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Antitumour Activity of *S*-p-bromobenzylglutathione Cyclopentyl Diester *in Vitro* and *in Vivo*.

INHIBITION OF GLYOXALASE I AND INDUCTION OF APOPTOSIS

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ABSTRACT. The glyoxalase I inhibitor diester, *S*-p-bromobenzyl-glutathione cyclopentyl diester ($BrBzGSHCp_2$), inhibited the growth of human leukaemia 60 (HL60) cells *in vitro*. The median growth inhibitory concentration GC_{50} value of $BrBzGSHCp_2$ was 4.23 ± 0.01 μM (n = 21), and the median toxic concentration TC_{50} value was 8.86 ± 0.01 μM (n = 21). $BrBzGSHCp_2$ inhibited DNA synthesis in the third hr of incubation: the median inhibitory concentration IC_{50} value was 6.11 ± 0.02 μM (n = 8). Incubation of HL60 cells with 10 μM $BrBzGSHCp_2$ delivered the diester into cells: de-esterification of the diester therein lead to formation of the *S*-p-bromobenzylglutathione, inhibition of glyoxalase I activity *in situ*, increase in the methylglyoxal concentration after 1 hr, and induction of apoptosis after 6 hr. $BrBzGSHCp_2$ (50–200 mg/kg) also inhibited the growth of murine adenocarcinoma 15A *in vivo*. Glyoxalase I inhibitor diesters may, therefore, inhibit tumour growth by inducing the accumulation of methylglyoxal in tumour cells, and induction of apoptosis. BIOCHEM PHARMACOL 51;10:1365–1372, 1996.

KEY WORDS. glyoxalase I; methylglyoxal; human leukaemia 60; apoptosis

Glyoxalase I (EC 3.2.1.6) catalyses the formation of *S*-D-lactoylglutathione from the hemithioacetal of methylglyoxal and reduced glutathione formed in a nonenzymatic pre-equilibrium reaction MeCOCHO + GSH ⇌ MeCOCH(OH) − SG $\xrightarrow{\text{GLYOXALASE I}}$ MeCH(OH)CO − SG. It is present in the cytosol of cells. Methylglyoxal is formed by elimination of phosphate from triose phosphates, the metabolism of acetone, and the catabolism of threonine; the rate of formation of methylglyoxal in human tissues is *ca.* 120 μM per day [1]. Methylglyoxal binds and irreversibly modifies proteins [2]. Methylglyoxal-modified proteins are bound by cell surface receptors on macrophages and undergo receptor-mediated endocytosis [3]. Methylglyoxal also binds and irreversibly modifies guanyl residues in DNA under physiological conditions forming 3-(2′-deoxy-β-D-*erythro*-pentafuranosyl)-6,7-dihydro-6,7-dihydroxy-6-methylimidazo[2,3-b]purine-9(8)one [4]. The modification of guanyl residues in DNA and RNA is thought to contribute to the antiproliferative activity of methylglyoxal, which is characterized by inhibition of DNA synthesis and inhibition of initiation of translation. Modification of DNA by methylglyoxal in cultured cells induced single-strand breaks, DNA-protein crosslinks, and cytotoxicity [5]. These processes are minimized under physiological conditions by the metabolism of methylglyoxal by the glyoxalase system [1, 5]. Pharmacological intervention to inhibit glyoxalase I was expected to lead to an increase in the cellular concentration of methylglyoxal in cell systems, and the development of methylglyoxal toxicity—particularly in proliferating cells and organisms [6].

BrBzGSH§ is a potent, competitive inhibitor of human glyoxalase I: the inhibition constant K_i value is 0.16 μM [7]. It is not, however, a potent antiproliferative agent; such glutathione-*S*-conjugates do not readily cross the cell plasma membrane. Rather, the diester derivative, BrBzGSH ethyl diester, was a potent antiproliferative agent. It inhibited the growth of human leukaemia 60 (HL60) cells *in vitro*; the median growth inhibitory concentration GC_{50} value was 8.3 μM [8]. It also inhibited the growth of the malaria parasite *Plasmodium falciparum in vitro*; the median growth inhibitory concentration IC_{50} value was 5.2 μM [9]. It was suggested that diesterification of BrBzGSH produced a prodrug that may readily cross the cell plasma membrane

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§ *Abbreviations:* BrBzGSH, *S*-p-bromobenzylglutathione; BrBzGSHCp, *S*-p-bromobenzylglutathione cyclopentyl monoester; $BrBzGSHCp_2$, *S*-p-bromobenzylglutathione cyclopentyl diester; BrBzGSHCx, *S*-p-bromobenzylglutathione cyclohexyl monoester.

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wherein nonspecific esterases liberate the glyoxalase I inhibitor; diesterification of BrBzGSH also confers resistance to extracellular degradation to BrBzGSH by γ-glutamyl transferase and dipeptidase on the extracellular surface of plasma membranes. We, now, describe the delivery of BrBzGSH into HL60 cells *in vitro* by the structural analogue, $BrBzGSHCp_2$, increase in the cellular concentration of methylglyoxal, inhibition of DNA synthesis, and induction of apoptosis. The inhibition of tumour growth *in vivo* by $BrBzGSHCp_2$ in a pilot study is also described.

MATERIALS AND METHODS

Materials

RNase A, proteinase K from *Tritirachium album*, Nonidet P-40, and Lambda Hind III digest DNA were purchased from Sigma Chem. Co., Ltd. (Poole, Dorset, U.K.). Tissue culture medium RPMI 1640 and foetal calf serum were purchased from Gibco Europe Ltd. (Paisley, Scotland). [*methyl*-^{3}H]Thymidine (80 mCi/mmol) was purchased from Amersham International (Amersham, Bucks., U.K.). Methylglyoxal was synthesised and concentrations of the stock aqueous solutions determined as previously described [10, 11]. BrBzGSHCp and diester derivatives were prepared and purified by methods similar to those described for the corresponding ethyl esters of BrBzGSH, except that acid-catalyzed esterification of BrBzGSH was performed in cyclopentanol for 7 days at room temperature [8]. BrBzGSHChx was used as the internal standard in the metabolite analysis of $BrBzGSHCp_2$ and was prepared and purified by similar acid-catalyzed esterification of BrBzGSH in cyclohexanol. ^{1}H and ^{13}C NMR spectroscopy and FAB mass spectrometry confirmed the structural assignments. Chromatographic R_f values on silica gel were: BrBzGSHCp 0.23, $BrBzGSHCp_2$ 0.90 (mobile phase chloroform, methanol, acetic acid, 8:1:1), and BrBzGSHChx 0.10 (mobile phase chloroform, methanol acetic acid, 18:1:1). The yields of BrBzGSHCp, $BrBzGSHCp_2$, and BrBzGSHChx were 27%, 71%, and 19%, respectively.

Antitumour Studies

HL60 cells were incubated at 37°C in RPMI 1640 media containing 10% foetal calf serum under an atmosphere of 5% CO_2 in air, 100% humidity [8]. Cells were seeded at an initial density of 5×10^4/mL and incubated with 1–50 μM $BrBzGSHCp_2$ and 10–500 μM BrBzGSHCp. Cell viability was judged by the ability of cells to exclude Trypan blue. The rate of DNA synthesis in HL60 cells was estimated by measuring the rate of incorporation of [^{3}H]thymidine into DNA. HL60 cells (5.0×10^4/mL) were incubated with 1–500 μM $BrBzGSHCp_2$ in RPMI 1640 with 10% foetal calf serum for 2 hr. [^{3}H]Thymidine (2.5 μCi, 2.5 μL) was added and the incubation continued for 1 hr. The DNA was then extracted and counted [12]. Similar experiments were performed with peripheral lymphocytes, isolated from venous blood samples of healthy human donors, where the rate of cell growth was determined by measuring the rate of incorporation of [^{3}H]thymidine into DNA [13]. Lymphocyte growth was stimulated by incubation of lymphocytes (1×10^5) in RPMI 1640 with 25% autologous plasma and 5 μg/mL of concanavalin A (final volume: 200 μL) for 72 hr. Thereafter, the lymphocytes were incubated for a further 4 hr with and without 20 μM $BrBzGSHCp_2$. [^{3}H]Thymidine in RPMI 1640 (5 μL; 0.2 mCi/mL) was then added, and the cell suspension incubated for a further 4 hr. DNA extracts were then prepared and counted. Similar incubations were performed with and without 20 μM $BrBzGSHCp_2$ for 8 hr, but without addition of [^{3}H]thymidine and cell morphology, and DNA fragmentation of the lymphocytes were examined as described below.

Morphological changes and DNA fragmentation induced by $BrBzGSHCp_2$ were investigated by incubation of HL60 cells (5×10^4/mL; 1×10^6) for 6–10 hr in the absence and presence of 524 μM methylglyoxal or 20 μM $BrBzGSHCp_2$. The HL60 cells were washed with phosphate-buffered saline (0.9% sodium chloride, 10 mM potassium phosphate buffer, pH 7.4; PBS), fixed in 95% ethanol for 5 min, and visualised by Giemsa staining. DNA fragmentation was analysed by agarose electrophoresis by modification of the method described [14].

The metabolites of $BrBzGSHCp_2$, BrBzGSHCp, and BrBzGSH, formed in the extracellular medium and cytosol of HL60 cells in culture, were analysed by HPLC. HL60 cells (5×10^4/mL; 5×10^6 cells) were incubated in RPMI 1640 with 10% foetal calf serum for 3 hr at 37°C with and without $BrBzGSHCp_2$. The HL60 cells were then sedimented by centrifugation (216 g, 5 min) and the supernatant removed; perchloric acid (0.6 M, 2 mL) was added to an aliquot of the supernatant (1 mL) and analysed for metabolites of $BrBzGSHCp_2$. The cell pellet was washed with 1 mL of PBS and perchloric acid (0.6 M, 2 mL) added to the cell pellet. The resulting perchloric acid extracts were stored at −196°C until analysed (<1 week). Sample storage validation studies showed that $BrBzGSHCp_2$, BrBzGSHCp, and BrBzGSH were stable under these conditions for ≤3 months.

The concentrations of $BrBzGSHCp_2$, BrBzGSHCp, and BrBzGSH were analysed by reversed-phase HPLC after partial purification by octadecylsilica solid-phase extraction (ODS-SPE). The internal standard (0.1 mg/mL of BrBzGSHChx in DMSO; 20 μL) was added to the perchloric acid extracts. They were centrifuged (6000 g, 10 min, 4°C), and the supernatant removed and neutralised to pH 2.3 by addition of 0.5 M Na_2HPO_4 (*ca.* 850 μL). The neutralised extract was then applied to an ODS-SPE cartridge (500 mg of ODS) previously equilibrated with 20 mM $NH_4H_2PO_4$, pH 2.3. The ODS-SPE cartridge was then washed with 5 mL of equilibration buffer, and the analytes collected by elution of the ODS-SPE cartridge with methanol (5 mL). The methanol was removed by centrifugal evaporation, the residual solid reconstituted in 200 μL of 30% acetonitrile in 50 mM trifluoroacetic acid, spin-filtered (0.2 μm) and analyzed by HPLC. A Waters HPLC

system (2 × 510 pumps, Lambda Max 481 LC spectrophotometer, with a 680 automated gradient controller) and a Kontron PC Integrator was used. The column was a Nova-Pak ODS 4 μm (0.8 cm × 10 cm) cartridge fitted with an ODS pre-column in an 8 × 10 radial compression unit. The mobile phase was: 50 mM trifluoroacetic acid with 30% acetonitrile from 0–5 min, and a linear gradient of 30–75% acetonitrile from 5–20 min. The flow rate was 2 mL/min and the eluate absorbance was monitored at 235 nm. The retention times of BrBzGSH, BrBzGSHCp, BrBzGSHChx, and $BrBzGSHCp_2$ (mean ± SD, $n = 12$) were 3.9 ± 0.4, 13.2 ± 0.5, 14.4 ± 0.4, and 16.9 ± 0.6 min, respectively. Calibration curves were produced by analysis of 1–15 μg of BrBzGSH, BrBzGSHCp, and $BrBzGSHCp_2$ ($n = 11$). The limits of detection were 0.45, 0.35, and 0.31 nmol, respectively; the recoveries in the working analytical range (mean ± SD %) were 63 ± 5, 57 ± 4%, and 62 ± 4, respectively. The concentration of methylglyoxal was assayed in HL60 cell suspensions incubated for 0–24 hr in RMPI 1640 with 10% foetal calf serum in the absence and presence of 10 μM $BrBzGSHCp_2$ [11].

The effect of $BrBzGSHCp_2$ on the growth of adenocarcinoma 15A cells *in vivo* was studied. Tumour cells (1×10^6) were implanted subcutaneously in mice. After 4 days, the mice were given one intraperitoneal injection of $BrBzGSHCp_2$ (0–200 mg/kg) in 20% Tween 80 in saline. After 1 week, the mice were killed and the tumour mass determined.

RESULTS AND DISCUSSION

When HL60 cells were incubated with $BrBzGSHCp_2$ (0–50 μM) for 3 days, there was a concentration-dependent decrease in cell growth and decrease in cell viability. The GC_{50} value was 4.23 ± 0.01 μM with an n value of 3.59 ± 0.01 ($n = 21$), Fig. 1a; the GC_{50} value of BrBzGSHCp was 392 ± 26 μM ($n = 7$), and the GC_{50} value of BrBzGSH was >500 μM [8]. The TC_{50} value of $BrBzGSHCp_2$ was 8.86 ± 0.01 μM with an n value of 2.14 ± 0.01 ($n = 21$), Fig. 1b. $BrBzGSHCp_2$ inhibited DNA synthesis in HL60 cells in the third hour of culture: the IC_{50} value was 6.11 ± 0.02 μM with an n value of 0.91 ± 0.01 ($n = 8$), Fig. 1c. $BrBzGSHCp_2$ did not induce terminal differentiation of HL60 cells. The effect of 4 μM $BrBzGSHCp_2$ on the HL60

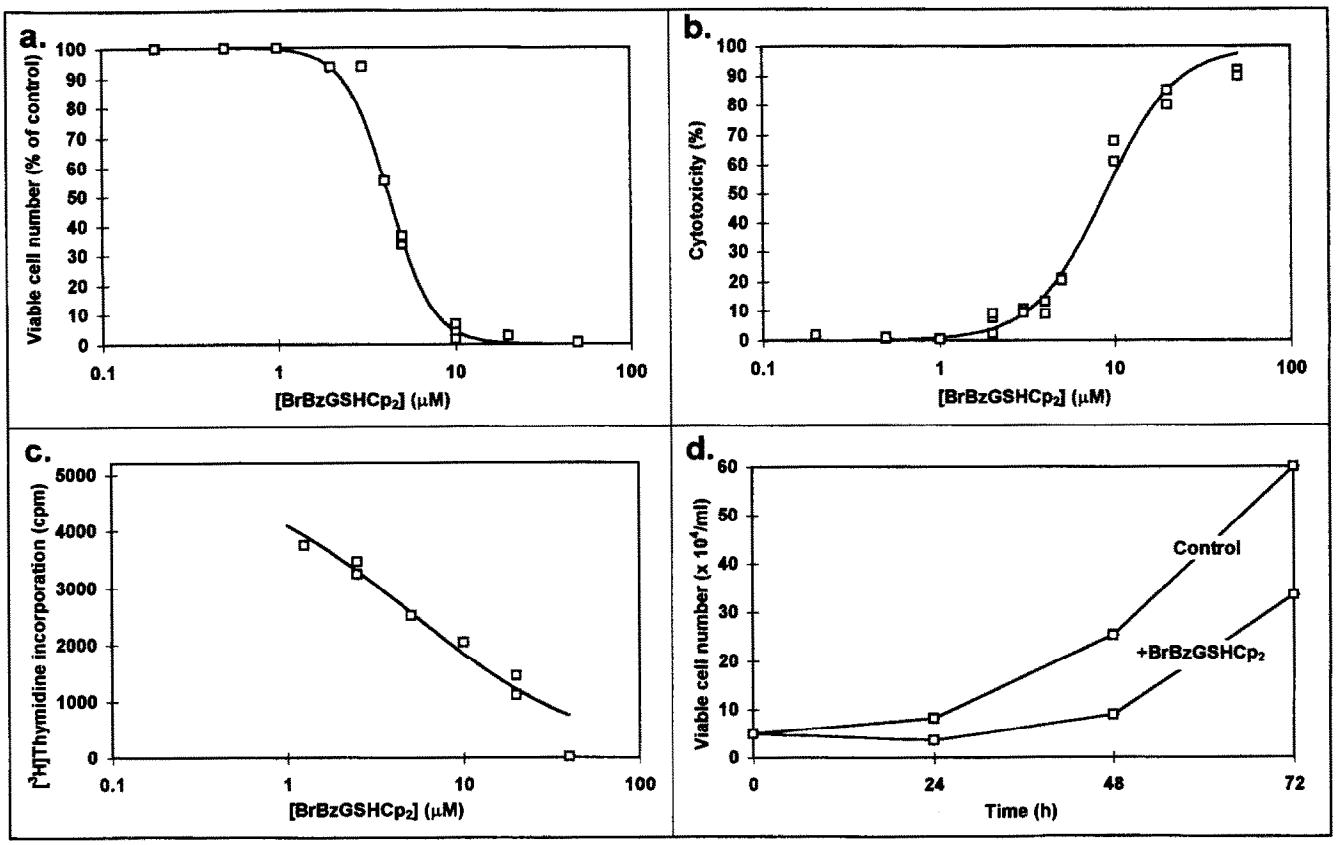

FIG. 1. Effect of S-p-bromobenzylglutathione cyclopentyl diester on HL60 cell growth *in vitro*. a. Dose-response curve and b. Effect on cell viability. HL60 Cells (5×10^4/mL) were incubated for 3 days with and without 0.1–20 μM $BrBzGSHCp_2$. GC_{50} = 4.23 ± 0.01 μM ($n = 21$), TC_{50} = 8.86 ± 0.01 μM ($n = 21$). c. Effect of $BrBzGSHCp_2$ on DNA synthesis in HL60 cells. IC_{50} = 6.11 ± 0.02 μM ($n = 8$); control rate of DNA synthesis was 5190 ± 280 cpm ($n = 4$). d. Cell growth curve. HL60 Cells (5×10^4/mL) were incubated for 3 days without (Control) and with (+$BrBzGSHCp_2$) 4 μM $BrBzGSHCp_2$. Data are the mean ± SD of 3 experiments.

growth curve indicated that growth inhibition and toxicity was induced in the initial 24 hr of culture; thereafter, residual surviving cells attained control growth kinetics, Fig. 1d. The cyclopentyl diester was chosen because it is resistant to extracellular cleavage by nonspecific esterases. $BrBzGSHCp_2$ was a more potent inhibitor of HL60 cell growth than the corresponding ethyl diester [8], and is the most potent diester studied to date.

The poly(ADP-ribose)polymerase inhibitor 3-aminobenzamide (2.5 mM) inhibited the decrease in HL60 cell growth induced by 4 μM $BrBzGSHCp_2$ in a 72-hr culture period, but not significantly (Table 1). Inhibitors of poly(ADP-ribose)polymerase (EC 2.4.2.30) may affect the potency of cytotoxic agents where modification of DNA is implicated in the mechanism of action [15, 16]—as is the case herein, where increased modification of DNA by methylglyoxal is expected in HL60 cells incubated with $BrBzGSHCp_2$. Poly(ADP-ribose)polymerase, however, is proteolytically degraded early in the development of apoptosis in HL60 cells incubated with DNA-modifying anticancer agents [17], which may explain the lack of effect of 3-aminobenzamide herein.

The aldose reductase inhibitor sorbinil (1 μM) did not inhibit the growth of HL60 cells, but potentiated the inhibition of HL60 cell growth by 4 μM $BrBzGSHCp_2$ significantly ($P < 0.001$). The aldose reductase inhibitor statil (1 μM), however, had no significant effect on either HL60 cell growth or the inhibition of HL60 cell growth by 4 μM $BrBzGSHCp_2$ (Table 1). Methylglyoxal may be detoxified in human tissues by the glyoxalase system [1] and by the nonspecific aldehyde reductase, aldose reductase (EC 1.1.1.21) [18]. Estimates of the ratio of *in situ* activities for the detoxification by glyoxalase I to aldose reductase suggested that glyoxalase I was the major pathway for the detoxification of methylglyoxal, except in the kidney where the high concentration of aldose reductase suggests that it is also important [5]. It is likely, however, that when glyoxalase I is pharmacologically inhibited, increased flux of methylglyoxal may be metabolised by aldose reductase. Pharmacologic inhibition of aldose reductase may, therefore, potentiate the cytotoxicity of $BrBzGSHCp_2$. The results were inconclusive because sorbinil (1 μM) significantly potentiated the toxicity of $BrBzGSHCp_2$ to HL60 cells, as expected, but statil (1 μM) potentiated the toxicity of $BrBzGSHCp_2$ to HL60 cells, although not significantly.

When human peripheral lymphocytes were stimulated with concanavalin A and incubated with 20 μM $BrBzGSHCp_2$, there was no significant cytotoxicity and/or fragmentation of cellular DNA observed. $BrBzGSHCp_2$ increased the rate of proliferation of concanavalin A-stimulated lymphocytes by 29% of control values, but the increase was not significant: [^{3}H]thymidine incorporation into lymphocyte DNA in the absence and presence of 20 μM $BrBzGSHCp_2$ was 1259 ± 294 cpm and 1595 ± 240 cpm ($n = 6$; $P > 0.05$), respectively. This suggests that the antiproliferative activity of $BrBzGSHCp_2$ was selective for HL60 cells.

When HL60 cells were incubated for 3 hr with 10 μM $BrBzGSHCp_2$, the extracellular concentration of $BrBzGSHCp_2$ decreased to 5.3 μM and low concentrations of BrBzGSHCp (0.48 μM) and BrBzGSH (0.81 μM) were formed. The de-esterification of $BrBzGSHCp_2$ may be catalyzed by serum and cell surface nonspecific esterases. In HL60 cells, $BrBzGSHCp_2$ and the de-esterified metabolites, BrBzGSHCp and BrBzGSH, were detected (Fig. 2a). This demonstrates that $BrBzGSHCp_2$ was an effective vehicle for the delivery of BrBzGSH into cells. When HL60 cells were incubated with and without 10 μM $BrBzGSHCp_2$ for 0–24 hr, there was an increase in the concentration of methylglyoxal in HL60 cell cultures incubated both with and without $BrBzGSHCp_2$ (Fig. 2b). The increase in methylglyoxal concentration in HL60 cells incubated with $BrBzGSHCp_2$, however, was greater than in the incubations of HL60 cells without $BrBzGSHCp_2$ over the total 1–24 hr (ANOVA; $P < 0.001$).

HL60 cells were incubated in the absence and presence of 20 μM $BrBzGSHCp_2$ for 6 hr and the cellular DNA

TABLE 1. Effect of 3-aminobenzamide, sorbinil, and statil on the inhibition of HL60 cell growth by *S*-*p*-bromobenzylglutathione cyclopentyl diester

Addition	HL60 Viable Cell Number (× 10^3/mL) (Mean ± SD)	(n)	*P*	*P'*
None (control)	623.2 ± 79.1	(19)	–	–
+ 4 μM $BrBzGSHCp_2$	295.2 ± 47.0	(6)	<0.001	–
+ 2.5 mM 3-Aminobenzamide	557.1 ± 44.9	(3)	>0.05	–
+ 4 μM $BrBzGSHCp_2$ and 2.5 mM 3-aminobenzamide	348.8 ± 26.1	(3)	<0.001	>0.05
+ 1 μM Sorbinil	530.9 ± 44.9	(6)	>0.05	–
+ 4 μM $BrBzGSHCp_2$ and 1 μM sorbinil	120.0 ± 13.9	(4)	<0.001	<0.001
+ 1 μM Statil	575.8 ± 53.2	(5)	>0.05	–
+ 4 μM $BrBzGSHCp_2$ and 1 μM statil	326.3 ± 45.4	(4)	<0.001	>0.05

P and *P'* indicate the level of significance (*t*-test) with respect to the control and cells incubated with 4 μM $BrBzGSHCp_2$, respectively.

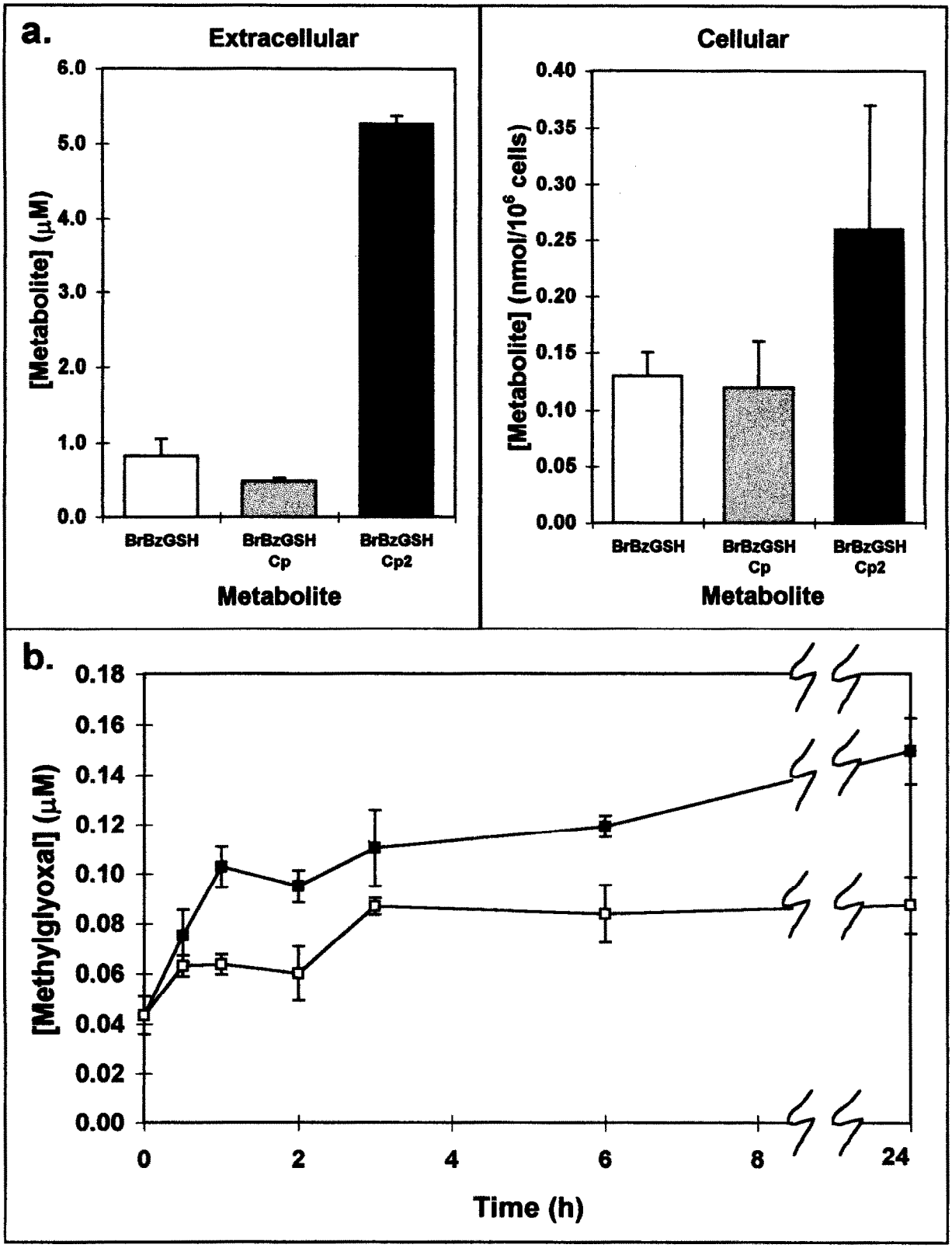

FIG. 2. Delivery of S-p-bromobenzylglutathione into HL60 cells by S-p-bromobenzylglutathione cyclopentyl diester and increase in the concentration of methylglyoxal. a. Extracellular and cellular concentrations of $BrBzGSHCp_2$ and metabolites BrBzGSHCp and BrBzGSH. HL60 cells (5×10^4/mL; 100 mL) were incubated for 3 hr with 10 μM $BrBzGSHCp_2$. Data are mean ± SD of 4 determinations. b. The concentration of methylglyoxal in HL60 cell suspensions (5×10^4/mL; 2 mL) incubated with (■) and without (□) 10 μM $BrBzGSHCp_2$ for the times indicated. Data are mean ± SD of ≥4 determinations.

stained with Giemsa stain, Fig. 3A, a and b, respectively. In HL60 cells incubated with $BrBzGSHCp_2$, there was condensation and fragmentation of nuclear DNA within cells that still had an intact plasma membrane, and budding of plasma membrane from cells; the buds containing fragments of DNA. When HL60 cells were incubated with 524 μM methylglyoxal, agarose gel electrophoresis of cellular DNA revealed the presence of DNA fragments in the range 23,000 to < 564 base pairs (Fig. 3B, lane 3) but not in control incubations (Fig. 3B, lane 2). In the low fragment range, 2027–564 base pairs, banding of the DNA fragments was evident. This is characteristic of apoptosis of HL60 cells induced by methylglyoxal [15]. When HL60 cells were incubated with 20 μM $BrBzGSHCp_2$ for 6 and 10 hr, DNA fragments in the range 23,000 to <2000 base pairs were formed (Fig. 3B, lanes 5 and 7). There was no fragmentation of cellular DNA in control incubations (Fig. 3B, lanes 4 and 6). This indicates that $BrBzGSHCp_2$ induced apoptosis in HL60 cells *in vitro*. DNA fragmentation was not present in incubations with 20 μM $BrBzGSHCp_2$ until after 6 hr whereas, with 524 μM methylglyoxal, DNA fragmentation was observed in HL60 cells after incubation for only 2 hr [19]. This delay in the appearance of DNA fragments with $BrBzGSHCp_2$ may be related to the time required for the $BrBzGSHCp_2$ to enter the HL60 cells, deesterification of $BrBzGSHCp_2$ to BrBzGSH, and increase in the concentration of methylglyoxal to cytotoxic levels.

Pilot studies to investigate the effect of $BrBzGSHCp_2$ on the growth of a subcutaneous implant of adenocarcinoma 15A cells in mice showed that doses of 50, 100, and 200 mg/kg of $BrBzGSHCp_2$ produced significant inhibition of tumour growth by 30, 33, and 40% of control values, respectively (Fig. 4). There was no indication of toxicity of $BrBzGSHCp_2$ to the mice at these doses.

$BrBzGSHCp_2$ may induce apoptosis in HL60 cells by a mechanism other than inhibition of glyoxalase I and accumulation of methylglyoxal, although transfection of murine NIH3T3 cells with glyoxalase I cDNA gave increased glyoxalase I activity and resistance to $BrBzGSHCp_2$ [20], further supporting evidence that cytotoxicity induced by $BrBzGSHCp_2$ was mediated by the inhibition of glyoxalase I. It is instructive to consider if the concentrations of BrBzGSH and methylglyoxal in HL60 cells were competent to inhibit glyoxalase I and modify DNA, respectively. The

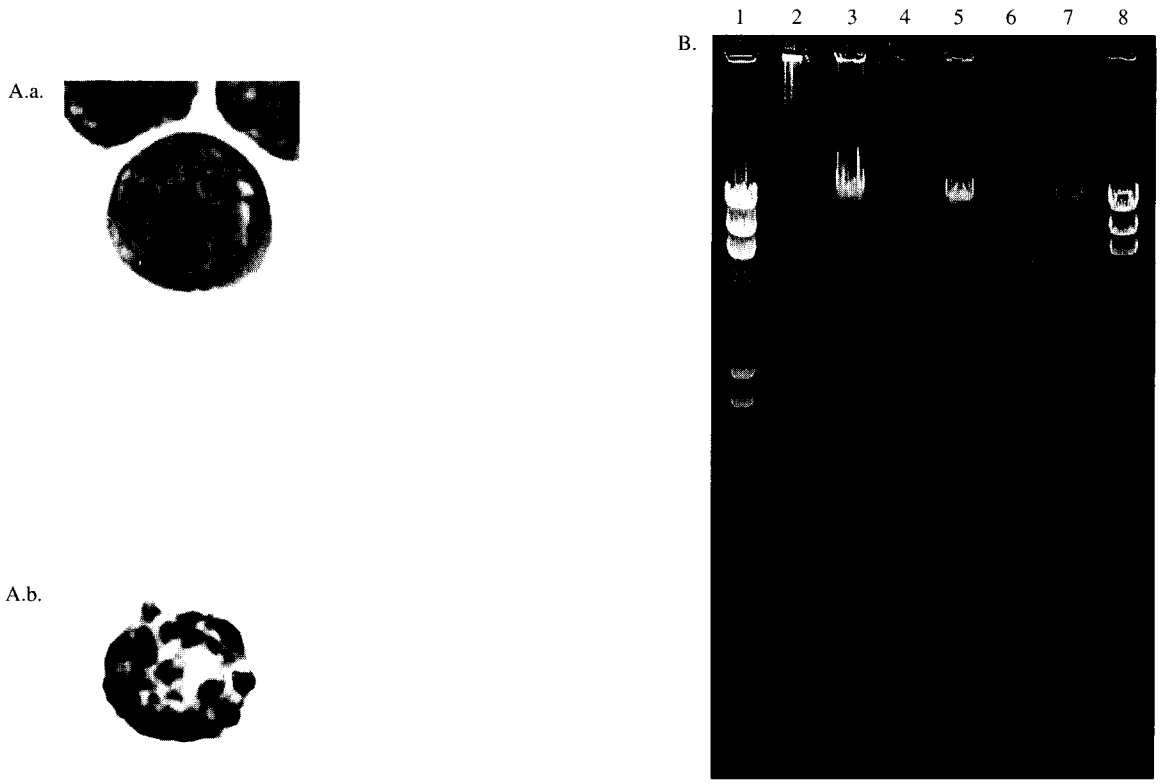

FIG. 3. The induction of apoptosis in HL60 cells by *S*-p-bromobenzylglutathione cyclopentyl diester. A. Morphological examination of HL60 cells; a. control HL60 cells; b. +20 μM $BrBzGSHCp_2$, incubated for 6 hr (magnification 1000×). B. DNA fragmentation in HL60 cells; Lane 1, DNA fragment standards; lane 2, control (6 hr); lane 3, +524 μM methylglyoxal (6 hr); lane 4, control (6 hr); lane 5, +20 μM $BrBzGSHCp_2$ (6 hr); lane 6, control (10 hr); lane 7, +20 μM $BrBzGSHCp_2$ (10 hr); lane 8, DNA fragment standards.

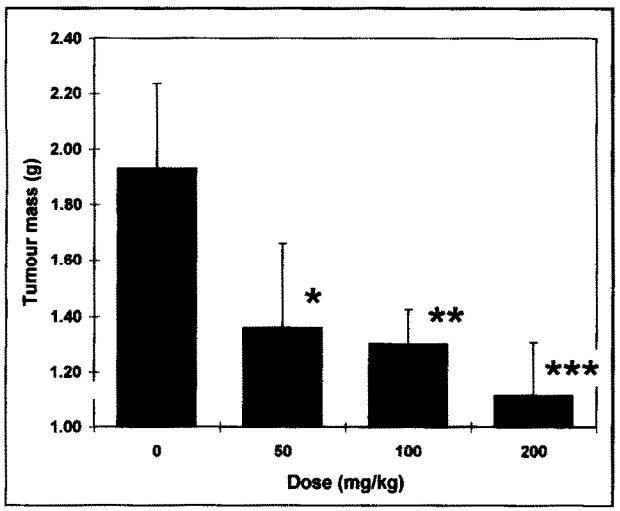

FIG. 4. Effect of S-p-bromobenzylglutathione cyclopentyl diester on the growth of murine adenocarcinoma 15A *in vitro*. Data are mean ± SD of 6 determinations. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

concentration of BrBzGSH found in HL60 cells after culture with 10 μM $BrBzGSHCp_2$ for 3 hr was *ca.* 0.13 nmol/10^6 cells, equivalent to a mean concentration of BrBzGSH in HL60 cells of *ca.* 140 μM (assuming the HL60 cell volume is 9.2×10^{-13} l [21]), which is sufficient to inhibit the metabolism of exogenous methylglyoxal [2]. The concentration of methylglyoxal in control cultures of HL60 cells increased from 43 nM to 87 nM after 24 hr. Methylglyoxal crosses cell plasma membranes readily—the methylglyoxal present in the HL60 cell suspension at time zero originated mainly from the foetal calf serum. As the incubation proceeds, methylglyoxal is formed in HL60 cells and may leak from the cells into the medium. The concentration of methylglyoxal in the extracellular medium will increase until it equilibrates with the concentration in HL60 cells. This may account for the increase in methylglyoxal concentration in HL60 cell suspensions in control incubations. The concentration of methylglyoxal in HL60 cell suspensions incubated with 10 μM $BrBzGSHCp_2$ increased more markedly than in control incubations. The method of assay of methylglyoxal concentration used determines both methylglyoxal in free solution (unhydrated and hydrated forms) and reversibly bound to protein, combined [11]. Greater than 90% of the concentration of methylglyoxal in HL60 cell suspensions is expected to be reversibly bound to proteins [2]. Recent studies of the incubation of methylglyoxal with plasmid DNA, in the absence of extraneous protein, and transfection of a lymphoblastoid cell line with the plasmid have shown a 100 nM methylglyoxal-induced mutation and abnormal gene expression [22]. The observed increase in the concentration of methylglyoxal in suspensions of HL60 cells incubated with 10 μM $BrBzGSHCp_2$ may, therefore, be sufficient to induce modification of DNA and activate apoptosis. Although methylglyoxal is known to cross cell plasma membranes readily, the high extent of reversible binding of methylglyoxal to cell proteins may have the effect of trapping methylglyoxal intracellularly. In this case, the concentration of methylglyoxal in HL60 cell suspensions reported herein may be an underestimate of intracellular methylglyoxal concentration.

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