigmented B16-BL6 melanoma cells (Fig. 5). In the presence of 100 μM BSO, the IC_{50} values for inhibition of *in situ* thymidylate synthase by GHB or I-GHB after a 24-hr exposure were <20 or <10 μM, respectively, and, in its absence, 142 ± 10 or 84 ± 4 μM, respectively. BSO significantly ($P < 0.005$) decreased the IC_{50} values of GHB or I-GHB inhibition of *in*

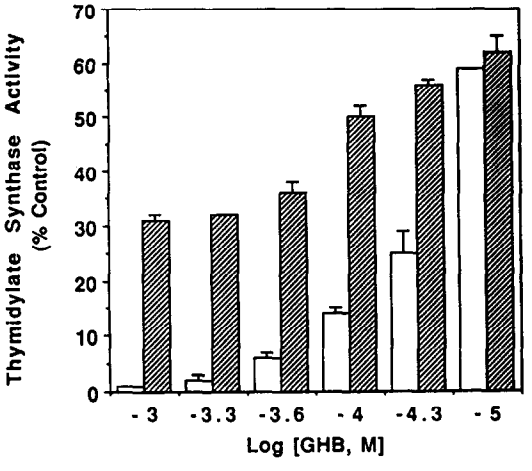

Fig. 4. Inhibition of thymidylate synthase activity by oxidized GHB and protection in the presence of glutathione. Glutathione (5 mM) was preincubated with oxidized GHB for 30 min before the addition of cell-free lysate containing thymidylate synthase. Inhibition of thymidylate synthase by oxidized GHB was determined in the absence of antioxidant (□) or in the presence of 5 mM reduced glutathione (▨). The control activities were 16,640 and 17,720 cpm released/mg protein/min, respectively. Values are means ± SD, N = 3.

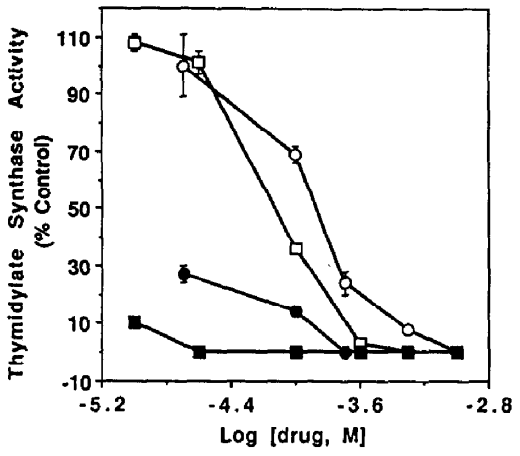

Fig. 5. BSO potentiation of GHB and I-GHB inhibition of *in situ* thymidylate synthase in B16-BL6 melanoma cells. Cells were preincubated for 24 hr with 100 μM BSO prior to the addition of GHB or I-GHB. After a 24 hr exposure of B16-BL6 cells to GHB alone (○), GHB + BSO (●), I-GHB alone (□), or I-GHB + BSO (■), percent inhibition of *in situ* thymidylate synthase activity was determined. The control activities for BSO-treated and non-treated groups were 8530 and 9810 cpm released/1 × 10^5 cells/hr, respectively. Values are means ± SD, N = 3.

situ thymidylate synthase by 80–90% in pigmented B16-BL6 melanoma cells. The IC_{50} values of GHB or I-GHB inhibition of *in situ* thymidylate synthase in pigmented B16-BL6 melanoma cells were significantly lower (63 or 35%, respectively; $P < 0.005$) than the IC_{50} values for the inhibition of cell growth. The IC_{50} value of I-GHB inhibition of *in situ* thymidylate synthase activity in B16-BL6 cells was significantly ($P < 0.005$; 41%) lower than that of GHB. GHB at a 1 mM concentration had no effect on *in situ* thymidylate synthase activity in the variant B16-F1 cell line with low γ-GTPase activity. At a 1 mM GHB concentration, *in situ* thymidylate synthase activity was equal to that of non-treated control cells.

DISCUSSION

We conclude that the novel melanin precursor, γ-L-glutaminyl-4-hydroxy-3-iodobenzene, has increased antitumor activity with human and murine melanoma cells relative to the parent compound γ-L-glutaminyl-4-hydroxybenzene. I-GHB was also more inhibitory to *in situ* thymidylate synthase in pigmented melanoma cells than GHB. Both compounds were substrates for tyrosinase and formed melanin in the presence of the enzyme. GHB was a better substrate for tyrosinase than I-GHB as determined by competitive inhibition of mammalian tyrosinase and the rate of melanin synthesis but was 3-fold less cytotoxic with human and murine melanoma cells.

Although the precise mechanism(s) of toxicity of GHB or I-GHB for melanogenic cells is unclear, the evidence is consistent with the hypothesis that specific cytotoxicity is related to active melanogenesis [15, 16, 31, 32]. Conceptually, GHB and I-GHB are activated by mammalian tyrosinase to quinone derivatives, which are toxic sulfhydryl reactive agents. This study suggests an alternative method by which phenolic amines may be activated to cytotoxic quinones. I-GHB and GHB may be cleaved by the enzyme γ-glutamyltranspeptidase, which has specific activity for γ-glutamyl compounds [33]. *In vitro* studies of others have shown that GHB and its metabolite, γ-L-glutamyl-3,4-dihydroxybenzene, undergo rapid cleavage by γ-glutamyltranspeptidase [12]. In this study, I-GHB was also found to be a substrate for γ-GTPase, and GHB and I-GHB were found to be 5- and 10-fold, respectively, more growth inhibitory in B16-BL6 cells with 20-fold higher γ-glutamyltranspeptidase activity than in variant B16-F1 cells. In B16-BL6 cells, the IC_{50} value for inhibition of thymidylate synthase by GHB was 142 μM but, in B16-F1 cells, the IC_{50} value was >1 mM. At a 1 mM GHB concentration no detectable inhibition of *in situ* thymidylate synthase was observed in B16-F1 cells. B16-BL6 and B16-F1 cells had similar tyrosinase activities. Increased γ-GTPase levels in B16-BL6 cells may lead to higher levels of 4-aminophenols generated from GHB and I-GHB than from B16-F1 cells. Thus, GHB and I-GHB could be cleaved by this enzyme to yield 4-aminophenol and 4-amino-2-iodophenol, respectively, which, upon oxidation in a melanogenic environment, will generate sulfhydryl-reactive quinones which are inhibitory to thymidylate synthase.

4-Aminophenol is selectively cytotoxic to ocular melanocytes in the phase of active melanogenesis [31]. It is also selectively cytotoxic to follicular melanocytes in the hair follicles of black C57BL/6J mice, whereas the keratinocytes appeared normal and were actively dividing [12]. These changes are identical to those reported earlier with GHB [30]. In our study, 4-aminophenol was observed to inhibit *in situ* thymidylate synthase activity in pigmented melanoma cells. Furthermore, tyrosinase was shown to generate the sulfhydryl-reactive 2-hydroxy-4-iminoquinone from 4-aminophenol and the formation of this compound was blocked by the antioxidant glutathione. HIQ may be at least partially responsible for the melanocytotoxic activity observed with these phenolic amines.

There is considerable evidence that the enzyme γ-GTPase is intimately involved in the process of melanogenesis and that it may contribute to the melanospecificity observed with GHB. γ-GTPase activity is present in melanocytes during their melanogenic stage, but absent from cells not engaging in melanogenesis [34, 35]. In B16 melanoma cells, light microscopic examination has revealed that the cytoplasm is fairly rich in γ-GTPase activity [34]. Mojamdar *et al.* [36], by subcellular distribution studies, revealed that tyrosinase and γ-GTPase co-exist within premelanosomes. Elevated serum γ-GTPase levels have been found in patients with metastatic melanoma [37]. Furthermore, the high γ-GTPase levels in the highly metastatic variant of B16 melanoma in relation to the weakly metastatic variant suggest a possible involvement of this enzyme in the complex process of metastasis.

Several protective mechanisms exist in melanoma

cells to diminish the hazards posed by the process of melanogenesis. The reactions are normally confined to specialized membrane-bound organelles, melanosomes, so that the reactants are segregated from the rest of the cytosol. Within melanosomes the potential cytotoxic hazard of intermediates generated by melanization is inhibited by polymerization and/or macromolecular capture of the reactive intermediates such as quinones. Low molecular weight thiols readily undergo reductive addition reactions to quinone products generated from tyrosinase [38]. It appears that cysteine is the major reactant present within melanosomes and nucleophilic addition of cysteine to dopaquinone gives predominantly 5-*S*-cysteinyldopa [39] which is regarded as an important branching point in the generation of pheomelanins [38]. Whereas reactions involving nucleophilic addition of glutathione are of little significance in normal melanogenesis [40], this reaction seems to constitute a mechanism which protects the cell from quinones that have leaked out of the melanosome. Glutathione-dopa has been found in melanogenic cells [41] and glutathione readily undergoes nucleophilic addition with quinones [38]. In this study, BSO was shown to enhance the cytotoxic activity of GHB and I-GHB in both human and murine melanoma cells, possibly by depleting glutathione which blocks the oxidized compound from expressing its inhibitory activity with sulfhydryl-dependent enzymes involved in DNA synthesis (e.g. thymidylate synthase, α-DNA polymerase).

A proposed mechanism of GHB action is tyrosinase generation of quinones which inhibit α-DNA polymerase, an enzyme essential to cell survival [17]. In this study, we demonstrated the *in situ* inhibitory effects of GHB on thymidylate synthase, another sulfhydryl-dependent enzyme involved in DNA synthesis. A consequence of the inhibition of DNA polymerase is elevated levels of deoxynucleoside triphosphate pools, specifically dTTP. The possibility that elevated dTTP pools feedback and inhibit thymidylate synthase has been ruled out by Reddy [42] who has shown that neither deoxythymidine monophosphate nor dTTP has any effect on thymidylate synthase activity in permeabilized cells. Thus, a primary site of inhibitory activity of these compounds seems to be thymidylate synthase.

In vitro and *in situ* analyses revealed that phenolic amine inhibition of thymidylate synthase required oxidation of the compound(s). In the presence of tyrosinase the IC_{50} value for inhibition of thymidylate synthase by GHB was 21 μM but, in its absence, $< 10\%$ inhibition was observed at a drug concentration of 1 mM. Glutathione partially blocked the inhibition of thymidylate synthase in cell-free extracts by oxidized GHB. BSO depletion of glutathione in pigmented melanoma cells increases inhibition of *in situ* thymidylate synthase by GHB and I-GHB. In cells of high oxidative potential, e.g. pigmented melanoma cells, the quinone-mediated mechanism of inhibition of DNA synthesis may be uniquely important in the expression of phenolic amine cytotoxicity.

Vogel *et al.* [15] demonstrated that GHB inhibited DNA synthesis in pigmented melanoma cells, whereas RNA and protein syntheses were largely unaffected. Since thymidylate synthase represents the sole *de novo* pathway for dTMP synthesis, blockade of its activity would have dramatic effects on proliferating cells. Indeed, inhibition of dTMP synthesis leads to a complex situation in which DNA synthesis is impaired while protein and RNA syntheses are not, a phenomenon termed "thymineless death" [19]. A proposed mechanism of cell death following thymidylate synthase inhibition (e.g. "thymineless death") is based on the accumulation of dUTP which ultimately leads to DNA damage and cell death [43, 44].

I-GHB is a substrate for mammalian tyrosinase which produces melanin. An alternate method of synthesis of I-GHB is currently underway, it will be adopted to the synthesis of radioiodinated GHB which will be tested for melanoma imaging capabilities in nude mice xenografted with human melanoma tumors. Furthermore, BSO will be tested for its ability to enhance the *in vivo* antimelanoma activity of GHB and I-GHB with human melanoma xenografts. These studies should aid in giving us a better understanding of the potential of I-GHB as an antimelanoma drug and the radioiodinated compound as a melanoma imaging agent.

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